RESEARCH COMMENTARY

Brain clocks for morning and evening behaviour

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Circadian clocks of fruit flies are multioscillatory, as many behavioural and physiological rhythms are timed by separate sets of oscillators. The basic rhythm-generating mechanisms are cellular, and involve an elaborate network of neurons. Different subgroups of cells that regulate the morning and evening components of activity are now being identified and are also known as the 'morning' and the 'evening' oscillators.

Life on earth has evolved under continuously changing day and night cycles caused by the rotation of the earth around its axis. Although day and night follow each other in a cycle of fixed length, their duration changes with season, latitude and altitude. A question then arises as to how organisms adjust their behavioural and physiological programmes to such ever-changing day and night cycles. We can think of two possible modes for such mechanisms: (i) the passive mode, where organisms mimic the cyclic environment by directly influencing their behaviour, and (ii) the active mode, where organisms adjust the phase of their circadian (*circa* = approximate, *dies* = a day) clocks through a set of mechanisms involving time cues in the environment, and the time-dependent sensitivity of the circadian clocks to such time cues. In addition, organisms seem to have developed a memory for day length imprinted on their clocks, often observed as the *aftereffects* in the circadian rhythms following exposure to day and night cycles. Such *aftereffects* may help organisms to track time under natural conditions, when information about day and night cycles is blurred owing to cloud cover or behavioural variations.

Circadian clocks operate in a variety of organisms on an approximately 24-hour scale. These clocks regulate the timing of a wide range of behavioural and metabolic processes. For example, in the fruit fly *Drosophila melanogaster*, circadian clocks time adult emergence, activity/rest cycle, egg-laying rhythm, olfactory and mating rhythms (reviewed in Sharma 2003). The pacemaker cells for some of these rhythms are located in the ventral lateral, dorsal lateral and dorsal region of the fly brain. In these neurons, a number of 'clock genes' such as *period* (*per*), *timeless* (*tim*), *cryptochrome* (*cry*) and a few others function in an orchestrated manner to generate an approximately 24-hour pattern in various behavioural and physiological processes. These clock genes operate in interlocking negative and positive feedback loops, and show robust oscillations in their transcripts and proteins levels. Before the advent of present-day molecular and genetic tools, the three primary questions that acted as the driving force for an enormous body of investigations in circadian biology were (i) how do circadian clocks work?, (ii) how do they get synchronized with *zeitgebers* (time cues) in the environment?, and (iii) what are the escapement (output) mechanisms that transduce information of time from circadian pacemaker to target organs?

At least two decades ago, a model of circadian clocks with two separate oscillators was implicated in tracking information of day and night. The model posited a 'morning oscillator' (M), which locks on to dawn and an 'evening oscillator' (E) that locks on to dusk (Pittendrigh and Daan 1976). The two main empirical observations that rendered support to this proposal were the occurrence of bimodal bouts of activity in a variety of organisms under periodic light/dark (LD) cycle, and splitting of the morning and evening components of activity under constant conditions. It was believed that bilaterally distributed clock cells in the brains of animals might serve as M and E oscillators. Indeed, unilateral lesions of the suprachiasmatic nucleus (SCN), the main mammalian clock, completely abolished or partially compressed one of the split components of activity in hamsters (Iglesia *et al*. 2000). The two SCN lobes in the brain of split hamsters simul-

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taneously synthesized transcripts of a clock gene, *mPer1*, in one of the two lobes, and transcripts of another clock gene, *Bmal1*, in the other, suggesting antiphasic nature of the two bilaterally symmetric SCN lobes. Further, it was proposed that, in mammals, the clock genes *mPer1* and *mCry1* serve as morning oscillator and *mPer2* and *mCry2* serve as evening oscillator. In fruit flies, *per* and *cry* genes were proposed as the molecular E oscillators, because flies that are null mutants in these two genes lack evening anticipation of locomotor activity under LD cycles (Emery 2000; Helfrich-Förster 2000). As the molecular mechanisms in fruit flies involve only one *per* and one *cry* gene, both involved in the regulation of evening component of activity, it would be interesting to look for the molecular M oscillators.

Although it is now quite clear that feedback loops involving transcription and translation of several clock genes regulate the circadian clocks in fruit flies, the neural mechanisms that host such molecular reactions remain a mystery. There are approximately 100 circadian clock neurons, bilaterally clustered in the lateral, ventral and dorsal regions of the cerebral lobes, in six distinct groups of cells. Two of the ventral lateral (LNv) groups of cells, each consisting of four or five neurons, have been implicated in regulation of adult emergence and locomotor activity rhythm in *D. melanogaster*, and, except for one of the five small LNv, express the *pigment dispersing factor* (*pdf*) gene. Although the LNvs are important for the locomotor activity rhythm in constant darkness (DD), as they are one of the key cellular coordinators that maintain rhythmic gene expression within the circadian network, recent studies have shown that ablation of LNvs affects only limited aspects of behavioural rhythms under LD conditions (Renn *et al*. 1999), suggesting that there are other clock neurons that control the locomotor activity rhythm under LD cycles.

The M and E oscillators for *D. melanogaster* have now been identified in two studies that appeared in two papers in the 14 October 2004 issue of *Nature*. In these studies Stoleru *et al*. (2004) and Grima *et al*. (2004) have shown that the morning and evening bouts of locomotor activity in *D. melanogaster* are regulated by separate groups of clock neurons in the brain. The two groups approached the issue in two different ways but arrived at similar conclusions. Stoleru *et al*. (2004) chose to analyse the locomotor activity behaviour of flies following genetic ablation of one or more groups of pacemaker neurons, while Grima *et al*. (2004) studied the locomotor activity behaviour of *per*-deficient flies following forced expression of *per* gene in one or more groups of cells. The fly clock network consists of three subgroups of dorsal neurons (DN1, DN2, and DN3), and three subgroups of lateral neurons, distributed bilaterally in the brain (figure 1). The genes *per* and *tim* are expressed in all clock neurons (PER^+ and TIM^+ cells), *pdf* is expressed in all but one LNvs (PDF^+ cells), and the circadian photoreceptor *cryptochrome* (*cry*) gene is expressed widely (all LNv, and LNd, DN1s, and one PDF^- LNv cell) (CRY^+ cells). Stoleru *et al*. (2004) and Grima *et al*. (2004) analysed the circadian clock network of fruit flies by extensively studying these neurons. Recent studies have also shown that genetic ablation of LNvs affects only the morning anticipation of locomotor activity rhythm under LD conditions, leaving the evening component intact, suggesting that there are other clock cells that control the evening component of locomotor activity rhythm. To identify these additional clock cells, Stoleru *et al*. (2004) chose to monitor locomotor activity rhythm of flies in which more than one group of neurons were genetically ablated. Using cleverly chosen *GAL4* driver lines they expressed the proapoptotic gene *hid* in one or more groups of neurons and subsequently monitored morning and evening bouts of locomotor activity. The activity patterns of the *cry-GAL4; UAS-hid* (UAS, upstream activating sequences) flies showed a complete loss of morning and evening anticipation, and in addition CRY^+ cells did not synthesize PER, suggesting that the CRY^+ cells serve as M and E oscillators. The authors hypothesized that the LNds, which are CRY^+ PDF^- cells, may serve as the E oscillators. In order to ablate the LNds, Stoleru *et al*. (2004) developed a novel expression system that is based on the MARCM system (mosaic analysis with a repressible cell marker), which allows spatial resolution of clock cell targeting. The authors developed

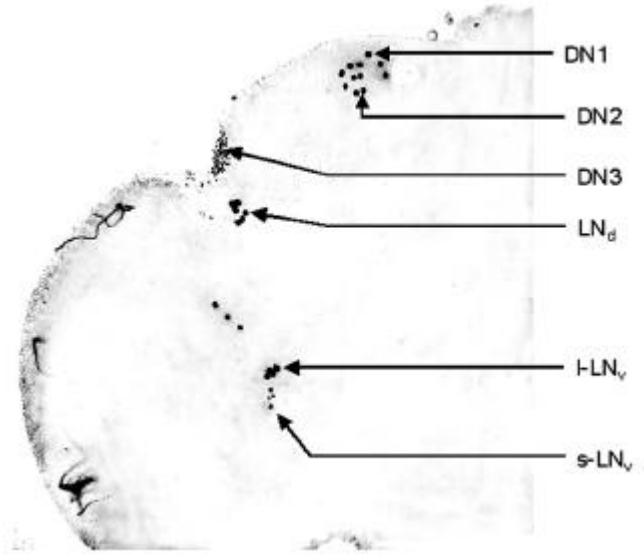

Figure 1. Clock neurons in the *Drosophila* brain: six group of neurons, namely the small and large ventral lateral neurons (s-LNv and l-LNv); dorsal lateral neurons (LNd); and three subgroups of neurons (DN1, DN2 and DN3). Stoleru *et al*. (2004) and Grima *et al*. (2004) have shown that the LNv and LNd neurons regulate the morning and evening bouts of locomotor activity in *D. melanogaster*.

transgenic fly lines that express *GAL80* under the control of various clock promoters, which were then crossed with lines in which clock promoters drive the expression of *GAL4*, a yeast transcriptional activator. This combination of three transgenes allows the visualization or targeted ablation or limited synthesis of proteins in neural circuits which were not clearly understood until now. Finally green flourescent protein (GFP) was used as a reporter to visualize the neurons.

A triple transgenic strain obtained by crossing *cry-GAL4*; *UAS-hid* with *Pdf-GAL80* then permitted behavioural as well as immunocytochemical comparison of flies in which only the LNd neurons and a fraction of DN1 neurons which are CRY^+ and PDF^- cells, are ablated. These flies did not show any evening anticipation while retaining fairly robust morning anticipation. This is the first instance of a selective disruption of evening anticipation. These results suggest that the E oscillators could in fact be the LNds. The absence of morning anticipation of the PDF^+ ablated lines was reminiscent of locomotor activity patterns in the pdf^{01}, reconfirming that the PDF^+ LNvs are indeed the M oscillators. The DD and LD behaviours of the ablated lines were consistent, and, as expected, in DD the LNv and LNd ablated lines displayed unimodal, apparently morning-only or evening-only peak of activity. The locomotor activity rhythm of PDF^+ ablated lines dampened in DD, while those of CRY^+ PDF^- strains continued unabated for several days. Stoleru *et al*. (2004) used an alternative strategy to demonstrate that the LNv and LNd are the M and E oscillators, by selectively disrupting the molecular oscillations in these groups of cells. A *UAS-per* transgene driven by a pan-neural *elav-GAL4* driver can rescue the behavioural defects of the per^0 mutants. They introduced *cry-GAL80* into a *per01*; *elav-GAL4*; *UAS-per* system to selectively block the *elav-GAL4*-mediated rescue of the CRY^+ cells. In subsequent experiments, they selectively blocked rescue of PDF^+ cells by using *pdf-GAL80* instead of *cry-GAL80*. These constructs mimicked the phenotype of CRY^+ and PDF^+ ablated lines, confirming that the LNvs and LNds indeed function as M and E oscillators in *D. melanogaster*.

In the other study Grima *et al*. (2004) sought to use targeted expression of *per* gene in the lateral neurons of per^0-flies. The authors used *cry-GAL4* lines to drive expression of *UAS-per* transgene in per^0 flies to restore wild-type behaviour (with robust morning and evening bouts of activity) by expressing *UAS-per* in the CRY^+ cells. Robust oscillation of PER could also be restored in the LNvs of per^0-flies when *pdf-GAL4* drove expression of *UAS-per* transgene, and unlike the *cry-GAL4; UAS-per* lines these flies exhibited just the morning anticipation activity. To further analyse the function of the LNvs the authors used *Mz520-GAL4* lines (enhancer-trap expression), in which expression of *per* is restricted only to the PDF^+ cells. Locomotor activity patterns with complete lack of evening component and robust PER oscillations were restored in the per^0-flies when *per* expression was driven in the LNvs using *Mz520-GAL4* driver. The authors then used two additional *GAL4* lines that restricted expression of *per* gene in specific groups of clock cells. In *C929-GAL4* lines, in which expression of the gene is driven only in the large LNv, morning and evening anticipation were not restored in per^0-flies, which suggests that the small LNvs alone regulate the morning anticipatory behaviour. Another enhancer-trap driver *Mai179-GAL4* restricted expression of clock genes in the five small LNv, a small number of large LNvs, and three or four of the six LNds. The *Mai179-GAL4*; *UAS-per* transgene rescued both morning and evening anticipation behaviour and PER oscillation in the small LNvs in the per^0-flies, which suggests that the PDF^- LNds function as the E oscillators, and the PDF^+ small LNvs as the M oscillators. Contrary to these findings, in a previous study on per^0-mutants the flies displayed morning anticipation of activity but no evening anticipation, similar to the flies with M oscillators. In a separate study on per^s- and per^L-flies, the evening peaks occurred earlier and later than those in the wild-type flies, while the morning peak remained largely unchanged (Helfrich-Förster 2001). These studies thus clearly suggest that the *per* gene is a key component of the molecular E oscillator. How do we reconcile the findings of Grima *et al*. (2004) and Helfrich-Förster (2001)? One possible explanation is that the levels of PER and activity in per^0-flies could be undetectably low under normal circumstances, especially during the morning hours, and a forced expression of *per* in the M oscillators could cause a significant enhancement in the amplitude of molecular and behavioural oscillations.

Thus, two independent strategies of genetic manipulation of circadian clock mechanisms led to a similar conclusion that the LNvs and LNds serve as M and E oscillators, respectively, in the *Drosophila* circadian timing system. The study of Grima *et al*. (2004) narrowed down the issue even further and made a finer distinction between the small and large groups of LNvs, as they demonstrate that the small LNv regulate the M component of activity and the LNds control the E component. The studies of Stoleru *et al*. (2004) and Grima *et al*. (2004) also conclude that the neurotransmitters that function as the messenger of timing information for the M and E oscillators may not be the same. While PDF appears to be the messenger for the M oscillator, the messenger for the E oscillator is still unknown. Although it is now clear that selective genetic ablation of clock neurons abolishes M or E component of activity, and forced gene expression can rescue them, it would be too simplistic to assume that the functional M and E oscillators have been discovered. For instance, we do not know the contributions of the dorsal neurons (DN1, DN2, and DN3), nor what couples the M and E oscillators and what the M and E messen-

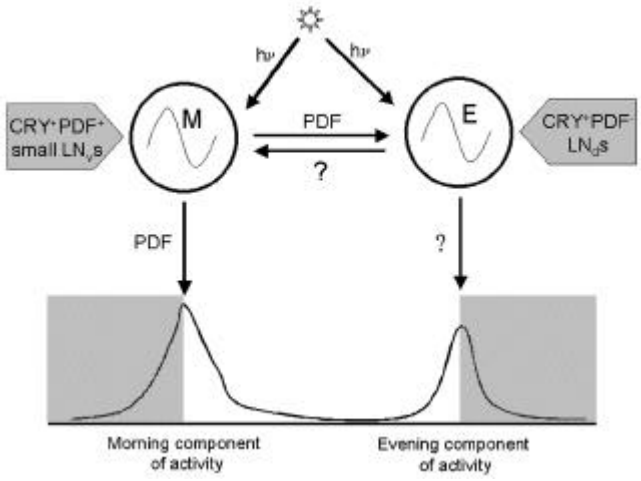

Figure 2. Model of *Drosophila* circadian clocks involving morning (M) and evening (E) oscillators. The M and E oscillators comprise $CRY^+ PDF^+$ small ventral lateral neurons (s-LNv), and $CRY^+ PDF^-$ dorsal lateral neurons, respectively. The M oscillator regulates the morning bout of activity via the neuropeptide pigment dispersing factor (PDF), and the E oscillator regulates the evening bout of activity through a yet unknown neuropeptide. Under normal circumstances M and E oscillators are believed to talk to each other via a host of neurotransmitters.

gers are (figure 2). Perhaps a more convincing demonstration for the M and E role for the clock neurons can come from genetic ablation studies on 'split' flies (Yoshii *et al*. 2004). These flies under specific environmental conditions of constant light show two bouts of activity, one of which exhibits a period of approximately 22 hours while the other exhibits an approximately 26-hour period. Following ablation of either the M or the E oscillator, these flies would be expected to display only one of the two bouts of activity, exhibiting either a 22-hour or a 26-hour periodicity.

What makes the field of circadian clocks so exciting is our ability to manipulate cellular and molecular cogs and wheels in this complex machinery and observe relatively simple behavioural outputs. Interestingly, clockwatchers, who initially described quite elegantly and elaborately the molecular mechanisms and the players by taking advantage of recent advancements in molecular genetic tools, are now getting interested in the functional significance of these mechanisms. It took almost four decades for them to realize that molecular mechanisms underlying circadian timekeeping machinery should be viewed in the light of the organism as a whole. Now the gap between organismal and molecular-biological views of circadian clocks appears to be getting bridged.

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