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Behavioural Processes 71 (2006) 211–225
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Review

Neural circuits underlying circadian behavior in *Drosophila melanogaster*

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Received 16 June 2005; received in revised form 2 October 2005; accepted 11 December 2005

Abstract

Circadian clocks include control systems for organizing daily behavior. Such a system consists of a time-keeping mechanism (the clock or pacemaker), input pathways for entraining the clock, and output pathways for producing overt rhythms in behavior and physiology. In *Drosophila melanogaster*, as in mammals, neural circuits play vital roles in all three functional subdivisions of the circadian system. Regarding the pacemaker, multiple clock neurons, each with cell-autonomous pacemaker capability, are coupled to each other in a network. The outputs of different sets of clock neurons in this network combine to produce the normal bimodal pattern of locomotor activity observed in *Drosophila*. Regarding input, multiple sensory modalities (including light, temperature, and pheromones) use their own circuitry to entrain the clock. Regarding output, distinct circuits are likely involved for controlling the timing of eclosion and for generating the locomotor activity rhythms. This review summarizes work on all of these circadian circuits, and discusses the broader utility of studying the fly's circadian system.
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Keywords: Circadian; *Drosophila melanogaster*; Eclosion; Entrainment; Locomotor activity; PDF

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doi:10.1016/j.beproc.2005.12.008

1. Introduction

1.1. The circadian clock

Every animal has a collection of neural circuits to generate its various behaviors. Clearly, it would be maladaptive for an animal to initiate a behavior at the wrong time (e.g. sleeping when all its rivals and potential mates are courting), or to initiate two incompatible behaviors at once (e.g. food foraging and sleeping). Therefore, an animal needs control systems to organize and optimize its behavior according to its environment and internal dynamics. The circadian clock is one such system.

Circadian clocks are biochemical entities that control the daily timing of physiological processes and behaviors as diverse as food foraging, sleep, and courtship (reviewed by Saunders, 1982; Dunlap et al., 2003), thus giving rise to the circadian rhythms seen in virtually all animals. As in other fields, the study of circadian biology occurs at many levels, from molecules, to cells, to circuits, to overt behaviors. Behavioral analysis first revealed the clock's existence and many of its properties (reviewed by Dunlap et al., 2003). Molecular analysis has revealed that circadian clocks are constructed from the actions of genes and proteins, forming intracellular, transcriptional feedback loops within "clock cells" (reviewed by Stanewsky, 2003).

The circadian system is most poorly characterized at the level of neural circuits. Although clock neurons important for circadian behavior have been identified and their anatomical projections crudely described (reviewed by Hall, 2003, 2005; Lee et al., 2003), the details of circadian clock circuitry remain mysterious. In order to regulate multiple behaviors, clock neurons must connect with the neural circuits governing those behaviors. Yet even the neurons immediately downstream of clock neurons are largely uncharacterized in any species.

The fly *Drosophila melanogaster* is an outstanding model organism for studying many areas of biology, including circadian rhythms (the history of which was chronicled by Weiner, 1999). There is a vast and still growing store of knowledge on *Drosophila* genetics, development, anatomy, physiology, and behavior. Moreover, researchers have access to powerful genetic and non-genetic tools for studying *Drosophila* (e.g. Duffy, 2002; Hall, 2005). The analysis of circadian circuits in *Drosophila* aims to bridge the gap between cells and behavior and shed light on the fundamental biology of behavior.

1.2. Organization of the circadian system in Drosophila

The circadian system is traditionally divided into three functional components: (1) the circadian clock itself (also called the pacemaker), responsible for generating an oscillation with a period of approximately 24 h, (2) input pathways responsible for synchronizing (or entraining) the pacemaker to external signals such as light, and (3) output pathways that enable the clock to produce overt rhythms in behavior and physiology (reviewed by Dunlap et al., 2003). Note that these functional subdivisions do not always correspond to anatomically separate structures. For example, one of the photoreceptors important for light input in *Drosophila* is a protein (CRYPTOCHROME, or CRY) found within most pacemaker neurons (reviewed by Hall, 2003, 2005). Thus an input pathway may be found in the same cells as the clock mechanism (although, as will be discussed later, the fly also possesses photoreceptors that are external to pacemaker neurons).

The basic clock mechanism is thought to be cell-autonomous (i.e. it does not rely on cell-to-cell communication). This is certainly true in single-celled organisms (reviewed by Lakin-Thomas and Brody, 2004) and has been demonstrated for dissociated snail neurons and dissociated vertebrate neurons in culture (e.g. Michel et al., 1993; Welsh et al., 1995). Furthermore, in both mammals and flies, the pacemaker mechanism deduced from genetic and biochemical work consists of transcriptional feedback loops and other intracellular regulatory pathways (reviewed by Stanewsky, 2003).

Some organisms possess a circadian system composed of a single cell; this circadian cell employs a cell-autonomous clock mechanism and input pathway and produces chemical outputs. This is true for unicellular organisms, of course (again, see Lakin-Thomas and Brody, 2004), and it is conceivable that some invertebrates may have cell-autonomous clocks. In support of this possibility, it has been found that individual basal retinal neurons of the marine snails *Bulla* and *Aplysia* are capable of circadian oscillation, entrainment, and output (via action potential-driven neurotransmitter release) (reviewed by Block et al., 1996). However, it remains likely that neural circuits play an important role in *Bulla* and *Aplysia* circadian rhythms. In any case, animals as diverse as flies and vertebrates possess a circadian system that is fundamentally multicellular; neural circuits remain essential to the production of normal circadian rhythms (Hall, 2005; Reppert and Weaver, 2001).

Therefore, to understand the circadian system, one must examine the cells involved and how they connect to form circuits. As mentioned above, many (perhaps most) of the cellular players in these circuits are unknown. The identity of pacemaker neurons, at least, are known, since they express (almost by definition) the genes involved in the circadian transcriptional feedback mechanism, such as *period* (*per*) and *timeless* (*tim*). In the adult *Drosophila* brain, there are a few dozen such pacemaker neurons, arranged in at least six bilaterally paired clusters. These clusters are named according to their anatomical location (and size): three clusters of dorsal neurons (DN1, DN2, and DN3), the dorsal lateral neurons (LN_d), the large ventral lateral neurons ($l\text{-}LN_v$), and the small ventral lateral neurons ($s\text{-}LN_v$). A seventh pair of neuron clusters, the lateral posterior neurons (LPN), also expresses the PER and TIM proteins and could prove to have pacemaker function, although this remains unknown (Kaneko and Hall, 2000; Helfrich-Förster, 2005; see Fig. 1).

This review discusses the functional connections among these pacemaker neurons, and their likely connections with input and output pathways.

2. Locomotor activity in *Drosophila*

Before grappling with circuit issues, it is important to introduce the behavioral assay that is used almost universally as the

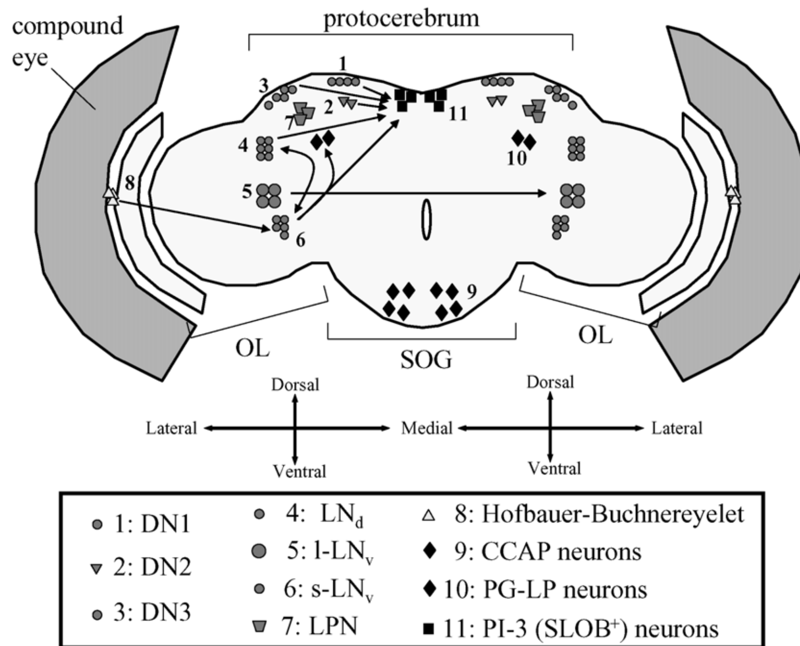

Fig. 1. Neurons in the adult *Drosophila* brain that have been implicated in circadian behavior. Six bilateral sets of brain neurons (numbered "1"–"6" on one side only) show rhythmic expression of the clock genes *period* and *timeless*: "1", "2", and "3" indicate three clusters of dorsal neurons (DN1, DN2, and DN3), "4" indicates the dorsal lateral neurons (LN$_d$s), "5" indicates the large ventral lateral neurons (l-LN$_v$s), and "6" indicates the small ventral lateral neurons (s-LN$_v$s). "7" indicates the lateral posterior neurons (LPN), which express PERIOD and TIMELESS and could be additional pacemaker cells. The s-LN$_v$s receive some (but not all) entrainment signals from "8" the Hofbauer–Buchner eyelet in the optic lobe. Neurons involved in the circadian timing of eclosion are also shown. "9" indicates the crustacean cardioactive peptide (CCAP)-expressing neurons of the suboesophageal ganglion; these cells show rhythmic expression of the RNA-binding protein LARK. The s-LN$_v$s seem to project to PG-LP neurons ("10"), which in turn project to the prothoracic gland (not shown), another key regulator of eclosion. At least some s-LN$_v$s, LN$_d$s, and DNs send projections to the dorsal medial protocerebrum; this is therefore a likely site for neurons important for circadian locomotor behavior. Within this region (specifically in the pars intercerebralis) are the PI-3 neurons ("11"), which express SLOB. The dorsal–ventral axis and the medial–lateral axis for the brain are shown. The optic lobes (OL), suboesophageal ganglion (SOG), and protocerebrum are labeled.

basis for assessing circadian function. This is the measurement of locomotor activity rhythms, which could be considered to be the *Drosophila* equivalent of sleep-wake cycles (reviewed by Shaw, 2003).

In essence, an individual fly is placed in a glass tube. The tube is plugged at one end with food and at the other end with cotton or some other porous material. The tube is placed in an apparatus that shines an infrared beam through the middle of the tube. A computer records every time the fly breaks the beam as it walks back and forth within the tube. This apparatus (and all the tubes it holds) may be placed in various light–dark cycles and at different temperatures to study fly behavior under those conditions (reviewed by Klarsfeld et al., 2003; Hall, 2005).

Measured in this way, *Drosophila*'s locomotor activity is rhythmic with what is sometimes called a crepuscular pattern of locomotion, having two peaks: one around "dawn" (usually an artificially set lights-on time) and one around "dusk" (lights-off) (reviewed by Klarsfeld et al., 2003). During daylight hours between dawn and dusk, there is a low level of activity (Klarsfeld et al., 2003); at night, these flies tend to enter a sleep-like rest state (Hendricks et al., 2000; Shaw et al., 2000).

It should be noted that in a light–dark cycle (LD), rhythmic activity may arise from the circadian system and/or as a direct response to the cyclic environmental conditions. Behavior of the latter category is called "masking" (reviewed by Dunlap et al., 2003), since such environmentally triggered rhythms may disguise the pattern (or absence) of circadian-driven behavior. Because of masking, one can only conclude that a rhythm is circadian if it persists under constant environmental conditions (e.g. constant darkness, which chronobiologists call "DD"). In *Drosophila*, however, the morning/evening activity peaks make it possible to detect circadian contributions to behavior even in LD, because the rise in activity leading to a peak occurs before the light–dark or dark–light transition. This anticipation of dawn or dusk requires some kind of timing system, and study of circadian clock gene mutants has revealed that the circadian clock is responsible. Of course, activity rhythms also persist in DD (e.g. Klarsfeld et al., 2003), confirming that the fly's locomotor activity is clock-regulated.

The locomotor activity assay described above is admittedly artificial. Thus, while it has the advantages of being convenient, reproducible, and easily analyzed, one might wonder whether it accurately reflects the fly's behavioral patterns in the wild. Although this is a valid concern for *Drosophila*'s behavioral ecology, I would argue that it is of little relevance to the fly's neurobiology. The behavior obtained by this artificial assay is driven by neurons and neural circuits in the fly, and the assay is therefore helpful in dissecting those pathways.

3. Pacemaker circuits

3.1. Coupled clocks

Beginning with studies of *per*, the first clock gene discovered (Konopka and Benzer, 1971), the expression pattern of clock genes and proteins suggested the existence of multiple circadian clock neurons. PER expression is found in compound eye photoreceptors, dozens of brain neurons, hundreds of glial cells, and diverse tissues outside the central nervous system (Liu et al., 1988; Siwicki et al., 1988). Likewise, the clock gene *timeless* (*tim*) is expressed in a similar (but not exactly overlapping) set of neurons, glia, and peripheral tissues (Kaneko and Hall, 2000). Both *per* and *tim* are rhythmically expressed in the six previously mentioned groups of clock neurons (DN1, DN2, DN3, LN_d, l-LN_v, and s-LN_v; reviewed by Hall, 2003), and possibly in the seventh as well (LPN; Kaneko and Hall, 2000). These intracellular molecular rhythms are among the main criteria for designating those cells as clock neurons (reviewed by Hall, 2003). If these multiple clock neurons control the same behavioral output, then cross-communication would almost certainly be necessary.

Neuroanatomical studies further support this near certainty, as labeling of the neurites of LN_vs show that each cluster of l-LN_vs sends projections to the vicinity of the contralateral l-LN_vs (e.g. Helfrich-Förster, 1995; Park et al., 2000). This labeling used antibodies that recognized pigment-dispersing factor (PDF), a neuropeptide present throughout the cytoplasm of the LN_vs (all of the l-LN_vs and four of five s-LN_vs) (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995; Park et al., 2000).

PDF is more than just a label, of course; it has profound functional importance as well. Ectopic expression of PDF in the brain using transgenes disturbs behavioral rhythms, often leading to arrhythmicity in DD (Helfrich-Förster et al., 2000). Moreover, flies with a null mutation in *Pdf* (Pdf^{01}), or transgenic flies in which these neurons were specifically ablated, gradually become arrhythmic over a few days in DD (Renn et al., 1999). In wild-type flies, PDF shows a circadian rhythm in abundance in neuronal terminals in the dorsal brain, suggestive of circadian-regulated peptide release (Park et al., 2000).

Since PDF would seem to be a peptide transmitter, it is likely important for mediating circadian output. That is, the PDF-positive (PDF^+) LN_vs likely regulate locomotor activity by synaptic signaling using PDF (see Section 5, below). However, PDF is also involved in cross-communication among clock neurons. In Pdf^{01} flies, various molecular circadian rhythms (particularly rhythms in abundance of clock gene RNA) in all clusters of clock neurons lose amplitude over 4 days in DD (Peng et al., 2003; Lin et al., 2004). The LN_vs seem to undergo phase dispersal; they grow increasingly desynchronized with each other in DD (Lin et al., 2004). Furthermore, the PER rhythms in LN_ds show a phase advancement relative to LN_vs (Lin et al., 2004). These data reflect coupling of the LN_vs with other LN_vs and with LN_ds and perhaps certain DNs. Moreover, the data suggest that coupling is required for synchronization of clock neurons and is at least partially dependent on PDF. This factor is likely to act directly on at least some clock neurons, since a PDF binding assay (with biotinylated PDF) shows binding in l-LN_vs and DN3 neurons, among other brain areas (Peng et al., 2003).

The required synchronization of clock neurons may explain the observation that electrical silencing of pacemaker neurons (using transgenes driving potassium channel expression in all PDF^+ cells) abolishes not only behavioral rhythms but also molecular rhythms in LN_vs (Nitabach et al., 2002). A similar study showed that potassium channel misexpression could also abolish molecular rhythms in larval clock neurons (Nitabach et al., 2005). The initial interpretation of these results was that electrical membrane activities are integral parts of the intracellular pacemaker mechanism (Nitabach et al., 2002, 2005). However, it may be that the LN_v clocks do not actually stop when the cells are electrically silenced, but rather become desynchronized. The molecular rhythms were measured by immunocytochemistry, which requires sacrificing different flies for each timepoint. Desynchronization of molecular rhythms could resemble arrhythmicity. Alternatively, the disruption of molecular rhythms could be due not to simple desynchronization, but rather to a more complex alteration of the circadian neural network. These re-interpretations of the Nitabach et al. (2002, 2005) studies are purely speculative and remain to be tested.

While on the subject of re-evaluating data from a multi-oscillator perspective, consider a recent study characterizing a new mutation in the *tim* gene, tim^{blind}. Flies with this mutation have abnormal locomotor activity rhythms but normal rhythms in eclosion, the emergence of the adult fly from metamorphosis (Wülbeck et al., 2005). However, it remains unknown how this mutation produces its effect. The original proposal is that *tim*, in addition to serving as a core pacemaker component, is also an output factor in a pathway specific for locomotor activity (Wülbeck et al., 2005). The presence of multiple coupled clock neurons offers an alternative explanation: perhaps different pacemaker cells make different contributions to eclosion and locomotor activity rhythms, and tim^{blind} affects pacemaker function to different degrees in different clock neurons. A similar situation appears to exist in wild-type flies for rhythms in oviposition (egg-laying) and eclosion, since these behaviors have different circadian periods in DD, a situation that could only occur in a multi-oscillator system (Paranjpe et al., 2004).

In summary, the adult *Drosophila* brain possesses six (or seven) distinct clusters of pacemaker neurons, and these neurons form a coupled network that together produce the fly's daily pattern of locomotor activity. PDF, probably a peptide transmitter of the LN_vs given that rhythmic secretion of PDF appears to occur at LN_v axon terminals (Park et al., 2000), plays a key role in the cross-communication among clock neurons. Studies of circadian neurobiology in *Drosophila* have largely focused on the PDF^+ LN_vs, perhaps because the relatively restricted expression of *Pdf* in those cells has provided both a convenient cellular label and a means for targeted transgene expression (e.g. Park et al., 2000). It is becoming increasingly clear, however, that other cells should not be neglected, since the PDF^+ LN_vs are neither necessary (e.g. Blanchardon et al., 2001) nor sufficient (e.g. Peng et al., 2003) for some degree of circadian locomotor behavior.

But what function is served by forming this network of clock neurons? One could imagine that this network merely provides amplification or improved stability of a clock output signal. However, the most current evidence suggests that different components of the network regulate different aspects of behavior.

3.2. Morning and evening oscillators

In 1976, chronobiologists Pittendrigh and Daan proposed that the circadian clock of nocturnal rodents involves a coupling of two oscillators, one for morning activity (near dawn) and one for evening activity (near dusk). The initial basis for postulating two oscillators was the phenomenon of "splitting", in which an animal's activity pattern, under the right conditions, could divide into two components, each with a different circadian period. Moreover, the activity pattern of nocturnal rodents (such as hamsters) include morning and evening activity peaks, and in LD these peaks occurred around lights-on and lights-off, respectively, regardless of the day-length (photoperiod). In other words, the interval between the peaks changed with changing photoperiod. Distinct (but coupled) morning and evening (M and E) oscillators could provide a simple mechanism for adapting to different photoperiods: the morning oscillator would entrain to the lights-on signal, while the evening oscillator would entrain to the lights-off signal (Pittendrigh and Daan, 1976).

Later work revealed that splitting in hamsters was due not to M and E oscillators, but rather to desynchrony of the left and right suprachiasmatic nuclei (SCN) (de la Iglesia et al., 2000). The SCN are generally acknowledged to be the site of the circadian clock(s) controlling most mammalian circadian rhythms (reviewed by Reppert and Weaver, 2001). In normal situations (e.g. a conventional LD cycle), the left and right SCN are synchronized in their molecular and physiological rhythms. The left and right SCN do not serve as M and E oscillators.

However, this does not completely invalidate Pittendrigh and Daan's (1976) idea. M and E oscillators may still exist in mammals, composed of subsets of cells imbedded in each SCN. These yet-to-be-discovered oscillator units may not have anything to do with splitting, but they could still play a role in adapting behavior to suit changing photoperiods.

In *Drosophila* the concept of M and E oscillators has recently been shown to explain the bimodal pattern of locomotor behavior found in this fly. Independent studies suggest that the evening and morning peaks in activity are controlled predominantly by separate sets of pacemaker neurons.

One study used transgenic expression of pro-apoptotic genes, *reaper* or *head involution defective* (*hid*), to ablate specific sets of clock neurons. Ablation of cells that were PDF^+ (all but one pair of LN_vs) abolished anticipation of dawn in LD, but did not affect anticipation of dusk. Conversely, ablation of cells that were CRY^+ but PDF^- (DNs, LN_ds, and the PDF^- LN_v) did not affect dawn anticipation, but abolished dusk anticipation (Stoleru et al., 2004).

A different study used flies with a null mutation in the *per* gene (*per*^{01}); transgenes were used to rescue (otherwise impaired) *per* expression (and thus clock function) in different subsets of clock neurons (Grima et al., 2004). This was done using the well-established GAL4-UAS system, which uses a pair of transgenes: one expresses the transcriptional activator GAL4 using any desired promoter, while the other uses GAL4-specific upstream activating sequences (UAS) in its promoter to drive a gene of interest (reviewed by Duffy, 2002), in this case (wild-type) *per*. When *per* expression was rescued only in PDF^+ LN_vs, dawn anticipation was restored but not dusk anticipation. When only s-LN_vs and LN_ds were rescued, anticipation of both dawn and dusk was restored (Grima et al., 2004).

Together, these two studies point to LN_vs (especially s-LN_vs) as the cells that drive the morning peak (and thus anticipation of dawn), while LN_ds (and perhaps other cells) control the evening peak (and thus anticipation of dusk). The role of PDF^+ cells in regulating the morning activity peak is supported by earlier studies as well. Ablation of LN_vs using the pro-apoptosis gene *bax* abolishes dawn anticipation (Blanchardon et al., 2001). Blockade of neurotransmission in LN_vs using tetanus toxin may reduce dawn anticipation (Kaneko et al., 2000), but not consistently (Blanchardon et al., 2001). Similarly, *Pdf*^{01} mutant flies show little or no anticipation of dawn, but robust anticipation of dusk (Renn et al., 1999).

The tidiness of this story is upset somewhat by the finding that if a *per* transgene is used in *per*^{01} flies to rescue all neurons *except* the PDF^+ cells, then anticipation of both dawn and dusk remains intact. In other words, the disruption of clock function selectively in PDF^+ neurons does not prevent dawn anticipation, even though the morning peak is supposedly driven by those cells (Stoleru et al., 2004). This remains to be explained conclusively, but it could reflect the coupling that is known to exist among the clock neurons. Perhaps the *per*-null LN_vs regain at least partial circadian function because they receive signals from functional clock neurons in the network (Stoleru et al., 2004).

The presence of M and E oscillators (in the form of LN_vs and LN_ds [plus perhaps DNs and PDF^- LN_vs], respectively) in *Drosophila* is now well supported. However, it remains unknown how different clock neurons entrain to different aspects of the LD cycle. This could reflect a difference in the light input mechanism to different sets of clock neurons, a difference yet to be fully detailed. Moreover, what molecular/cellular differences within M and E neurons might account for their distinct functions? Part of the answer has been found with the aid of flies with a hypomorphic mutant form of the photoreceptor protein *cry* (*cry*^{b}). These flies, under certain conditions, show splitting of locomotor activity: two behavioral components with different periods (Yoshii et al., 2004). Immunohistological analysis (of PER and TIM) revealed that s-LN_vs in these flies have short-period molecular rhythms while LN_ds and other neurons have long-period molecular rhythms (Yoshii et al., 2004). These period differences are consistent with their roles as M and E oscillators, as predicted by the Pittendrigh–Daan model. In other words, differential light responses in the LN_vs versus LN_ds may result in different period lengths, M and E activity peaks and, possibly, photoperiod adaptation. However, these hypotheses demand further testing. In particular, it remains to be demonstrated whether the M and E cellular oscillators actually enable the fly to adapt to changing photoperiods, as originally proposed by Pittendrigh and Daan (1976).

It is worth mentioning that a recent study has suggested a different model for photoperiodism in *Drosophila*, in which photoperiod responses depend on intracellular molecular mechanisms involving the *tim* gene (Shafer et al., 2004). Of course, a multicellular mechanism involving M and E oscillators and a *tim*-dependent intracellular process are not mutually exclusive; both may contribute to the fly's photoperiod adaptation.

One observation worth noting is that some of the studies above used different combinations of transgenes to target different subsets of clock neurons. For example, in order to ablate CRY^+ PDF^- cells, Stoleru et al. (2004) used a *cry-gal4* transgene to drive UAS-*hid* (or *reaper*) in clock neurons, and *Pdf-gal80* to block UAS-*hid* expression in PDF^+ cells. The combinatorial use of transgenes (driven by different fly promoters) is valuable because most cell types are distinguished from other cell types not by a single molecular marker, but rather by a unique combination of expressed genes. Thus, as *Drosophila* geneticists continue to dissect neuronal circuits (or more generally attempt to target more specific cell types), the use of multiple-transgene combinations is likely to become increasingly common.

3.3. The dorsal neurons

A simplified view of the M and E oscillator story would be that LN_vs drive the M peak while LN_ds drive the E peak. However, this simplification ignores the contributions of the DNs, which seem to play a role in E activity even in the studies already discussed (Stoleru et al., 2004).

The importance of DNs is also supported by an earlier study involving a luciferase transgene (Veleri et al., 2003). Luciferase (LUC) is an enzyme derived from the firefly beetle, *Photinus pyralis*, that produces light by oxidizing its substrate, luciferin (de Wet et al., 1987). The enzyme, substrate, and reaction are all non-toxic to *Drosophila*; thus transgenic luciferase expression allows the measurement (via the light produced) of luciferase activity in living flies (Brandes et al., 1996). Fusing LUC with PER allows real-time tracking of the abundance of the modified PER protein, albeit indirectly (Stanewsky et al., 1997); importantly, the addition of luciferase to the C-terminus of PER does not seem to impair PER's circadian functions (Veleri et al., 2003). Thus, while the usual histological or biochemical methods of assaying molecular rhythms, in which an individual brain could only provide data for one timepoint, require multiple brains to assess a circadian rhythm, the PER–LUC transgene product provides a molecular readout of clock function from single living brains.

Veleri et al. (2003) expressed a promoterless transgene encoding a fusion protein of PER and luciferase in per^{01} flies. This transgene expresses in DNs and occasionally LN_ds, but not in LN_vs, thus rescuing clock function only in a subset of pacemaker neurons (Veleri et al., 2003). Although the DN-restricted expression of PER–LUC is not sufficient to produce robust behavioral rhythms in DD in a per^{01} genetic background, it does restore anticipation of the E peak in LD (Veleri et al., 2003).

The flies with DN-restricted PER–LUC expression display both robust bioluminescence rhythms and behavioral anticipation of lights-off in LD. However, as mentioned above, these flies are behaviorally arrhythmic in DD. Thus the DNs seem to contribute to the evening peak of the locomotor activity rhythm, but are insufficient to drive behavioral rhythms on their own (Veleri et al., 2003).

The data discussed above also show that the DNs are bona fide pacemaker cells, as opposed to being cells whose molecular rhythms are driven by LN_vs and/or LN_ds. This conclusion is further supported by studies of *disconnected* (*disco*) mutant flies. The *disco* gene encodes a zinc finger transcription factor involved in various developmental processes; the *disco* mutation causes numerous neuroanatomical defects, including hypomorphic optic lobes (Heilig et al., 1991; Lee et al., 1991). By preventing the development or maintenance of LN_vs and LN_ds (Helfrich-Förster, 1998), the *disco* mutation disrupts behavioral circadian rhythms (Dushay et al., 1989; Dowse et al., 1989; Helfrich-Förster, 1998). However, DN3s are present in *disco* flies, and continue to produce molecular oscillations even in the absence of LN_vs and LN_ds (Veleri et al., 2003).

Among the DNs, the DN2s deserve special mention because in DD their molecular clock oscillates in antiphase to that of every other pacemaker neuron in the brain (Veleri et al., 2003). It is unclear, however, what function these "antiphase" cells perform. Nevertheless, an important function is likely because in the brains of larvae there are also DNs that oscillate in antiphase (Kaneko et al., 1997). This desynchronization is made possible by the lack of CRY expression in the larval antiphase DNs, allowing them to entrain to the environment in a completely different way from other cells (Klarsfeld et al., 2004). These larval DNs could be the DN2s at an earlier stage in development; if so, the DN2s would be among the few clock cells that do persist through fly development, perhaps an indication of their value. s-LN_vs (but not l-LN_vs) also have larval equivalents, expressing both PDF and core pacemaker genes like *per* (Helfrich-Förster, 1997).

3.4. Overlapping circadian and non-circadian circuits

The pacemaker neurons discussed above are usually treated as though they were dedicated for circadian function. However, there is evidence in both larvae and adults that the same neurons that regulate circadian behavior may also be components of neural circuits involved in non-circadian functions.

In larvae, the PDF^+ pacemaker neurons (which will later become the PDF^+ s-LN_vs) receive light input (in part) from Bolwig's organ (the larval eye). Thus light information from Bolwig's organ seems to be used to entrain the clock and thus regulate the timing of larval circadian behavior (e.g. Malpel et al., 2002). However, Bolwig's organ also feeds into circuits controlling the larva's light avoidance behavior, or photophobicity (Sawin-McCormack et al., 1995). Recently it was discovered that both the circuit controlling photophobicity and the circuit controlling circadian behavior include the PDF^+ pacemaker cells (Mazzoni et al., 2005). Ablation of the PDF^+ cells (using diphtheria toxin) causes the same abolishment of photophobicity as ablation of the Bolwig's organ itself (using *hid*). A similar disruption of the photophobic behavior is seen in per^{01} and tim^{01} mutants, but not in cyc^0 or Clk^{Jrk} mutants, even though

all of these mutations disrupt circadian clock function. Thus, while the photophobic behavior depends on the PDF$^+$ pacemaker cells, it does not require their time-keeping function to remain intact. However, the photophobic response does vary over the course of a day in wild-type flies, indicating that the clock regulates this acute light response (Mazzoni et al., 2005). This also neatly explains why some clock mutants affect the photophobic response while others do not: some mutations (i.e. *per^{01}* and *tim^{01}*) "jam" the clock in a state resembling daytime, which is associated with reduced photophobicity, while others (i.e. *cyc^{0}* and *Clk^{Jrk}*) jam the clock in a night-like state, associated with strong photophobicity (Mazzoni et al., 2005). It should be reiterated, however, that while the clock cells regulate photophobic behavior, they also play another role, as a component of the photophobicity circuit itself, transmitting light information from Bolwig's organ to downstream neurons in the acute light response (Mazzoni et al., 2005).

In adults, there is overlap between circadian circuits and those involved in the fly's response to cocaine. A variety of circadian gene mutations (e.g. *per^{01}*, *Clk^{Jrk}*, and *cyc^{01}*) cause a defect in cocaine sensitization (the increase in severity of drug response due to prior exposure to the drug), while a few (e.g. *tim^{01}*) do not (Andretic et al., 1999). Similarly, some circadian gene mutations (e.g. *per^{01}*) cause an increase in cocaine sensitivity (dose-dependent responsiveness to the drug without prior exposure), while others (e.g. *Clk^{Jrk}* and *cyc^{01}*) do not (Andretic et al., 1999). All of the mutations just mentioned (*per^{01}*, *Clk^{Jrk}*, *cyc^{01}*, and *tim^{01}*) virtually eliminate circadian clock function, and yet they affect *Drosophila*'s cocaine responses in different ways. It thus appears as though normal cocaine responses are dependent on circadian clock genes without being dependent on a functioning circadian clock (Andretic et al., 1999).

It now appears that this is true because some circadian clock cells, namely the LN_vs, play a role in cocaine response circuits (Tsai et al., 2004). This discovery involved the study of flies with mutations in the *LIM-only* (*Lmo*) gene, a presumed transcription factor involved in development. Loss-of-function mutations in *Lmo* increases the fly's sensitivity to cocaine (i.e. these flies display aberrant walking patterns or akinesia [no walking at all] at lower doses of cocaine), while overexpression of *Lmo* reduces sensitivity to cocaine. The loss-of-function *Lmo* phenotype is rescued by expression of wild-type *Lmo* only in PDF$^+$ neurons, whereas overexpression of *Lmo* only in PDF$^+$ neurons (in otherwise wild-type flies) causes reduced sensitivity to cocaine, mimicking the ubiquitous overexpression of *Lmo*. Furthermore, ablation (via *hid* expression) or blockade of synaptic transmission (via tetanus toxin light chain) of PDF$^+$ neurons causes reduced cocaine sensitivity. Although there are no published histological studies showing that LN_vs normally express *Lmo*, these genetic experiments support that hypothesis. Loss-of-function *Lmo* mutations also disrupt circadian locomotor activity rhythms, causing a higher proportion of arrhythmicity in DD. However, the acute effects of cocaine do not vary over the course of a day and do not depend on PDF. Thus, although PDF$^+$ LN_vs appear to be involved in cocaine sensitivity, this acute behavioral drug response is not a circadian output and does not seem to utilize the same neural circuitry (Tsai et al., 2004). Circadian clock proteins (or their absence) within LN_vs may affect cocaine responses not through a circadian mechanism, but through a process (involving *Lmo*) that affects the LN_vs function in the cocaine-response circuitry.

4. Input circuits

4.1. Entrainment to light

I have thus far discussed the various clock neurons in the brain, how they are coupled and contribute in different ways to locomotor activity rhythms, and how PDF$^+$ pacemaker cells are also components of circuits regulating non-circadian behaviors. Attention is now turned to factors involved in entraining the clock neurons. Light is the most powerful and most obvious (at least to highly visual primates such as ourselves) timing cue for entrainment. Thus let us consider how clock neurons sense light.

As mentioned previously, many of the adult fly brain's pacemaker neurons express an intrinsic photoreceptor, CRY. Flies with the *cry^{b}* hypomorph mutation show clear abnormalities in their behavioral responses to light, as do flies overexpressing CRY protein (e.g. Stanewsky et al., 1998; Emery et al., 1998; Egan et al., 1999; reviewed in more detail by Hall, 2005). If that were the whole story, then no special input circuits would be necessary.

However, the circadian clocks of *cry^{b}* flies are still able to entrain to light. One might argue that because *cry^{b}* is not a null allele (Stanewsky et al., 1998), residual CRY function could explain this ability. However, photic entrainment can be much more severely disrupted by combining *cry^{b}* with mutations affecting the development or function of compound eyes and/or other photoreceptors, indicating that the primary reason that *cry^{b}* flies can entrain is because they possess additional photic input pathways that do not require CRY. One such mutation is *norpA^{P41}*, a null allele of the phospholipase C-encoding gene involved in rhodopsin signal transduction (Stanewsky et al., 1998). Another is *glass^{60j}* (Helfrich-Förster et al., 2001), a loss-of-function allele of a gene encoding a zinc finger transcription factor. The *glass^{60j}* mutation causes disorganization of compound eye photoreceptors and the absence of the Hofbauer–Buchner eyelet (Hofbauer and Buchner, 1989), which is an extraretinal cluster of rhodopsin-containing neurons that is derived from Bolwig's organ and that projects to the s-LN_vs (Helfrich-Förster et al., 2002). Notably, the *glass^{60j} cry^{b}* combination abolishes photic entrainment more completely than the *norpA^{P41}*; *cry^{b}*; combination, suggesting that there exists a circadian photoreceptor whose function is abolished by the *glass^{60j}* mutation but not the *norpA^{P41}* mutation (Helfrich-Förster et al., 2001; Malpel et al., 2002; Mealey-Ferrara et al., 2003). The identity of this photoreceptor remains unknown.

These and other studies (reviewed in greater detail by Hall, 2005) indicate that the circadian system in adult flies receives light input from several sources: CRY, the compound eyes, the ocelli, the Hofbauer–Buchner eyelet, and perhaps other, unidentified photoreceptors. CRY operates cell autonomously, but the other photoreceptive pathways involve neural circuits that are distinct for each photoreceptor. These various photic input path-

ways are semi-redundant, but they are not exactly equivalent. For example, the compound eyes seem to play a more prominent role in entrainment to extreme photoperiods (Rieger et al., 2003). Furthermore, the circuits likely provide different signals to different pacemaker neurons, as suggested by the fact that the DN2s oscillate in antiphase to LN_vs and other pacemaker cells (Veleri et al., 2003; Klarsfeld et al., 2004).

Thus circuits exist connecting the compound eyes, ocelli, and Hofbauer–Buchner eyelet to the pacemaker cells, allowing those cells to entrain to light–dark cycles even in the absence of functional CRY (e.g. Stanewsky et al., 1998). While some of the anatomy of these circuits is known (e.g. the projections of both the compound eye photoreceptors and l-LN_vs into the medulla of the optic lobe), much work is needed to characterize the functional dynamics of light entrainment through these circuits.

4.2. Non-photic entrainment

Light is not the only modality that can entrain the clock. Non-photic entrainment, unless cell autonomous, obviously requires different circuits than those conveying information from the eyes or other photoreceptive structures.

The best studied non-photic entrainment signal is temperature. It has long been known that temperature cycles can entrain circadian clocks (e.g. Wheeler et al., 1993 and references therein). In *Drosophila*, one might suppose that clock neurons receive temperature information from the antennae, which are the site of thermosensors involved in detecting spatial thermal gradients or non-nociceptive heat intensities (Sayeed and Benzer, 1996; Zars, 2001). However, a recent study discovered that the antennae are in fact dispensable for thermal entrainment of circadian behavior. Physical removal of the antenna or use of a genetic mutation that eliminates thermal gradient sensing (*bizarre*, or *biz*) does not affect circadian entrainment to thermal cycles (Glaser and Stanewsky, 2005). Two mutations do impair circadian thermal entrainment: $norpA^{P41}$ and a novel allele of a yet unidentified gene, *nocte* (Glaser and Stanewsky, 2005).

Future studies will no doubt clarify the nature of the non-antennal circadian thermosensor and any circuits involved. It should be noted that while the antennae are dispensable, they could still make a contribution to thermal entrainment. This argument is based on analogy with photic entrainment (discussed above): elimination of the compound eyes does not prevent photic entrainment (reviewed by Hall, 2003); nevertheless the compound eyes do contribute to circadian light responses (e.g. Rieger et al., 2003). Following this analogy, a circuit could exist connecting the antennal thermosensors to circadian pacemakers.

There is another modality for which antennal involvement has been established: pheromonal social cues from other flies. Flies monitored individually for locomotor activity show phase dispersal in DD; the slight individual differences in period and phase entrainment cause the flies to become more and more desynchronized (Levine et al., 2002). However, flies that were previously group-housed show less dispersal than flies housed in isolation, suggesting that flies are able to synchronize their clocks. Wild-type flies housed with clock-defective per^{01} mutant flies show increased phase dispersal in locomotor activity in DD, suggesting that signals from the per^{01} flies "confused" the clocks of the wild-type flies (Levine et al., 2002). These signals are olfactory in nature; isolated flies in DD exposed to air from a group of flies in LD show less dispersal than flies exposed to "neutral air" (air from a food bottle lacking flies). Furthermore, mutant flies defective in olfaction ($para^{sbl\text{-}1}$ and $para^{sbl\text{-}2}$) are impervious to the confusing signals produced by per^{01} flies (Levine et al., 2002). It remains unclear, however, how olfactory neurons in the antennae connect with locomotor activity circuits. These olfactory neurons themselves contain a circadian oscillator (Krishnan et al., 1999) that may play an important role in social entrainment. It is unclear how the pacemaker neurons in the brain are involved in this process. Furthermore, it remains possible that tactile and/or auditory cues contribute to social entrainment in addition to olfactory signals (Levine et al., 2002).

4.3. Circuits that modulate the sensitivity of input circuits

Besides circuits that actually convey entrainment cues to clock neurons, there also seem to be circuits that modulate the responsiveness of clock neurons to those cues. Recently, a role for serotonin (also known as 5-hydroxytryptamine or 5-HT) in modulating circadian entrainment has been well demonstrated (Yuan et al., 2005; Hamasaka and Nässel, 2006). Histological studies show that serotonergic neurons send neurites that terminate in close proximity to LN_vs in both larvae and adults (Hamasaka and Nässel, 2006; Yuan et al., 2005). Moreover, LN_vs show immunostaining for the d5-HT1B serotonin receptor, and a transgene driven by the *d5-HT1B* promoter is expressed in LN_vs (Yuan et al., 2005).

Functionally, PDF^+ cells in larvae were shown to be responsive to serotonin in cell culture (Hamasaka and Nässel, 2006). As mentioned earlier in this review, *Drosophila* larvae possess PDF^+ neurons that are the cellular precursors of the adult fly's s-LN_vs. Transgenes can be used to express green fluorescent protein (GFP) specifically in PDF^+ cells. GFP fluorescence may then be used to find the PDF^+ neurons among dissociated cells from the larval brain grown in culture. Once these neurons have been found, they can be injected with a calcium imaging dye (Fura-2) used for measuring the levels of intracellular calcium ions. It turns out that these calcium levels decrease when the larval LN_vs are exposed to serotonin (Hamasaka and Nässel, 2006).

In adult flies, drugs that augment serotonergic neurotransmission (a serotonin precursor, 5-HTP, and a reuptake inhibitor, Prozac) reduce the circadian phase shift in response to a light pulse, as does overexpression of d5-HT1B in clock neurons (Yuan et al., 2005). Meanwhile, reduction of d5-HT1B transcript levels using RNA interference (RNAi) increases circadian photosensitivity (Yuan et al., 2005). Endogenous serotonin levels decrease and circadian photosensitivity increases under DD conditions, apparently as an adaptation to the darkness. It thus seems as though a serotonergic circuit is involved in modu-

lating circadian light responses, allowing the flies to adapt to environments of varying light intensity (Yuan et al., 2005). Serotonin seems to affect circadian photosensitivity by binding the d5-HT1B receptor, which, via second messenger pathways, increases phosphorylation of SHAGGY, a kinase known to play a role in regulating TIM turnover and thus clock function (Yuan et al., 2005; Martinek et al., 2001).

Other neurons, using the neurotransmitter gamma-aminobutyric acid (GABA) may also regulate the LN_vs (Hamasaka et al., 2005). Histologically, neurites from GABAergic neurons and neurites from PDF^+ LN_vs are found in close apposition in both larval and adult brains (Hamasaka et al., 2005). In the same larval LN_v primary culture system described above, GABA, like serotonin, was shown to decrease LN_v calcium levels (Hamasaka et al., 2005). Moreover, GABA was shown to act on larval LN_vs through a specific GABA receptor, $GABA_B$, since the effect of GABA could be mimicked by a specific agonist of $GABA_B$ receptors, and it could be inhibited by an antagonist of $GABA_B$ receptors (Hamasaka et al., 2005). Although the effect of GABA on circadian behavior has not yet been studied, it is likely that GABAergic neurons do modulate LN_vs, at least in *Drosophila* larvae.

Thus LN_vs are modulated by serotonergic neurons via d5-HT1B receptors, and are likely to be regulated also by GABAergic neurons via $GABA_B$ receptors (Yuan et al., 2005; Hamasaka et al., 2005). However, both serotonin and GABA are widely used neurotransmitters, and it remains a challenge to analyze the specific cells involved in modulating circadian entrainment, and determine how they are regulated in turn. Other circuits are likely to play a role as well; histological analysis has also suggested that some DNs might interact with dopaminergic and histaminergic neurons (Hamasaka and Nässel, 2006).

Entrainment in the fly clearly involves a complex arrangement of multiple circuits, from sensory structures of varying modality, possibly possessing intricate interconnections, and with neurochemically diverse regulatory circuits. While genetic, anatomical, and pharmacological work has provided handles for dissecting these circuits, the major part of the circuit dissection remains untouched. A similar assessment may be made for circuits involved in circadian output, i.e. the circuits by which pacemaker neurons produce overt behavioral rhythms.

5. Output circuits

5.1. How do clock neurons control behavior?

Presumably, circadian clock neurons regulate locomotor activity by sending signals to locomotor circuits and sleep regulation circuits. These signals would fall into the broad category of neuromodulation, and could involve both neurohormones and synaptic neurotransmitters.

There is an old study in which transplantation of per^+ brains rescued behavior in per^{01} mutant flies, suggesting that a neurohormone may be involved in circadian control of locomotor activity (Handler and Konopka, 1979). However, the neurohormone involved (if such a molecule exists) remains unidentified.

PDF does not seem to be a candidate for this putative neurohormone, since misexpression of PDF in the main neurosecretory center of the brain (the pars intercerebralis, associated with the main neurohoromal release site, the corpora cardiaca), which would provide easy access of PDF to the hemolymph, does not alter locomotor activity (Helfrich-Förster et al., 2000). Meanwhile, misexpression of PDF in other regions of the brain using various *gal4* transgenic strains did perturb locomotor activity rhythms. When the expression profiles of these *gal4* lines were compared, it was found that two pairs of neurons (that are normally PDF^-) were targeted by all of the lines where behavior was abnormal (Helfrich-Förster et al., 2000). These neurons both send projections to the dorsal brain of the fly. Thus, rather than serving as a neurohormone, it seems likely that PDF acts as a transmitter on specific neural targets in the dorsal brain to regulate locomotor activity. Of course, this does not rule out other brain regions as being important as PDF targets and/or as substrates for circadian control of locomotion (Helfrich-Förster et al., 2000).

Besides PDF, there is evidence that at least one other neuropeptide is involved in controlling locomotor activity rhythms. Most *Drosophila* neuropeptides require C-terminal α-amidation, a process involving the enzyme PHM (Taghert et al., 2001, and references therein). When *phm*-null mutant flies were rescued with various *gal4* lines driving UAS-*phm*, it was found that one such line, c929-*gal4*, rescued viability but locomotor activity remained arrhythmic. The combination of c929-*gal4* and *Pdf-gal4* still did not rescue rhythms, but c929-*gal4* and *tim-gal4* together did rescue rhythms (Taghert et al., 2001). Thus, there is a population of TIM^+ PDF^- neuropeptide-producing cells required for circadian locomotor activity. The identity of the non-PDF neuropeptide(s) involved remains unknown.

It remains unclear what PDF, additional neuropeptides, the putative neurohormone, and other output signals sent by the clock actually do to their targets. How do clock output signals produce rhythms in overt behavior? One simple model would be that PDF either inhibits or stimulates neuronal excitability of its targets. Thus, differential release of PDF (or other signals) over the course of a day would cause daily variation in firing rate of the target neurons. Another possibility is that the target cells themselves contain circadian oscillators, and that output signals from the main pacemaker neurons synchronize or entrain these peripheral clocks.

And peripheral clocks are very widespread. Although the focus of this review is on circadian function in brain neurons, it should be noted that such function is not restricted to neurons or glia. Numerous peripheral tissues (e.g. the wing, legs, and antennae) possess circadian oscillators capable of entraining to light and maintaining rhythmicity even when isolated from the central nervous system (Emery et al., 1997; Hege et al., 1997; Plautz et al., 1997). The question remains: even though these peripheral clocks are capable of running on their own, are they functionally regulated by the brain's pacemakers to regulate behavioral timing? The answer seems to be "yes" in at least one case: the prothoracic gland, an endocrine structure involved in eclosion (Myers et al., 2003; see below).

5.2. Eclosion circuits

Besides locomotor activity, the most frequently assayed circadian behavior in *Drosophila* is the daily timing of eclosion (e.g. Konopka and Benzer, 1971). Although any individual fly can only emerge from its pupal case once in its lifetime, an eclosion rhythm may be observed in a population of pupae whose circadian clocks are (more or less) synchronized; different flies will eclose on different days, but always in the morning. Eclosion involves a sequence of behavioral events, including pre-ecdysis maneuvers (ecdysis refers to the shedding of an old cuticle; it applies to molting as well as eclosion) such as inflation of the head tracheae and extension of the ptilinum to rupture the pupal cuticle, and the eclosion motor program itself, involving head movements and abdominal muscular contractions to propel the body out of the pupal case (summarized nicely by McNabb et al., 1997).

The timing of eclosion is controlled by a cascade of hormones (reviewed by Mesce and Fahrbach, 2002; Ewer and Reynolds, 2002). Ecdysosteroids (like ecdysone) are produced by the prothoracic gland (PG), a component of the ring gland surrounding the heart, to regulate many aspects of metamorphosis. About two days prior to eclosion, the ecdysosteroid levels fall. This triggers secretion of eclosion hormone (EH, a peptide hormone produced by a pair of brain neurons) and ecdysis triggering hormone (ETH, a peptide hormone produced by Inka cells in the tracheae). EH and ETH each stimulate the other's production, resulting in a positive feedback loop and a sharp spike in EH and ETH levels. The effects of these hormones are complex, but ETH seems to be primarily involved in stimulating pre-ecdysis behaviors, while EH stimulates secretion of another peptide hormone, crustacean cardioactive peptide (CCAP), which stimulates ecdysis behaviors. These hormonal events are best characterized in large moths, on which many types of physiological and biochemical studies are easier to perform than on tiny drosophilid flies. However, similar events do seem to take place in regulating *Drosophila* eclosion (reviewed by Mesce and Fahrbach, 2002; Ewer and Reynolds, 2002).

In many insects, the timing of eclosion is restricted to specific times of day, and this restriction (or "gating") of eclosion is controlled by the circadian clock. This is true for silk moths (e.g. Truman and Riddiford, 1970) as well as for *Drosophila* (e.g. Konopka and Benzer, 1971). In populations of animals (originally entrained to the same LD regimen), this gating is seen as a rhythm. The circuits involved have not been thoroughly characterized, but in *Drosophila*, there is evidence that the pacemaker neurons regulate two sets of cells involved in eclosion behavior: PG cells and CCAP neurons.

The PG possesses its own circadian oscillator, with molecular rhythms in PER and TIM levels (although PER rhythms are weak in DD) (Emery et al., 1997; Myers et al., 2003). The functioning of the PG oscillator seems to be essential for eclosion behavioral rhythms, since overexpression of TIM (a treatment that stops clock function—Yang and Sehgal, 2001) specifically in PG cells abolishes eclosion rhythms (Myers et al., 2003). Ablation of PDF$^+$ neurons (either using the *disco* mutation or targeted expression of *hid*) abolish TIM rhythms in the PG, suggesting that LN_vs influence the PG clock (Myers et al., 2003). PDF$^+$ LN_v ablation also cause eclosion rhythms to become arrhythmic, but only after a couple days in DD, suggesting that like locomotor activity, eclosion timing may be regulated by a network of pacemaker cells that become desynchronized in the absence of PDF (Myers et al., 2003). It remains unclear whether pacemaker cells are required to drive the PG oscillator, or whether they synchronize the oscillators ticking in PG cells, allowing the production of a tissue-level oscillation.

In any case, the involvement of LN_vs is further supported by earlier studies. One study showed that overexpression of PER specifically in LN_vs disrupt eclosion rhythms (Blanchardon et al., 2001). Another study showed that at least some s-LN_vs have synaptic terminals in the immediate vicinity of neurons (called PG-LP neurons; see Fig. 1) that project to the PG (Siegmund and Korge, 2001). Thus it is possible that a circuit connects s-LN_vs to PG-LP neurons to the PG, allowing circadian regulation of ecdysosteroid release, which in turn might regulate the timing of other hormones and thus eclosion.

However, this is almost certainly not the only means by which clock neurons control the timing of eclosion. The circadian system also communicates with CCAP neurons, which are found in the suboesophageal ganglion of the brain and in the ventral nerve cord (McNeil et al., 1998; Zhang et al., 2000; see Fig. 1). Ablation of CCAP neurons (using *reaper*) causes defects in both execution and circadian timing of eclosion behaviors, confirming their importance in the eclosion pathway (Park et al., 2003). Moreover, within CCAP neurons, an RNA-binding protein called LARK oscillates with a circadian rhythm (McNeil et al., 1998; Zhang et al., 2000). Although CCAP neurons do not express PER themselves, the LARK oscillation is abolished in *per*^{01} flies, indicating that CCAP neurons receive timing signals from PER$^+$ pacemaker cells (Zhang et al., 2000). The circuit connecting pacemaker cells to CCAP cells remains uncharacterized.

The function of LARK is presumed to be the translational regulation of the mRNA of certain genes. The *Ccap* message does not appear to be one of LARK's targets, since CCAP immunoreactivity is not rhythmic. It is thought that LARK may target a factor that in turn affects CCAP release (Zhang et al., 2000). A null mutation in the *lark* gene causes a phase advance in the eclosion rhythm (in heterozygotes; the mutation is homozygous lethal), without affecting locomotor activity (Newby and Jackson, 1993). Curiously, overexpression of LARK in CCAP neurons also causes a phase advance in eclosion rhythms (Schroeder et al., 2003). Discovering the RNA targets for LARK should clarify its role on controlling CCAP release and eclosion timing.

5.3. Locomotor activity circuits: what are the targets of pacemaker neurons?

As mentioned above, the locomotor activity rhythm in *Drosophila* is roughly equivalent to our sleep–wake cycles. In brief, *Drosophila* engages in a rest state that is (like vertebrate sleep) controlled both by the circadian clock and by a homeostatic system. (Hendricks et al., 2000; Shaw et al., 2000, 2002).

I have already discussed how different pacemaker neurons may contribute to regulating locomotor activity. I now turn to the cells in the locomotor activity circuit that are downstream of the pacemaker neurons. These target cells are likely to reside in the dorsal brain, as suggested by the projection pattern of pacemaker neurons: s-LN_vs, LN_ds, and DNs all send projections to the dorsal brain (reviewed by Hall, 2003, 2005). Also recall that PDF immunoreactivity is rhythmic at nerve terminals in the dorsal brain (Park et al., 2000) and that ectopic expression of PDF in neurons projecting to the dorsal brain causes behavioral abnormalities (Helfrich-Förster et al., 2000). However, a rough idea of the anatomical location of the target cells is not enough to characterize their function. Molecular markers for these cells are needed in order to understand how locomotor activity rhythms are produced.

5.4. Molecules involved in locomotor output

The putative PDF receptor (PDFR) is almost certainly expressed in the cells that are the primary targets of PDF^+ LN_vs. Recently, the gene encoding this receptor has been identified by three independent groups, each of which christened it with a different name: *han* (Hyun et al., 2005), *groom-of-PDF* (Lear et al., 2005), and *pdfr* (Mertens et al., 2005). I will use *pdfr* to refer to the gene, since that is the most intuitive name. Not surprisingly, the encoded protein is a G-protein coupled receptor (GPCR), as is the case for all known neuropeptide receptors (see Hewes and Taghert, 2001). What is the evidence linking this particular GPCR with PDF? First, binding assays in cultured cells show that PDF is indeed a ligand of the PDFR protein (Mertens et al., 2005; Hyun et al., 2005), although other neuropeptides may also activate PDFR (Mertens et al., 2005). Second, disruption of the *pdfr* gene (by retrotransposon insertion or P-element mutagenesis) causes a defect in circadian rhythms resembling the Pdf^{01} mutation (Lear et al., 2005; Hyun et al., 2005; Mertens et al., 2005), although there are other, non-circadian phenotypes as well, including alteration in temperature preference (Hyun et al., 2005) and geotaxis behavior (Mertens et al., 2005). Finally, the expression pattern of PDFR includes cells where PDF is expected to have circadian functions: a subset of clock neurons and neurons in the optic lobe and dorsal brain, regions where PDF^+ LN_vs are known to project (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). In summary, it is clear that the newly identified gene does encode the PDFR, and that further study of the function of both the gene and the cells where it is expressed will lead to a better understanding of the circuits involved in circadian rhythms, as well as other behaviors.

Nevertheless, it is important to look at molecular markers other than the PDFR, because the PDFR seems to be expressed in three types of cells: clock neurons (or pacemaker neurons), clock output neurons (downstream of the pacemaker neurons), and neurons not involved in circadian behavior. Using the *pdfr* as a molecular handle for manipulating output neurons would be complicated by the gene's expression in cells other than output neurons. To target specific cells involved in clock output, it might be useful to examine other genes and undertake a combinatorial transgene approach (similar to those used to examine different sets of clock neurons—see Section 3.2 above). Below I discuss three of the genes that come to mind because of studies implicating them in circadian output: *Neurofibromatosis-1*, *Slowpoke-binding protein*, and *ebony*.

Neurofibromatosis-1 (*Nf1*) encodes a member of the Ras-specific GTPase activating protein (Ras-GAP) family, and is involved in cyclic AMP (cAMP)/protein kinase A (PKA)- and mitogen-activated protein kinase (MAPK)-signaling pathways (The et al., 1997; Guo et al., 2000; Williams et al., 2001). An *Nf1* mutation disrupts circadian rhythms in locomotor activity but does not affect molecular oscillations typical of circadian clock cells (Williams et al., 2001). Thus, *Nf1* is involved in circadian output, but apparently not in the clock mechanism itself. Specifically, the circadian function of NF1 appears to involve periodic inhibition of MAPK in the vicinity of LN_v nerve terminals; thus NF1 is likely to be expressed in the primary targets of the LN_vs (Williams et al., 2001). In support of this possibility, a cell culture experiment showed that the presence of NF1 improves the ability of PDFR to activate adenylate cyclase (Mertens et al., 2005). If NF1 is expressed in a circadian-relevant subset of $PDFR^+$ neurons, then the combination of these two molecular handles might allow more specific analysis of circadian output circuitry.

Slowpoke-binding protein (*Slob*) encodes a cell-signaling kinase that forms a complex with various other proteins, including the SLOWPOKE potassium channel (Zhou et al., 1999). Independent microarray studies showed that *Slob* mRNA oscillates with a circadian rhythm (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Lin et al., 2002; Ueda et al., 2002). Histological analysis revealed that *Slob* is expressed in "PI-3" neurons of the dorsal median brain, but not in *period*-expressing clock neurons (Jaramillo et al., 2004; see Fig. 1). Furthermore, pan-neuronal overexpression of *Slob* causes abnormalities in circadian behavior (Jaramillo et al., 2004). Thus, *Slob* is another good candidate for a molecular marker of pacemaker target cells.

ebony encodes β-alanyl-dopamine synthetase, an enzyme that can join β-alanine to dopamine or histamine (e.g. Richardt et al., 2003). By converting dopamine or histamine into its β-alanine-conjugated form (which cannot function as a neurotransmitter), the EBONY protein inhibits dopaminergic and histaminergic neurotransmission (Wright, 1987; Hovemann et al., 1998; Borycz et al., 2002). The majority of flies homozygous for the $ebony^1$ (loss-of-function) allele lack locomotor activity rhythms at temperatures cooler than 24° C. However, their circadian timing of eclosion (emergence of the adult fly from the pupa case) is normal, suggesting that *ebony* mutations do not disrupt the circadian clock itself, but rather interfere with a subset of downstream processes (Newby and Jackson, 1991). Alternatively, eclosion and locomotor activity could be controlled by distinct combinations of oscillator cells (as discussed for tim^{blind} in Section 3.1, above).

Ebony is interesting because its expression is largely (perhaps primarily) glial (Richardt et al., 2002). $EBONY^+$ cells are therefore unlikely to be the primary targets of pacemaker neurons, which presumably form synapses with other neurons. However, glial cells could still play important roles in the function of circadian circuits. Indeed, analysis of per^+ and per^{01}

mosaic animals revealed that glial expression of *per* was sufficient for some degree of behavioral rhythmicity (Ewer et al., 1992). As mentioned before, a large cohort of glia express *per* and *tim* (e.g. Kaneko and Hall, 2000). It is currently unknown whether EBONY$^+$ glia are also PER$^+$ and/or TIM$^+$. In any case, further study of glial contributions to circadian behavior is certainly warranted. Indeed, such studies in rodents have revealed that SCN glia, specifically astrocytes, are important for regulating the SCN's circadian rhythms in neuronal activity (van den Pol et al., 1992; Prosser et al., 1994) and are pacemaker cells themselves (Prolo et al., 2005). It is likely that in both flies and mammals, glial cells are vital circadian circuit components alongside neurons.

6. Utility of studying *Drosophila* circadian circuits

6.1. Advancing Drosophila biology

The study of *Drosophila*'s circadian circuits is still in its early stages. Considerable work remains to be done in characterizing how pacemaker neurons regulate one another, how input pathways of different modalities entrain pacemaker neurons, and how behavioral rhythms are generated. The analysis of these circuits will not only elaborate a fascinating mechanism for controlling behavioral timing, but will also provide inroads to understanding the fundamental basis of various behaviors in the fly. For example, the circuit from circadian pacemakers to locomotor activity will eventually intersect with sleep-promoting circuits, which at the moment are completely mysterious.

Other behaviors whose mechanisms will be illuminated by tracing circadian circuits are eclosion, oviposition, pheromone signaling, feeding and foraging, mating, and courtship; all of these behaviors have documented circadian influences (reviewed by Hall, 2005).

The study of neural circuits will also inevitably include tracing the developmental processes giving rise to those circuits and their constituent cells. This will provide numerous examples from which biologists studying neurodevelopment may deduce more general principles. Furthermore, learning more about the genetic control of development and differentiation could help in designing strategies for transgenic targeting of specific cells in the circuits of interest. Combinatorial transgenes for targeting a cell type could be inspired by nature's use of combinatorial genes to generate that cell type in the first place.

6.2. Extrapolating from the model system

Drosophila has a long history as a model organism; many biochemical pathways first discovered in this fly have since proven to be conserved in diverse species, including mammals. A sizable proportion of the *Drosophila* genome shares homology with the genomes of vertebrates; thus genetic and biochemical studies in the fly will no doubt continue to provide useful information on molecular mechanisms that are homologous to processes found in (for example) humans.

In the realm of neural circuits, the utility of *Drosophila* is less clear. The newly cloned PDF receptor is actually homologous to mammalian neuropeptide receptors important for circadian rhythms, suggesting the possibility of some fly-mammal homology in circadian circuits (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). However, even the most casual comparison of the fly brain and any vertebrate brain would reveal a huge divergence in neuroanatomy and circuitry. It is doubtful that very much circuitry in the fly could be said to be directly homologous to any mammalian circuit.

However, analogy could still be useful in the absence of strong homology. For example, while the circadian clock of the bread mold *Neurospora crassa* does not possess molecular components that are strongly homologous to human clock proteins, its mechanism is pervaded by processes that are analogous to human clock processes. Among these analogous processes are negative feedback loops involving inhibition of transcriptional activators, circadian-regulated phosphorylation and regulated protein turnover (reviewed by Dunlap et al., 2003). Likewise, dissecting the functional properties of circadian circuits in *Drosophila* could provide insights that neurobiologists may apply to circuits in the much more complex mammalian brain. This area of neuroscience—understanding the dynamic properties of neural circuits and how they actually generate behavior in the whole animal—is such a young enterprise, it has barely seen the first light of dawn. Studying the humble fly will be invaluable to making it through the rest of the day.

Acknowledgements

I would like to thank Jeffrey C. Hall and Ralf Stanewsky for reading, correcting, and improving this manuscript.

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