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INVITED REVIEW

Even a stopped clock tells the right time twice a day: circadian timekeeping in *Drosophila*

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Abstract "Even a stopped clock tells the right time twice a day, and for once I'm inclined to believe Withnail is right. We are indeed drifting into the arena of the unwell... What we need is harmony. Fresh air. Stuff like that." *Bruce Robinson (1986, ref. 1)*. Although a stopped *Drosophila* clock probably does not tell the right time even once a day, recent findings have demonstrated that accurate circadian time-keeping is dependent on harmony between groups of clock neurons within the brain. Furthermore, when harmony between the environment and the endogenous clock is lost, as during jet lag, we definitely feel unwell. In this review, we provide an overview of the current understanding of circadian rhythms in *Drosophila*, focussing on recent discoveries that demonstrate how approximately 100 neurons within the *Drosophila* brain control the behaviour of the whole fly, and how these rhythms respond to the environment.

Keywords Circadian rhythms · *Drosophila* · Clock inputs · Clock neural circuits

Introduction

Intuitively, an organism could optimize its behaviour and physiology by responding to daily and seasonal changes in the environment. Yet virtually all organisms from *Cyanobacteria* to humans have an internal circadian clock that allows them to anticipate daily environmental changes and to alter their behaviour and physiology accordingly.

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The roles of these internal clocks in our lives can perhaps most clearly be understood by seeing what happens when our clocks become *de*synchronized from the environment. In Major League Baseball, the effect of jet lag on West Coast teams that travel to the East Coast (but not vice versa) increases the chance of East Coast teams winning home games. This effect decreases as the visiting team acclimatizes during the course of a three- or four-game series. This small, yet statistically significant effect, as recorded from 1991–1993, may even have accounted for the Atlanta Braves winning their division by one game from their West Coast rivals in 1991 and 1993 [2].

In addition to controlling the timing of sleep/wake cycles and thus influencing alertness, circadian clocks in mammals have been shown to control rates of drug detoxification, bone growth, liver regeneration and cell division [3–5]. Circadian rhythm disruptions can lead to depression, obesity and higher incidences of cancer [6–10] while normal rhythms control the sensitivity of an organism to drugs of abuse [11–13]. In other words, circadian rhythms are very important for the normal well-being of an animal because they enable an organism to anticipate and respond to environmental changes before they happen. In contrast, reindeer that live in the Arctic abandon daily activity rhythms during summers of constant light and winters of constant darkness since an endogenous circadian clock is apparently unnecessary without daily environmental changes to anticipate [14].

The beginnings of circadian research

The existence of an endogenous clock was first reported by French geophysicist Jean-Jacques d'Ortous de Mairan in 1729 [15]. Having observed a daily cycle of leaf opening

and closing in heliotrope plants, he asked what would happen to this rhythm in the absence of environmental cues by moving the plants to his dark wine cellar. He found that leaves continued to show daily cycles of opening and closing even in constant darkness (DD), indicating the existence of an internal clock. Since these rhythms run with an approximately 24 h repeating period under constant conditions, they are termed circadian—'about a day'.

Many of the pioneering experiments on circadian rhythms were performed in *Drosophila*. In one classic paper [16], Colin Pittendrigh demonstrated that the clock that drives rhythms in eclosion (hatching of adults from their pupal case) free-runs with a circadian rhythm in constant darkness, can be reset by light delivered during darkness and runs with an ~24 h period over a 10°C temperature range—a phenomenon known as temperature compensation. This last feature of molecular clocks is important not just for cold-blooded animals like *Drosophila,* but also for mammals given the daily fluctuations in body temperature, and especially for hibernating animals. It was the identification of the first clock gene mutants by Konopka and Benzer [17] in *Drosophila* that opened the door which ultimately led to a detailed molecular understanding of how intracellular clocks tick and how they are reset by light. However, the mechanisms underlying temperature compensation remain mysterious. More recently, this molecular understanding has been used to move towards an understanding of the neural circuits driving circadian behaviour. So what have we learnt from *Drosophila*?

Circadian rhythms in *Drosophila*

When Konopka and Benzer [17] initially screened mutant flies for disrupted circadian rhythms, others were sceptical that mutating a single gene would have much effect on a complex behaviour. But Konopka and Benzer succeeded in identifying the first three clock mutants, all in the *period (per)* gene: a null allele (per^{01}) that led to a complete loss of rhythmic behaviour, and two alleles that left rhythms intact but gave flies either short-period (19 h) or long-period (27 h) rhythms in DD.

The ensuing 35 years of circadian biology have made circadian rhythms the best understood behaviour at the molecular level. A number of factors have probably contributed to this, including: (1) Circadian rhythms are not essential in the lab, although they presumably do contribute to survival in the wild [18, 19]. (2) The first four clock genes identified are non-essential genes with few roles outside the clock. Indeed, they seem dedicated to rhythmic processes, as their other reported effects include altered courtship song rhythms and recovery from sleep deprivation [20, 21]. This can be contrasted with the first two learning and memory genes identified in *Drosophila*, *dunce* and *rutabaga*, which are essential genes with general roles in cyclic AMP signal transduction [reviewed in 22]. (3) Circadian rhythms are robust and easily quantifiable at the single fly level. This allowed screens of single F_1 progeny [23] rather than having to generate lines of flies—and the same approach was successful in identifying clock mutations in mice [24]. (4) Functional clocks in *Drosophila* are found not only in the pacemaker cells in the brain that drive behavioural rhythms but also in peripheral oscillators in sensory neurons (e.g. photoreceptor cells in the eye and olfactory neurons in antennae) that probably drive daily rhythms in sensory sensitivity [25]. The number of photoreceptor cells per fly make the eye clock ideal for quantitative analysis of RNA and protein level oscillations and circadian regulation of post-translational modifications such as phosphorylation. One screen for clock mutants even used a *per*-luciferase reporter gene with the signal coming largely from the eye to identify mutants that no longer rhythmically produced luciferase [26]. (5) Finally, the absence of many of the core clock components in *Drosophila* cell lines has allowed the reconstitution of certain aspects of the clock in vitro (see later).

The techniques available to a field of research influence its direction, and the relative ease of biochemically studying the clock means that we now have a good molecular understanding of clock genes. As genetic tools have become available that make it possible to study and manipulate individual neurons, circadian researchers are now able to explore how individual clock cells communicate, and how the clock translates environmental inputs to modulate behavioural outputs. In this review, we highlight some recent findings, focussing first on the molecular clock and then moving towards a more neurobiological understanding of circadian behaviour.

The *Drosophila* molecular clock

All circadian clocks characterized to date are based on negative feedback loops. In the current model of the *Drosophila* molecular clock [reviewed more extensively in 27], the basic helix-loop-helix transcription factors CLOCK (CLK) and CYCLE (CYC) activate expression of *per* and *timeless* (*tim*, see Fig. 1). PER requires TIM for stabilization, but TIM is light sensitive, so PER and TIM levels do not rise until dark. PER/TIM dimers then accumulate in the cytoplasm, and enter the nucleus ~6 h after lights off. PER and TIM dissociate inside the nucleus, and PER represses transcription by preventing the CLK/CYC dimer from binding the *per* and *tim* promoters [28, 29]. The length of the delay before nuclear entry of PER/TIM is in part dependent on the activity of the Glycogen Synthase Kinase

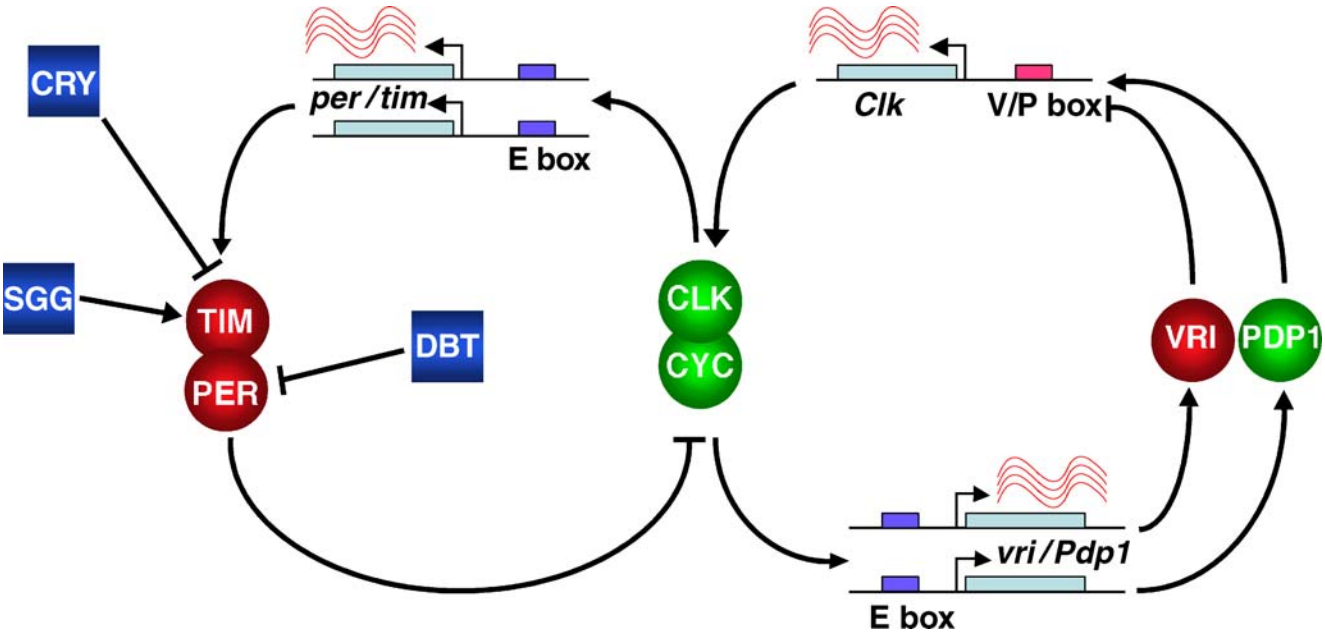

Fig. 1 The two loops of the *Drosophila* molecular clock. In the first loop, CLK and CYC directly activate transcription of *per* and *tim*. CRY mediates light-dependent degradation of TIM and is partly responsible for preventing the accumulation of PER/TIM heterodimers during the day. At night, PER and TIM accumulate in the cytoplasm and enter the nucleus, where PER inhibits CLK/CYC activity. The timing of nuclear entry and the stability of PER are controlled by SGG phosphorylation of TIM and DBT phosphorylation of PER. In the second loop, PDP1ε acts as an activator and VRI a repressor of *Clk* transcription. As *Pdp1ε* and *vri* are direct targets of CLK/CYC, this creates a second feedback loop

ortholog SHAGGY (SGG) [30]. SGG overexpression leads to hyperphosphorylated TIM and the PER/TIM heterodimer translocates into the nucleus ~4 h early, shortening the period as CLK/CYC activity is inhibited prematurely [30]. Many other factors contribute to this loop, including DOUBLE-TIME (DBT), a Casein Kinase Iε homologue [23], Casein Kinase II [31, 32], Protein Phosphatase 2A (PP2A) [33], the F-box protein SLIMB [34, 35], and the blue light photoreceptor CRYPTOCHROME (CRY), that also doubles as a transcriptional repressor in some clock cells [26, 36, 37]. Attention has largely focussed on how these proteins regulate the stability of PER and TIM, although DBT and PP2A also regulate CLK activity [38], with hypophosphorylated CLK associated with maximal *per* transcription [29, 38].

A second, interlocked loop regulates rhythmic expression of *Clk* and probably of *cry*. Here, CLK/CYC activates transcription of *vrille* (*vri*) and *PAR-domain protein 1ε (Pdp1ε)*, which encode related basic leucine zipper transcription factors [39–41]. VRI and PDP1ε regulate *Clk* transcription, with VRI acting as a repressor and PDP1ε an activator of transcription, causing *Clk* RNA levels to cycle in opposite phase to *per*, *tim*, *vri* and *Pdp1ε* [39, 40]. *vri* RNA and protein levels peak ~3–6 h before those of *Pdp1ε*, presumably underpinning the daily rhythm in *Clk* expression [40].

A two-loop clock is found in flies, mammals and *Arabidopsis*, suggesting that it is an optimal system for accurate, temperature-compensated 24 h timekeeping. Although rhythms can be achieved with much less (see below), this complexity may be required so that the clock can be fine-tuned to the environment, and to facilitate the regulation of outputs at different times of day by altering the transcriptional properties of the cell through daily oscillations in transcription factor activity (e.g. CLK/CYC and VRI/PDP1ε).

Reconstructing the clock

A recent experiment in *Cyanobacteria* found that temperature-compensated 24 h rhythms in KaiC phosphorylation were observable for at least three days when purified KaiA, B and C proteins were mixed together in a test tube. Thus, just three *Cyanobacteria* clock proteins are sufficient for generating accurate rhythms, and rhythmic transcriptional regulation is unnecessary [42].

Similarly, PER and TIM transfected into *Drosophila* S2 cells replicate the long delay between their cytoplasmic accumulation and nuclear entry as observed in vivo [43]. Fluorescence resonance energy transfer (FRET) levels were measured between CFP-labelled PER and YFP-labelled TIM proteins to reveal the dynamics of their interaction and accumulation. Maximum levels of FRET were observed from ~30 min after transfection, demonstrating that PER and TIM interact almost immediately. PER and TIM

accumulated in speckled foci in the cytoplasm, where they remained together for ~5 h. Importantly, using Konopka and Benzer's original long-period PER mutant extended the delay in nuclear entry of PER and TIM, as seen in vivo [44]. Thus, PER and TIM dynamics generate an accurate 'interval timer' even in naive S2 cells, a delay which presumably contributes to the 24 h molecular clock.

Interestingly, high levels of FRET disappeared just before nuclear entry, and the onset of PER nuclear accumulation often preceded TIM. Thus, PER and TIM may not enter the nucleus together, as also suggested by in vivo studies where PER was detected in the nucleus before TIM [45]. Foci of transfected CRY protein were previously observed in the cytoplasm of S2 cells, and these aggregations were dependent on PER, TIM and light [46]. Care has to be taken with interpreting data from S2 cells, as equivalent foci have not been identified in vivo and also since there are contradictory conclusions in the literature between the findings from S2 cells and arrhythmic pacemaker neurons [47, 48]. Nevertheless, S2 cells seem to be useful for studying specific portions of the molecular clock, and the ability to mimic the dynamics of PER/TIM nuclear entry in S2 cells should help understand the biochemistry of this interval timer.

Sensory inputs to the clock: light

The primary input to the circadian clock is light, with flies active during the day and asleep at night. There are two ways by which *Drosophila* clock cells receive light information—through the endogenous blue light photoreceptor, CRY, and, for cells with appropriate neural connections, from the eye: Projections from the eye photoreceptor cells contact the l-LN$_v$s, whereas the Hofbauer–Buchner eyelet contacts the s- and l-LN$_v$s [reviewed in 49]. The clocks of mutant flies lacking photoreceptors and CRY are not entrainable by light [50].

The best characterized effect of light on the *Drosophila* clock is on TIM degradation, and this rapid response enables the molecular clock to respond to daily and seasonal changes in light. At the behavioural level, a light pulse during darkness delays or advances the timing of onset of activity on the next day, depending on the timing of the light pulse. At the molecular level, a light pulse in the early evening degrades cytoplasmic TIM, which delays PER accumulation and thus, progression of the molecular clock. Consequently, the timing of activity on the next day is also delayed. Conversely, a light pulse late at night degrades nuclear TIM, freeing PER to repress CLK/CYC activity earlier than normal, advancing timing of the onset of activity on the next day.

The importance of light as a clock input is underlined by the effect of prolonged light. Whereas flies show molecular and behavioural rhythms in constant darkness, they become arrhythmic in constant light (LL). Strangely, *Drosophila* cry^b mutants show both molecular and behavioural rhythms in LL. This is surprising given that cry^b flies can still detect light via the visual system and can entrain to LD cycles [51–53], but is probably partly due to increased TIM stability in cry^b mutants in some clock neurons.

CRY-dependent TIM degradation involves the recently identified F-box protein JETLAG (JET) [54]. Like cry^b mutants, *jet* mutants are rhythmic in LL, have normal DD behaviour and show reduced responses to light pulses, suggesting a defect in the light input pathway [54]. This effect is dependent on the genetic background, as rhythmic behaviour is only observed in jet^c mutants when they have one of two naturally occurring *tim* alleles that differ by 23 AA at the N-terminus [55, 56]. The functional difference between these two TIM isoforms remains to be determined.

Sensory inputs to the clock: temperature

Quite sensibly, *Drosophila* have a mid-day siesta and avoid activity during the hottest part of the day. At colder temperatures, this siesta is reduced as the evening activity peak moves earlier in the day. This response is controlled by the splicing of an intron within the 3′ UTR of *per* [57]. Regulation of *per* splicing integrates seasonal information, as it responds to both temperature and light [57–59]. At low temperatures, or under the short photoperiods associated with colder days, *per* splicing levels are increased at least in photoreceptor cells where this phenomenon has largely been studied. This leads to earlier processing of the *per* transcript and earlier accumulation of PER protein, resulting in an earlier phase of evening activity, and so reducing the mid-day siesta. This allows the behaviour of a fly to be fine-tuned to any given day across the seasons [57–59]. Rescue of per^{01} mutants with a *per* transgene where the intron cannot be spliced fails to rescue this adaptive response [57]. This suggests that *per* splicing is regulated in the same way in pacemaker neurons as in the eye, and that regulated *per* splicing is responsible for the shift in mid-day siesta in response to temperature changes.

Information regulating *per* splicing is received through a signalling pathway involving the NorpA Phospholipase-C [58, 59]. Furthermore, *NorpA* has subsequently been shown to be a general factor involved in temperature entrainment of behaviour, as *NorpA* mutants fail to entrain behavioural rhythms to temperature cycles in LL. *NorpA* likely acts as part of a signalling cascade relaying temperature information to the clock, possibly through PER [60]. A second, as yet un-cloned mutation, *nocte*, also specifically abolishes temperature entrainment, whilst leaving light entrainment intact [60].

Temperature entrainment of the clock and temperature compensation appear to be independent of one another. Neither the regulation of *per* splicing nor the temperature entrainment roles of *NorpA* and *nocte* seem to contribute to temperature compensation [58, 60]. Instead, naturally occurring polymorphisms in a Threonine–Glycine (Thr-Gly) repeat region in the *per* gene may provide a clue. 99% percent of wild-type *Drosophila* strains have a *per* gene encoding 14, 17, 20 or 23 Thr-Gly repeats [61]. These alleles affect temperature compensation: $(Thr\text{-}Gly)_{20}$ display a slightly short ~23.7 h period that remains constant over a wide range of temperatures [62]. $(Thr\text{-}Gly)_{17}$ has a period closer to 24 h than $(Thr\text{-}Gly)_{20}$ at higher temperatures, but overall is less well temperature compensated. This difference in temperature compensation explains the highly significant cline in $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$ allele distribution in wild populations through Europe [61]. $(Thr\text{-}Gly)_{20}$ is more common in the North where temperatures are more variable, whereas $(Thr\text{-}Gly)_{17}$ is more common in Southern Europe where temperatures are warmer and therefore an allele giving a 24 h period at higher temperatures is ideal [62]. Several long- and short-period mutations of *per* and *tim* also affect temperature compensation [63–66], although this may simply reflect that these alleles generate temperature-sensitive proteins rather than affecting parts of the temperature compensation mechanism per se.

Social inputs to the *Drosophila* clock

Although *Drosophila* is not generally considered a social animal, its circadian clock is influenced by social signals, as are the clocks of bees, rodents, fish and humans. Flies pre-housed together show greater synchrony in behaviour when allowed to free run individually than those pre-housed apart [67]. Adding arrhythmic per^{01} mutants to a group of rhythmic flies reduces (but does not abolish) synchronization. Furthermore, adding a small number of 'visitor' flies with an earlier phase of activity than their hosts advances the phase of the hosts' activity, demonstrating that flies communicate circadian information. These social signals are airborne, as pumping air from a vial containing flies in LD to flies in DD helped synchronize flies individually housed in DD. Furthermore, mutants lacking normal olfactory responses are unaffected by per^{01} visitors.

One important result was that per^{01} visitor flies do not disrupt synchronization of flies in which *per* expression is limited to a subset of central brain neurons, the Lateral Neurons (LNs, see below). One interpretation of this result is that social entrainment is mediated via the peripheral clock in the antenna. However, another possibility is that social entrainment is mediated via non-LN central brain pacemaker clocks such as the Dorsal Neurons (DNs, see below). The identification of the relevant neural and molecular substrates for social entrainment of the clock will be very exciting.

Functional groups of clock neurons

Clock protein oscillations can be detected in six major groups of neurons in the *Drosophila* brain which have been anatomically divided into Lateral and Dorsal Neurons (LNs and DNs; see Fig. 2). The LNs are subdivided into large and small ventral lateral neurons (l- and s-LN_vs), which produce the neuropeptide Pigment Dispersing Factor (PDF), and dorsal lateral neurons (LN_ds). DNs are subdivided into three groups (DN_{1-3}), although it was recently found that DN_1s are a heterogeneous group consisting of 2 DN_{1a} anterior neurons that produce the neuropeptide IPNamide, and the more posterior DN_1s whose transmitter(s) remain unknown [68]. The clocks in all of these neurons oscillate in wild-type flies in DD; even the l-LN_vs, while initially arrhythmic, regain rhythms after several days [69]. Overall, clock gene oscillations in ~100 neurons control rhythmic behaviour of the whole organism [70]—but what are the roles of these individual groups of clock neurons?

Three initial studies suggested the importance of the s-LN_vs in driving behavioural rhythms: (1) the presence of just one s-LN_v in a *disconnected* mutant is sufficient for a fly to display some rhythms, while flies with no s-LN_vs remaining are arrhythmic [71]; (2) cry^b mutants are rhythmic in DD, with the s-LN_vs the only cells to show rhythms in TIM protein levels, at least in LD cycles [26]; and (3) PDF is only produced in LN_vs, and *Pdf* null mutants become arrhythmic after 1–2 days in DD [72]—thus LN_vs are important for rhythms. However, the persistence of rhythms for the first 2 days in DD in *Pdf* mutants and in flies in which PDF cells have been ablated indicates that other cells are also required to drive normal behavioural rhythms.

Morning and evening peaks are controlled by separate clock neuron groups

Although flies can sustain behavioural rhythms for weeks in DD, they normally live in a constantly changing environment. A long-standing prediction of Pittendrigh and Dann [73] was that separate morning and evening oscillators would help an organism adapt to seasonal changes. In an LD cycle, *Drosophila* activity peaks in the morning and evening, anticipating lights on and off. Pdf^{01}

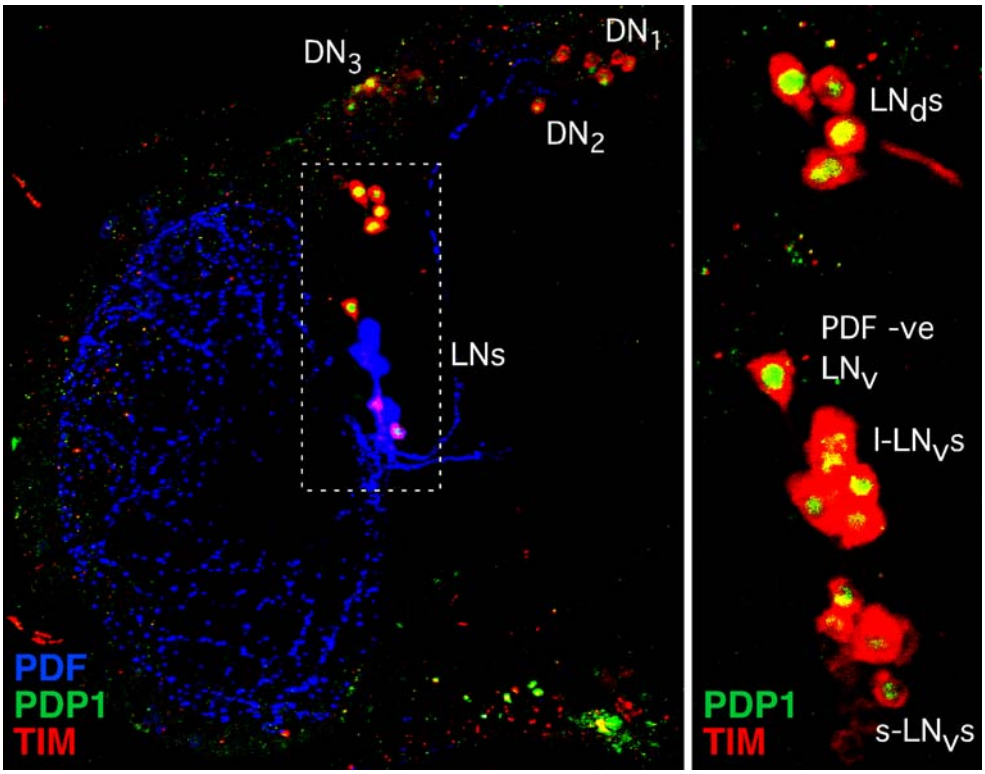

Fig. 2 Clock neurons in the *Drosophila* brain. One lobe of an adult per^{01} mutant brain stained for PDF (*blue*), PDP1ε (*green*) and TIM (*red*). Levels of PDP1ε and TIM are high in all six groups of clock cells as a result of the per^{01} mutation. *Inset* on *right* shows a closer view of LNs stained for PDP1ε (*green*) and TIM (*red*)

mutants or flies with PDF cells ablated are rhythmic in LD, but they only anticipate dusk (the "evening peak") not dawn (the "morning peak") [72]. Thus, the LN_vs are a good candidate for the Morning oscillator (M) cells.

To identify the Evening (E) cells, locomotor activity rhythms were tested in flies which had subsets of clock neurons ablated [74] or had *per* restored in an otherwise per^{01} mutant background [74, 75]. The presence or absence of a morning and/or evening peak was then correlated with the presence or absence of a functional clock within each neuronal group. This is summarized in Fig. 3. These approaches confirmed that the PDF-expressing LN_vs control the morning peak, as any genotype lacking clock function in these cells shows no anticipatory morning peak (with one exception, see below). Furthermore, clock function in the s-LN_vs alone is necessary and sufficient for the morning peak, making the s-LN_vs the M cells [74, 75].

Conversely, the LN_ds, a PDF-negative LN_v, and possibly a small number of DN_1s contribute to the evening peak (E cells). The neurotransmitter(s) controlling the evening peak is unknown, but signalling by the neuropeptide IPNamide, recently shown to be expressed in a subclass of DN_1 neurons that project to the s-LN_vs [68], may be involved in this process.

Under constant conditions, the function of the M and E cells is slightly different. Pittendrigh and Dann's [73] model predicted that in constant light, the M oscillator will free run with a short period and the E oscillator a long period. cry^b flies show a weak morning and a strong evening activity peak in LL [53]. After a few days, the evening peak splits into a long (25.2 h) and a short (22.5 h) period component; the short component has a similar period to the weak morning activity peak. A single LN_d and the 5th PDF-negative s-LN_v are the only neurons with a long period molecular clock under these conditions, making them strong candidates for the E oscillator neurons. Similarly, the s-LN_vs display a short period, fitting their role as the M cells. The splitting of the evening peak in LL into both a short and long component suggests that the M cells also contribute to the evening peak of activity, an addition to the original model, suggesting that these cells should be named 'Main' rather than 'Morning' neurons [53].

The relationship between M and E cells was probed further by over-expressing *sgg* in the LN_vs, shortening the period by ~3 h in DD [74]. This advanced *tim* RNA accumulation in both the s-LN_vs and E cells and the timing of both morning and evening peaks. Over-expression of *sgg* in the E cells advanced the timing of *tim* accumulation only in the DN_2s and l-LN_vs, and not in the LN_ds or 5th LN_v. Presumably, a signal from the s-LN_vs overrides the endogenous period of the E cells. This confirms that the s-LN_vs are the 'Main' oscillator, necessary and sufficient for rhythmic activity in DD and for determining the period of DD rhythms [76]. But if the s-LN_vs contribute to both morning and evening peaks, what is the function of the E cells?

The LN_ds and possibly the 5th s-LN_v also show robust oscillations in DD, but are unable to drive rhythmic

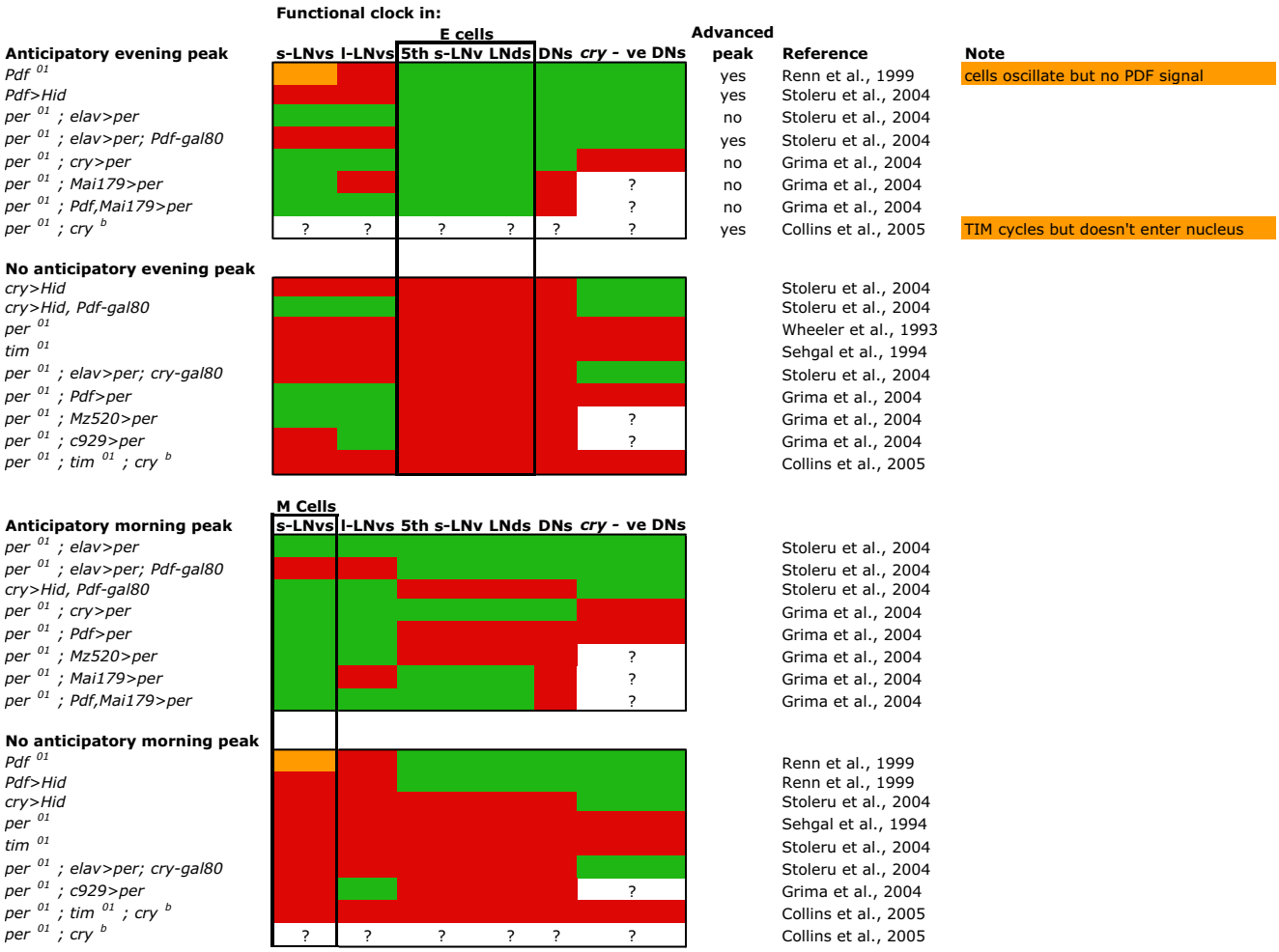

Fig. 3 Morning/Main and Evening cells. Presence (*green*) or absence (*red*) of a functional clock in each group of clock cells in different genetic backgrounds. Cells in *orange* retain some clock function. "?" represents unknown clock function

locomotor activity independent of the s-LN$_v$s. To determine the function of the E cells in DD, the period of the E cells was accelerated by over-expression of *sgg*. In contrast to expression in the M cells, this did not affect the free-running period. However, it did reduce the time between morning and evening peaks by ~2 h; expression in M cells alone had no effect on this interval [76]. Thus, the M cells set the overall period of the system and presumably send a daily resetting signal to the E cells, which then drive an evening peak in response to this signal. The time between the signal from the M cells and the appearance of an evening peak is dependent on the period of the E cells' clock: If it runs fast, then the evening peak is earlier [76]. In this way, changes in temperature or day length that tend to affect the evening and not the morning peak [77] can be accommodated by the clock without disrupting the underlying 24 h period. As the LN$_d$s' clock is advanced in *Pdf*^{01} mutants, this suggests that PDF signals normally maintain phasing and amplitude of LN$_d$ rhythms perhaps by delaying the timing of PER nuclear entry [78]. Thus, PDF may be the daily resetting signal from M to E cells.

These experiments, together with the lack of anticipatory peaks in clock-gene mutants such as *per*^{01}, suggest that anticipatory activity in LD is clock-dependent. However, this is confounded by the observation of anticipatory evening activity in *per*^{01}; *cry*^{b} double mutant flies that should lack a functional clock in all clock cells [79]. Anticipation is TIM-dependent, as both *per*^{01}; *tim*^{01}; *cry*^{b} triple mutants and *tim*^{01}; *cry*^{b} double mutants showed no anticipation. *per*^{01}; *cry*^{b} mutants are arrhythmic in DD and LL, so rhythmicity is dependent on an LD cycle. One possibility is that the *cry*^{b} mutation prevents the immediate degradation of TIM in response to light. The resultant light-driven oscillation in TIM then restores enough clock function (to the E cells?) to generate rhythmic evening activity in LD. However, TIM does not enter the nucleus of either the M or E cells in *per*^{01}; *cry*^{b} mutants, making it unclear exactly how much clock function is sufficient to drive LD rhythmicity [79].

Clock neural networks

In order for different groups of clock neurons to control a single behaviour, they need to form a single network. Although the PDF-expressing LNs can be considered the 'Main' oscillator cells, they need the support of the rest of the clock network to control locomotor behaviour.

There are several pieces of evidence that support a model of networked clock neurons. Firstly, although *Pdf* null mutants become arrhythmic in DD, rhythms of PER oscillation persist in pacemaker LNs [72, 78]. However, the timing of PER nuclear entry between individual *Pdf* mutant s-LN_vs becomes desynchronized in DD [78]. The importance of PDF for synchronization is likely reduced when individual neurons can receive light via CRY, hence the relatively normal LD behaviour of Pdf^{01} mutants. PDF could normally keep the s-LN_vs synchronized either by direct signalling or via the whole clock circuit. A circuit explanation seems more likely because the s-LN_vs do not produce the PDF Receptor (PDFR) [80–82]. Thus, a PDF signal from the s-LN_vs is relayed back to the other s-LN_vs via at least one other clock neuronal group.

Secondly, although the morning and evening oscillators function independently, the LN_ds, DN_1s and the PDF-negative LN_v can drive normal LD rhythms (even including the morning peak) in per^{01} flies where *per* expression is restored everywhere except the PDF cells [75]. The restoration of the morning peak suggests that rhythmic activity of E cells is sufficient to drive the appropriately timed release of PDF from M cells. However, when the PDF expressing cells are ablated or do not produce PDF, no anticipatory morning peak is observed [72].

Finally, the ability of a functional clock in PDF neurons to drive rhythmic behaviour depends on the clock state of the rest of the network. PDF-cell specific expression of *per* in a per^{01} mutant background rescued both molecular and behavioural rhythms [75]. Similarly, molecular rhythms in only LN_vs are sufficient for behavioural rhythms in cry^b mutants [26]. Conversely, PDF-cell specific expression of *cyc* in a *cyc* mutant background rescued molecular oscillations of *tim* RNA in the s- and l-LN_vs, but failed to restore behavioural rhythms of flies [69]. How can these results be reconciled? One idea is that the status of the clock in the remainder of the clock neuron circuit (see below) may or may not render them permissive to respond to rhythmic outputs of the PDF cells. Certainly the molecular functions of PER and CRY as transcriptional repressors are different enough from CYC (a transcriptional activator) to leave the basal states of mutant cells very different. What this means at the molecular and electrophysiological level remains to be explored.

In summary, the ability of a fly to remain highly rhythmic for weeks in DD is probably the result of a network of clock neurons keeping each other synchronized rather than the highly accurate cell autonomous timekeeping of individual clocks in neurons.

Outputs: neurotransmitters and membrane excitability

In contrast to the relatively detailed understanding of the molecular clock and the beginnings of an understanding about the roles of the different clock neurons, very little is known about the step in between: how a clock is coupled to neuronal outputs. There are two specific questions here: What are the neurotransmitters released by individual clock neuron groups? And how does an intracellular clock control neuronal activity to convey a circadian message?

Of the six main groups of clock neurons in the central brain, we know embarrassingly little about their neurotransmitters: We know only that PDF is produced in the LNs, and IPNamide in a subset of DN_1s. Mutations that eliminate PDF production phenocopy ablation of LN_vs, suggesting that PDF is the major signal from LN_vs to control locomotor behaviour. However, neuropeptides are often co-produced in neurons with a more classical small molecule neurotransmitter, and these remain to be identified in clock neurons. Certainly, additional signalling molecules must be present to explain how LN_vs control light avoidance in larvae and cocaine sensitivity in adults independently of PDF [11, 83].

How a molecular clock controls rhythmic neuronal activity (as would be predicted by analogy with the mammalian suprachiasmatic nucleus [84]) is presumably tied to its rhythmic transcription. Indeed the transcriptional state of the s-LN_vs seems to determine their output levels, at least in one simple system: light avoidance by larvae.

Drosophila larvae are intrinsically photophobic: When wild-type larvae are aligned down the middle of a half-covered Petri dish, ~70% of larvae will be on the dark side after 15 min [83]. Ablation of the larval visual system, Bolwig's Organ (BO), causes larvae to distribute randomly between light and dark ('blind'). BO projects to the larval LN_vs, and ablation of LN_vs also causes larvae to distribute randomly, indicating that the LN_vs are necessary for light avoidance, and suggesting that they transmit a signal they receive from BO. Light avoidance is under circadian control: Wild-type larvae are most sensitive to light at dawn and least sensitive at dusk, and this is clock gene-dependent, since per^{01} and tim^{01} mutants (high CLK/CYC activity) are constitutively blind, whereas Clk^{Jrk} and cyc^0 mutants (low CLK/CYC activity) are constantly highly photophobic. Clock gene modulation is dependent on the LN_vs, as the larval photoreceptor cells that make up BO have no clock [83], in contrast to adult photoreceptors.

The blindness of *per* and *tim* mutants can be rescued by increasing the light intensity—presumably increasing the amount of transmitter released by BO—whereas LN_v-ablated larvae remain blind even under bright light conditions. Conversely, *Clk* and *cyc* mutant larvae are still photophobic at low light levels where wild-type larvae are blind. Thus, clock modulation of larval light avoidance is dependent on the transcriptional state of the LN_vs, consistent with the excitability of LN_vs being under clock control. The time of day at which larval LN_vs are most sensitive to light (dawn) corresponds well with their presumed activity as adult morning cells. Pupal LN_vs drive the daily rhythm in eclosion, and this also peaks at dawn, suggesting that morning behaviour is under the control of the same intracellular mechanisms in the s-LN_vs throughout a fly's lifetime. This could be achieved by circadianly regulated transcription of genes that alter the neuronal activity of the cell and/or the production of the appropriate output signal. Such genes could include ion channels and their modulators and/or enzymes involved in transmitter production.

The M and E neurons control behavioural outputs at opposite times of day. This could mean that they fire in antiphase—and this would occur despite the oscillations of clock proteins appearing broadly similar between these cells. To explain this, we can borrow an analogy from mammals, which have clocks in many tissues outside the nervous system including the liver and the heart. Although the molecular clocks in these tissues are similarly organized, they are coupled to rhythmic expression of genes with tissue-specific functions (e.g. Alcohol dehydrogenase in the liver and Fibrillin-1 in the heart [85]). Perhaps the output pathways downstream of the molecular clocks in different clock neurons in flies are also very different, enabling them to be active at different times of day.

Finally, there is increasing evidence that clocks are not just housed within neurons, but that the neuron itself is a part of the clock [86, 87]. Electrically silencing the LNs stops their free-running rhythms—thus membrane electrical activity is coupled to the molecular clock. Again, this remains to be understood at the molecular level. A circuit explanation could be invoked here too, as the molecular clock in electrically silenced LN_vs runs down in DD rather than stopping immediately.

Conclusion

Researchers studying circadian rhythms in *Drosophila* are leaving their molecular biology roots and are moving towards a more holistic understanding of how flies keep accurate time. There are clearly unanswered questions that will occupy the field for many years, some of which have been mentioned here. Presentations at recent meetings highlight this trend, with researchers reporting the effects of more natural lighting conditions on the clock (including moonlight) and trying to tie together temperature and light changes over 24 h. Ultimately, we should have a good understanding of how flies keep in internal harmony with their continuously changing external environment.

References

1. Robinson B (1986) Withnail and I. Bloomsbury Publishing Film Script
2. Recht LD, Lew RA, Schwartz WJ (1995) Baseball teams beaten by jet lag. Nature 377:583
3. Gorbacheva VY, Kondratov RV, Zhang R, Cherukuri S, Gudkov AV, Takahashi JS, Antoch MP (2005) Circadian sensitivity to the chemotherapeutic agent cyclophosphamide depends on the functional status of the CLOCK/BMAL1 transactivation complex. Proc Natl Acad Sci USA 102:3407–3412
4. Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H (2003) Control mechanism of the circadian clock for timing of cell division in vivo. Science 302:255–259
5. Fu L, Patel MS, Bradley A, Wagner EF, Karsenty G (2005) The molecular clock mediates leptin-regulated bone formation. Cell 122:803–815
6. Lewy AJ, Lefler BJ, Emens JS, Bauer VK (2006) The circadian basis of winter depression. Proc Natl Acad Sci USA 103:7414–7419
7. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J (2005) Obesity and metabolic syndrome in circadian clock mutant mice. Science 308:1043–1045
8. Hansen J (2001) Increased breast cancer risk among women who work predominantly at night. Epidemiology 12:74–77
9. Davis S, Mirick DK, Stevens RG (2001) Night shift work, light at night, and risk of breast cancer. J Natl Cancer Inst 93:1557–1562
10. Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Colditz GA (2001) Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. J Natl Cancer Inst 93:1563–1568
11. Tsai LT, Bainton RJ, Blau J, Heberlein U (2004) *Lmo* mutants reveal a novel role for circadian pacemaker neurons in cocaine-induced behaviors. PLoS Biol 2:e408
12. Andretic R, Chaney S, Hirsh J (1999) Requirement of circadian genes for cocaine sensitization in *Drosophila*. Science 285:1066–1068
13. Abarca C, Albrecht U, Spanagel R (2002) Cocaine sensitization and reward are under the influence of circadian genes and rhythm. Proc Natl Acad Sci USA 99:9026–9030
14. van Oort BE, Tyler NJ, Gerkema MP, Folkow L, Blix AS, Stokkan KA (2005) Circadian organization in reindeer. Nature 438:1095–1096
15. de Mairan JJ (1729) Observation botanique. Histoire de l'Academie Royale des Sciences, pp 5–36
16. Pittendrigh CS (1954) On temperature independence in the clock system controlling emergence time in *Drosophila*. Proc Natl Acad Sci USA 40:1018–1029
17. Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci USA 68:2112–2116
18. Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in *Cyanobacteria*. Proc Natl Acad Sci USA 95:8660–8664
19. DeCoursey PJ, Walker JK, Smith SA (2000) A circadian pacemaker in free-living chipmunks: essential for survival? J Comp Physiol [A] 186:169–180

20. Kyriacou CP, Hall JC (1980) Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. Proc Natl Acad Sci USA 77:6729–6733
21. Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, Pack AI (2000) Rest in *Drosophila* is a sleep-like state. Neuron 25:129–138
22. Waddell S, Quinn WG (2001) Flies, genes, and learning. Annu Rev Neurosci 24:1283–1309
23. Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83–95
24. Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS (1994) Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science 264:719–725
25. Tanoue S, Krishnan P, Krishnan B, Dryer SE, Hardin PE (2004) Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. Curr Biol 14:638–649
26. Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC (1998) The cry^b mutation identifies Cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692
27. Hardin PE (2004) Transcription regulation within the circadian clock: the E-box and beyond. J Biol Rhythms 19:348–360
28. Lee C, Bae K, Edery I (1999) PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. Mol Cell Biol 19:5316–5325
29. Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev 20:723–733
30. Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. Cell 105:769–779
31. Akten B, Jauch E, Genova GK, Kim EY, Edery I, Raabe T, Jackson FR (2003) A role for CK2 in the *Drosophila* circadian oscillator. Nat Neurosci 6:251–257
32. Lin JM, Kilman VL, Keegan K, Paddock B, Emery-Le M, Rosbash M, Allada R (2002) A role for casein kinase 2α in the *Drosophila* circadian clock. Nature 420:816–820
33. Sathyanarayanan S, Zheng X, Xiao R, Sehgal A (2004) Posttranslational regulation of *Drosophila* PERIOD protein by Protein Phosphatase 2A. Cell 116:603–615
34. Ko HW, Jiang J, Edery I (2002) Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. Nature 420:673–678
35. Grima B, Lamouroux A, Chelot E, Papin C, Limbourg-Bouchon B, Rouyer F (2002) The F-box protein Slimb controls the levels of clock proteins Period and Timeless. Nature 420:178–182
36. Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated Cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679
37. Collins B, Mazzoni EO, Stanewsky R, Blau J (2006) *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441–449
38. Kim EY, Edery I (2006) Balance between DBT/CKIε kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. Proc Natl Acad Sci USA 103:6178–6183
39. Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE (2003) VRILLE feeds back to control circadian transcription of clock in the *Drosophila* circadian oscillator. Neuron 37:249–261
40. Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, Hardin PE, Young MW, Storti RV, Blau J (2003) *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329–341
41. Blau J, Young MW (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell 99:661–671
42. Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T (2005) Reconstitution of circadian oscillation of Cyanobacterial KaiC phosphorylation in vitro. Science 308:414–415
43. Meyer P, Saez L, Young MW (2006) PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. Science 311:226–229
44. Curtin KD, Huang ZJ, Rosbash M (1995) Temporally regulated nuclear entry of the *Drosophila* Period protein contributes to the circadian clock. Neuron 14:365–372
45. Shafer OT, Levine JD, Truman JW, Hall JC (2004) Flies by night: effects of changing day length on *Drosophila's* circadian clock. Curr Biol 14:424–432
46. Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553–556
47. Nawathean P, Menet JS, Rosbash M (2005) Assaying the *Drosophila* negative feedback loop with RNA interference in S2 cells. Methods Enzymol 393:610–622
48. Cyran SA, Yiannoulos G, Buchsbaum AM, Saez L, Young MW, Blau J (2005) The Double-time protein kinase regulates the subcellular localization of the *Drosophila* clock protein Period. J Neurosci 25:5430–5437
49. Helfrich-Forster C (2005) Neurobiology of the fruit fly's circadian clock. Genes Brain Behav 4:65–76
50. Helfrich-Forster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. Neuron 30:249–261
51. Emery P, Stanewsky R, Hall JC, Rosbash M (2000) A unique circadian-rhythm photoreceptor. Nature 404:456–457
52. Yoshii T, Funada Y, Ibuki-Ishibashi T, Matsumoto A, Tanimura T, Tomioka K (2004) *Drosophila* cry^b mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. J Insect Physiol 50:479–488
53. Rieger D, Shafer OT, Tomioka K, Helfrich-Forster C (2006) Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. J Neurosci 26:2531–2543
54. Koh K, Zheng X, Sehgal A (2006) JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. Science 312:1809–1812
55. Rosato E, Trevisan A, Sandrelli F, Zordan M, Kyriacou CP, Costa R (1997) Conceptual translation of Timeless reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res 25:455–458
56. Peschel N, Veleri S, Stanewsky R (2006) *Veela* defines a molecular link between Cryptochrome and Timeless in the light input pathway to *Drosophila's* circadian clock. Proc Natl Acad Sci USA 103:17313–17318
57. Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219–230
58. Collins BH, Rosato E, Kyriacou CP (2004) Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. Proc Natl Acad Sci USA 101:1945–1950
59. Majercak J, Chen WF, Edery I (2004) Splicing of the *period* gene 3′-terminal intron is regulated by light, circadian clock factors, and phospholipase C. Mol Cell Biol 24:3359–3372
60. Glaser FT, Stanewsky R (2005) Temperature synchronization of the *Drosophila* circadian clock. Curr Biol 15:1352–1363
61. Costa R, Peixoto AA, Barbujani G, Kyriacou CP (1992) A latitudinal cline in a *Drosophila* clock gene. Proc Biol Sci 250:43–49

62. Sawyer LA, Hennessy JM, Peixoto AA, Rosato E, Parkinson H, Costa R, Kyriacou CP (1997) Natural variation in a *Drosophila* clock gene and temperature compensation. Science 278:2117–2120
63. Hamblen MJ, White NE, Emery PT, Kaiser K, Hall JC (1998) Molecular and behavioral analysis of four *period* mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 149:165–178
64. Matsumoto A, Tomioka K, Chiba Y, Tanimura T (1999) tim^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343–4354
65. Rutila JE, Zeng H, Le M, Curtin KD, Hall JC, Rosbash M (1996) The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron 17:921–929
66. Huang ZJ, Curtin KD, Rosbash M (1995) PER protein interactions and temperature compensation of a circadian clock in *Drosophila*. Science 267:1169–1172
67. Levine JD, Funes P, Dowse HB, Hall JC (2002) Resetting the circadian clock by social experience in *Drosophila melanogaster*. Science 298:2010–2012
68. Shafer OT, Helfrich-Forster C, Renn SC, Taghert PH (2006) Reevaluation of *Drosophila melanogaster's* neuronal circadian pacemakers reveals new neuronal classes. J Comp Neurol 498:180–193
69. Peng Y, Stoleru D, Levine JD, Hall JC, Rosbash M (2003) *Drosophila* free-running rhythms require intercellular communication. PLoS Biol 1:E13
70. Kaneko M, Hall JC (2000) Neuroanatomy of cells expressing clock genes in *Drosophila*: transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian pacemaker neurons and their projections. J Comp Neurol 422:66–94
71. Helfrich-Forster C (1998) Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: a brain-behavioral study of *disconnected* mutants. J Comp Physiol [A] 182:435–453
72. Renn SC, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802
73. Pittendrigh C, Dann S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 106:333–355
74. Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. Nature 431:862–868
75. Grima B, Chelot E, Xia R, Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431:869–873
76. Stoleru D, Peng Y, Nawathean P, Rosbash M (2005) A resetting signal between *Drosophila* pacemakers synchronizes morning and evening activity. Nature 438:238–242
77. Hall JC (2003) Genetics and molecular biology of rhythms in *Drosophila* and other insects. Adv Genet 48:1–280
78. Lin Y, Stormo GD, Taghert PH (2004) The neuropeptide Pigment Dispersing Factor coordinates pacemaker interactions in the *Drosophila* circadian system. J Neurosci 24:7951–7957
79. Collins BH, Dissel S, Gaten E, Rosato E, Kyriacou CP (2005) Disruption of Cryptochrome partially restores circadian rhythmicity to the arrhythmic *period* mutant of *Drosophila*. Proc Natl Acad Sci USA 102:19021–19026
80. Hyun S, Lee Y, Hong ST, Bang S, Paik D, Kang J, Shin J, Lee J, Jeon K, Hwang S, Bae E, Kim J (2005) *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron 48:67–278
81. Lear BC, Merrill CE, Lin JM, Schroeder A, Zhang L, Allada R (2005) A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. Neuron 48:21–227
82. Mertens I, Vandingenen A, Johnson EC, Shafer OT, Li W, Trigg JS, De Loof A, Schoofs L, Taghert PH (2005) PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. Neuron 48:13–219
83. Mazzoni EO, Desplan C, Blau J (2005) Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response. Neuron 45:93–300
84. Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:97–706
85. Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. Nature 417:78–83
86. Nitabach MN, Blau J, Holmes TC (2002) Electrical silencing of *Drosophila* pacemaker neurons stops the free-running circadian clock. Cell 109:485–495
87. Nitabach MN, Sheeba V, Vera DA, Blau J, Holmes TC (2005) Membrane electrical excitability is necessary for the free-running larval *Drosophila* circadian clock. J Neurobiol 62:1–13