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A Plastic Clock: How Circadian Rhythms Respond to Environmental Cues in *Drosophila*

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Abstract Circadian clocks synchronize the physiology and behavior of most animals with the day to night cycle. A fundamental property of the molecular pacemakers generating circadian rhythms is their self-sustained nature: they keep oscillating even under constant conditions, with a period close to, but not exactly, 24 h. However, circadian pacemakers have to be sensitive to environmental cues to be beneficial. They need to be reset every day to keep a proper phase relationship with the day to night cycle, and they have to be able to adjust to seasonal changes in day length and temperature. Here, we review our current knowledge of the molecular and neural mechanisms contributing to the plasticity of *Drosophila* circadian rhythms, which are proving to be remarkably sophisticated and complex.

Keywords Circadian rhythms · Behavior · Neural network · Circadian entrainment · Seasonal adaptation · Circadian photoresponses · Circadian thermoresponses · Molecular mechanisms underlying circadian rhythms

Introduction

Most organisms face major adaptive challenges every day. They have to cope with dramatic cyclical variations of the physical properties of their environment, such as light intensity and temperature. The ecological environment changes too. For example, the activity of prey or predator species is usually determined by the time of day. The role of circadian rhythms is to help organisms coping with these daily challenges. They time cell biochemistry, body physiology, and behavior to maximize the chances of survival. The molecular pacemaker generating circadian rhythms is a self-sustained molecular feedback loop that oscillates with a period that approximates 24 h. Since this pacemaker does not exactly match the 24-h day length, it needs to be resynchronized (re-entrained) every day. Environmental cues such as light intensity, temperature variation, availability of food, and social interactions can synchronize circadian clocks and are therefore called Zeitgeber (time giver in German).

The task of circadian clocks is complicated at most latitudes by changes in the length of the day light period (photoperiod) and in overall temperature. In addition, weather changes can result in short-term variations of temperature and light intensity. Therefore, circadian clocks have to be plastic, but at the same time need to be able to discriminate between erratic and cyclical variations. We review here the mechanisms that are believed to contribute to the plastic nature of the circadian clock in *Drosophila melanogaster*. It is becoming clear that circadian plasticity occurs at multiple levels, from cell-autonomous mechanisms to interaction between circadian neurons.

Circadian Rhythms in *Drosophila*

D. melanogaster is a remarkable organism to study circadian rhythms. First, it shows robust circadian behavior. Eclosion (emergence of adult flies out of the pupal case) was initially used to genetically dissect circadian rhythms, but the most commonly used output is currently locomotor

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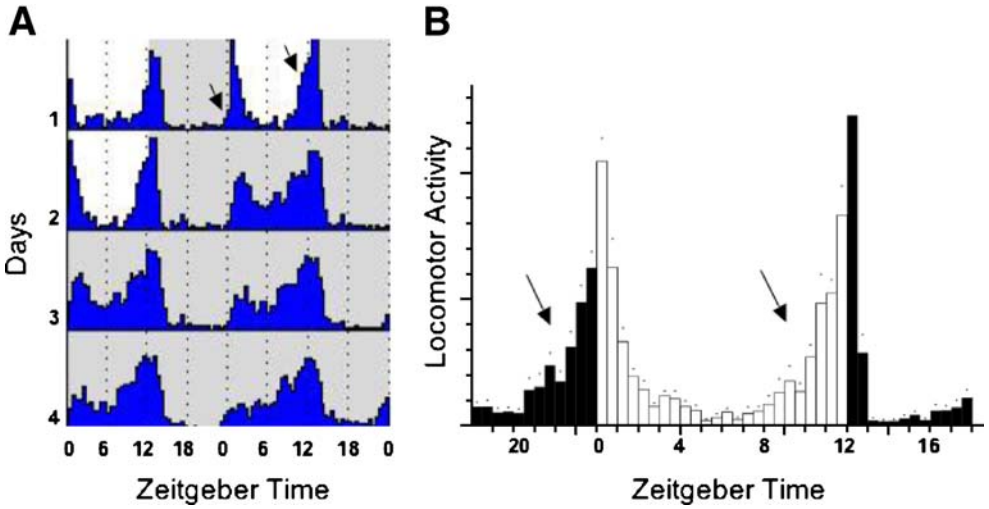

Fig. 1 Locomotor activity of *Drosophila* in LD and in DD. **a** Double-plotted actograms showing the average locomotor activity of a group of individually monitored wild-type male flies entrained to a 12:12 h light to dark cycle (LD), and then released in constant darkness (DD). Days 2–4 are plotted twice: first on the *right half* of the actogram and then on the *left half* of the next line. *Gray shading* indicate darkness. Note the persistence of approximately 24-h period locomotor rhythms in DD. The *arrows* show the increase in behavioral activity that anticipates the light-on and light-off transitions. **b** This anticipatory behavior is best visualized with an histogram showing only the average activity during the 12:12 h LD cycle. *X*-axis—Zeitgeber time. *Y*-axis—relative locomotor activity

activity rhythms (Fig. 1), which can persist under constant conditions in individual flies for weeks. Second, the powerful genetic arsenal available in *Drosophila* has been instrumental for the identification of "clock" genes. Molecular and biochemical studies of clock proteins have resulted in a detailed understanding of the molecular mechanisms underlying circadian rhythms. Third, the recent development of genetic tools to ablate or manipulate specific groups of circadian neurons is beginning to reveal how circadian behavior is neurally controlled. A comprehensive picture of the control of circadian locomotor behavior is therefore emerging in *Drosophila*, from biochemical to neural circuit mechanisms. In addition to eclosion and locomotor behavior, circadian rhythms also influence courtship behavior, which in males is more pronounced at night [1]. They also modulate olfactory sensitivity [2] and must influence many other sensory and physiological processes since circadian rhythms can be detected in many organs and body parts of the flies, including wings, legs, gut, and Malpighian tubules [3–5].

The *Drosophila* Circadian Pacemaker

The core of the *Drosophila* pacemaker is a negative transcriptional feedback loop [6] (Fig. 2). CLOCK (CLK) and CYCLE (CYC) proteins heterodimerize and bind to E boxes present in the *per* and *tim* promoters to activate these genes [7–11]. PERIOD (PER) and TIMELESS (TIM) form a complex with TIM protecting PER from proteasomal degradation [12–15]. The PER/TIM dimer enters into the nucleus and inhibits *per* and *tim* transcription by blocking the activity of the CLK/CYC heterodimer, probably by reducing its affinity for its DNA binding sites [9, 16, 17].

The precise timing of activation and repression of *per* and *tim* transcription is regulated by posttranslational modification of PER, TIM, and CLK. A set of kinases and phosphatases regulates the activity and the stability of these three proteins and determine for PER and TIM the timing of their entry into the nucleus. The kinase DOUBLETIME (DBT) is particularly important since it regulates PER and CLK phosphorylation, their stability, and activity [17–20]. In fact, DBT stays attached to PER during the translocation of the PER/TIM complex into the nucleus [21] and can thus regulate CLK activity. In addition to DBT, PER phosphorylation is regulated by casein kinase II and protein phosphatase 2A [22–24]. The latter one also regulates CLK phosphorylation levels [20]. TIM phosphorylation is regulated by the kinase SHAGGY/GSK3β and by protein phosphatase 1 [25, 26]. How the activity of these kinases and phosphatases is coordinated to precisely time the progressive phosphorylation of CLK, PER, and TIM remains unclear. *per/tim* transcription is fine-tuned by a second transcriptional feedback loop: the *clockwork orange* (*cwo*) gene is a direct target of CLK/CYC, and encodes a bHLH transcription factor that competes with CLK/CYC for E-box binding [27–30]. An additional interlocked feedback loop regulates *Clk* transcription, but its function remains unclear [31–34].

Thus, the circadian pacemaker in *Drosophila* is a combination of transcriptional and posttranscriptional regu-

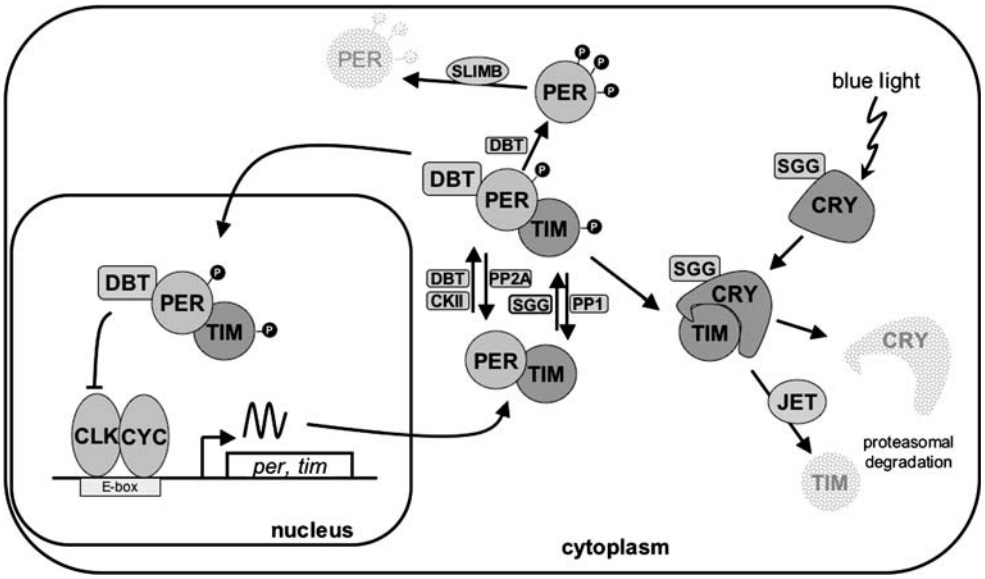

Fig. 2 The *Drosophila* circadian pacemaker and the CRY light input pathway. See main text for details. *Speckled shapes* indicate proteins undergoing proteasomal degradation. *P* phosphate groups

lation. This basic principle is valid in most organisms studied so far. Interestingly, the molecular architecture of the *Drosophila* and mammalian circadian feedback loops are very similar [35]. Homologs of PER, DBT, CLK, and CYC are key elements of the mammalian clock.

As mentioned above, although its oscillations can persist for weeks, the circadian molecular pacemaker has to be reset every day to remain properly phased with the day/night cycle. As will be discussed below, light, temperature, and even social/olfactory cues can synchronize *Drosophila* circadian rhythms. First, however, we need to describe the neural network controlling circadian behavior.

Neural Control of Circadian Locomotor Behavior

Circadian locomotor behavior is usually monitored in *Drosophila* by placing single flies in small tubes and by monitoring the occurrence of disruption of an infrared beam to detect locomotor activity. Monitoring is performed in environmental incubators with light and temperature control. The natural light to dark (LD) cycle is extremely difficult to reproduce in a laboratory context, because at dawn and dusk, light intensity progressively increases and decreases respectively, and spectral composition of light also changes gradually. In laboratory conditions, the natural light to dark cycle is usually grossly mimicked by turning on and off the lights of the incubator. Thus, the so-called laboratory dusk is a light-off transition, while the dawn is a light-on transition. In such an experimental LD cycle, with a day and night length of 12 h and with temperature kept constantly at 25°C, *Drosophila* typically displays a bimodal locomotor activity (Fig. 1). Flies anticipate dawn by increasing their activity about 2 h before the light-on transition and also anticipate by 3–4 h the light-off transition. The sharp peaks detected at the light-on and light-off transitions are due to noncircadian responses to these transitions (startle response or positive masking), since they are also found in mutant flies with no circadian rhythms. In DD (constant darkness) and at 25°C, the morning activity peak tends to disappear, leaving only what appears to be an evening peak of activity.

per and other clock genes are expressed in approximately 150 neurons in the fly brain [36–39] that are thus candidates for controlling circadian behavior (Fig. 3). These circadian neurons form clusters originally named according to their anatomical location. These names therefore do not necessarily reflect functional entities. They are divided into six major groups: the ventrolateral neurons (LN_vs), the dorsolateral neurons (LN_ds), three groups of dorsal neurons (DN1s,2s,3s), and the recently described lateral-posterior neurons (LPNs). These neurons can be further subdivided according to their soma size and specific genes they express.

The LN_vs can be divided in three subgroups. Four small LN_vs (s-LN_vs) and four to five large LN_vs (l-LN_vs) express the neuropeptide pigment-dispersing factor (PDF). A single additional PER-expressing neuron is located in the vicinity of the other LN_vs but does not express PDF. This neuron is already present in the larval brain together with the PDF-positive s-LN_vs and is therefore called the "fifth s-LN_v" [40]. The other groups of circadian neurons do not express PDF. The dorsolateral neurons (LN_ds) form a heterogeneous group of six cells. One cell is larger than the other, and only three to four LN_ds express detectable amount of the photoreceptor CRYPTOCHROME (CRY) [39, 41, 42]. The DN1s can also be divided into at least two subgroups [38]. Two DN1s, called anterior DN1s ($DN1_a$s) based on

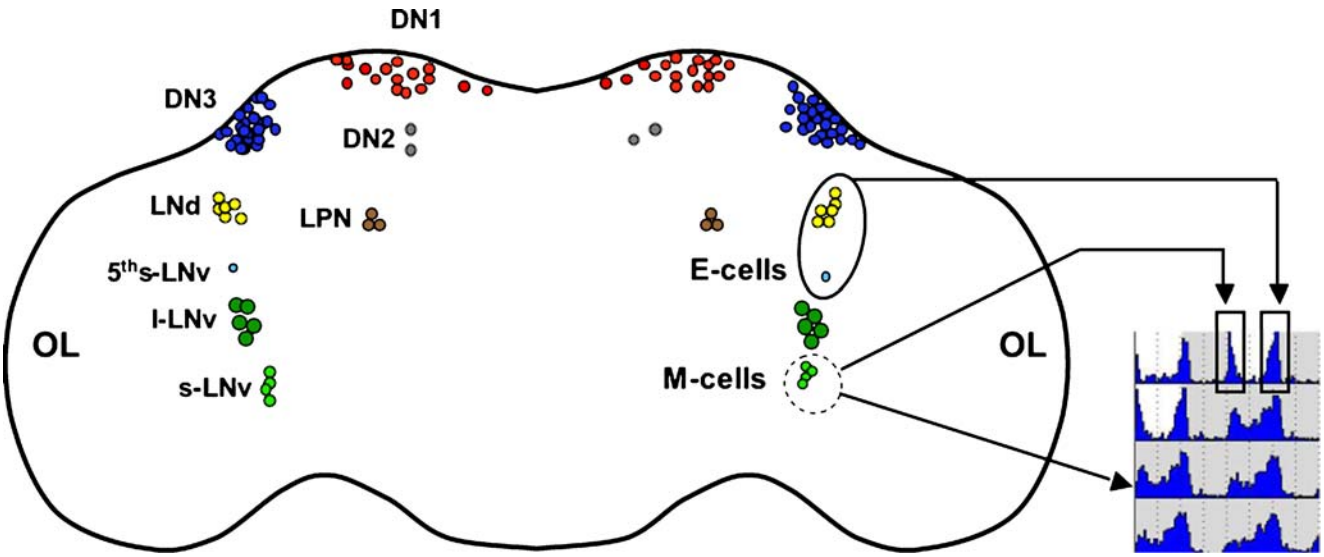

Fig. 3 The circadian clock neurons in the Drosophila brain. The small ventrolateral (s-LN_vs) and the large ventrolateral neurons (l-LN_vs) are the only PDF-positive cells. The fifth s-LN_v, the dorsolateral neurons (LN_ds), the lateral posterior neurons LPNs, and the three groups of dorsal neurons (DN1s, 2s, and 3s) do not express PDF. The s-LN_vs are frequently called morning cells (M-cells), since they are necessary for the morning peak of activity under LD conditions. They are also necessary for free-running locomotor activity rhythms in DD. The fifth s-LN_v and the LN_ds generate evening activity under LD conditions and are therefore called evening cells (E-cells). *OL* Optic lobe

their planar location compared to the other DN1s, are the only circadian neurons expressing the neuropeptide IPNamide [43]. The 15 posterior DN1s ($DN1_p$s) are developmentally distinct since they are not present in larvae unlike the $DN1_a$s. They all express the GLASS transcription factor, which is necessary for visual photoreceptor differentiation [44]. The $DN1_a$s and apparently only a subset of $DN1_p$s express CRY, indicating that the $DN1_p$s are a heterogeneous cell population [42]. There are in addition two DN2s, approximately 40 DN3s that appear heterogeneous in size, and 3 LPNs. This complex organization suggests that specific neurons have dedicated roles in the control of circadian rhythms.

In the past decade, researchers have addressed the question of the hierarchy and function of different groups of circadian neurons in the control of locomotor activity. These studies took advantage of the GAL4/upstream activator sequence (UAS) binary system [45] (Table 1). In this system, transgenic flies expressing the yeast transcription factor GAL4 under the control of a *Drosophila* promoter are crossed to a second transgenic line carrying a gene of interest controlled by GAL4 binding sites (upstream activator sequence). The progeny of these flies, carrying both transgenes, will express the gene of interest in a spatial pattern determined by the promoter controlling GAL4. GAL4-driven expression can be repressed by

Table 1 GAL4 driven gene expression pattern obtained with various combinations of *GAL4* and *GAL80* transgenes

Driver/repressor transgenes	s-LN_vs (4)	l-LN_vs (5)	5ths-LN_v (1)	LN_ds (6)	$DN1_s$(~17)	$DN2_s$ (2)	$DN3_s$ (~40)
pdf-GAL4	++	++	–	–	–	–	–
cry_{13}-GAL4	++	++	++	++	+/−2	–	–
cry_{13}-GAL4 pdf-GAL80	–	–	++	++	+/−2	–	–
tim-GAL4	++	++	++	++	++	++	++
tim-GAL4, pdf-GAL80	–	–	++	++	++	++	++
Mai179-GAL4	++	–	++	+/−3	–	–	–
C929-GAL4	–	++	–	–	–	–	–

For each cell type, the total number of neurons per hemisphere is indicated. For *Mai-179*, weak expression was occasionally detected in one l-LN_v [47]. For *cry-GAL4*, we present in this table the pattern of expression originally reported by Stoleru et al. [48]. Very similar expression pattern was obtained by Busza et al. [91]. However, Shafer et al. observed *cry-GAL4* driven GFP expression in the two IPNamide positive $DN1_a$s, two additional DN1s and 2 DN3s [38]. The LPNs have not been systematically studied and are thus not included in this table.
++ Expression in all neurons of the cluster, +/− expression in a subset of neurons; the number of neurons with expression is indicated, − no expression

GAL80 [46]. Thus, different promoters can be used to express GAL4 and GAL80 to refine the expression pattern of the gene of interest (see Table 1). These tools were used to rescue PER expression in arrhythmic per^0 flies in specific groups of clock neurons [47] or to ablate specific circadian cells by expressing the proapoptotic gene *head involution defective* (*hid*) [48, 49]. Ablation of the PDF-positive circadian neurons (the LN_vs) with *pdf-GAL4* combined with *UAS-hid* results in arrhythmic behavior in DD and the loss of morning anticipation [48, 49]. On the other hand, *per* expression restricted specifically to these neurons in a per^0 background rescues both light-on anticipation and rhythmicity in DD [47]. The LN_vs are thus necessary and their circadian molecular pacemaker sufficient to maintain rhythmic behavior in constant darkness and to control the morning peak of activity in LD. They are therefore now frequently referred to as morning cells (M-cells). It should be noted that rescuing CYC expression in the LN_vs of cyc^0 flies with *pdf-GAL4* does not restore rhythmic behavior and morning anticipatory behavior [50]. These results appear to contradict those obtained with per^0 rescue. However, *pdf-GAL4* might not be expressed early enough during development to rescue the morphological defects affecting the LN_vs of cyc^0 flies (abnormal projections) [51].

The M-cells requires the neuropeptide PDF for their function, since pdf^0 mutant flies have a phenotype similar to flies without M-cells [49]. PDF appears to be secreted rhythmically through dorsal projections of the s-LN_vs, in the vicinity of dorsally located neurons [51]. The receptor for PDF is expressed in at least a subset of these dorsal neurons [52–54], and a recent elegant study based on real-time cAMP imaging demonstrates that actually most dorsal neurons (and the s-LN_vs themselves) do respond acutely to the PDF neuropeptide [55]. Since rhythms decrease rapidly in amplitude and coherence when PDF is absent, it seems likely that rhythmic secretion of PDF is crucial for the synchronization of different groups of circadian neurons and thus for persistent rhythms in DD [50, 56]. Further evidence implicating the s-LN_vs as M-cells comes from the use of a GAL4 driver expressed in the s-LN_vs and three LN_ds, but not in the l-LN_vs (*Mai179-GAL4*). Morning anticipation is restored when this driver is used to rescue PER expression in per^0 flies, but it is not if a l-LN_v-specific GAL4 driver is used (*C929-GAL4*) [47].

Ablation of the LN_ds, the PDF-negative fifth s-LN_v, and 2 DN1s results in the total absence of evening anticipation [48]. When PER expression is rescued in 3 LN_ds and the fifth s-LN_v in an otherwise per^0 fly, the evening peak is restored [47]. In addition, the phase of PER and TIM oscillations in the larger LN_d and the fifth s-LN_v has been associated with the phase of the evening peak [39, 57]. Based on this set of results, we will only include in the evening cell (E-cell) group the LN_ds and the PDF-negative fifth s-LN_v, although a subset of DNs are sometimes also considered to be part of the E-cell group [48, 58].

Entrainment of the *Drosophila* Circadian Clock

Circadian Light Input Pathways

The *Drosophila* circadian clock is very sensitive to light input. LD cycles with light intensities corresponding to quarter-moon light (0.03 lux) during the light phase can synchronize circadian behavior [57]. Brief high-intensity light pulses (as short as 1 min) given during the night or the "subjective" night (if flies are in constant darkness prior to the light pulse) can phase shift circadian behavior by several hours [59, 60]. The directionality of the phase shift is dependent on the time at which the light pulse is administered: phase delays are observed with early night light pulses, while phase advances are triggered by late-night light pulses. During the subjective day, flies are not responsive to light pulses. A phase response curve can be obtained by plotting the magnitude and directionality of the phase shift as a function of the time at which the light (or temperature) pulse is administered [59, 61–63].

Most tissues with circadian rhythms are light sensitive, and light is most likely perceived cell autonomously [4]. Tissues such as wings and legs can be cultured, and light-entrained circadian rhythms can be detected with the help of a *per promoter–luciferase* reporter transgene. CRY is the intracellular photoreceptor that synchronizes the circadian clock in most tissues of the fly, including the circadian neurons controlling locomotor behavior [60, 64–69]. CRY binds directly to TIM in a light-dependent manner and triggers proteasomal degradation of TIM [70–72] (Fig. 2). TIM degradation exposes PER to phosphorylation and subsequent proteasomal degradation [14, 15, 73, 74], which results in the resetting of the circadian pacemaker. In cry^b mutant flies, which carry a point mutation affecting an amino acid residue crucial for flavin binding and hence for CRY photoreceptive function, TIM is not degraded upon light exposure, and this leads to severe disruption of circadian light responses [64, 66, 75]. cry^b flies remain rhythmic under constant light (LL), a condition in which wild-type flies become rapidly arrhythmic, presumably because wild-type CRY is constantly activated by light and TIM continuously degraded. Moreover, short light pulses administered during the night do not phase shift cry^b flies. The recent behavioral analysis of a complete amoprhic *cry* allele (cry^0) confirms the crucial role of CRY in circadian light responses [76].

The precise mechanism by which CRY triggers TIM degradation is still unclear. It might involve the phosphory-

lation of TIM tyrosine residues [71]. Recently, it has been shown that the F-box protein JETLAG (JET)—which is part of an SCF E3 ubiquitin ligase complex—can promote TIM degradation [77, 78]. TIM light-dependent degradation is reduced in *jet* mutants. Moreover, JET expression is necessary in the *Drosophila* embryonic S2 cell line to recapitulate CRY-dependent TIM degradation after light exposure. In addition, like cry^b flies, *jet* mutants are rhythmic in LL, and their ability to respond to short light pulses is diminished, although not abolished. Importantly, the *jet* phenotypes are only observed when the *jet* mutations are combined with a specific *tim* allele, *ls-tim* [78]. In this variant, the presence of two alternative start codons results in expression of a long and a short form of TIM [79–81]. The long form contains 23 extra N-terminal amino acids and is much less sensitive to light because it binds poorly to CRY (for a detailed review on the functional implications of *tim* variants and their selection in natural populations of *Drosophila*, see [82]). In the *s-tim* background, in which only the short highly light-sensitive form of *tim* is produced, the jet^C mutation does not result in rhythmicity in LL. Thus, while JET certainly promotes TIM degradation, it remains unclear whether it is actually necessary for TIM degradation in vivo. Since the two known *jet* alleles are point mutations affecting only one amino acid [77], it is possible that the mutant proteins retain TIM-ubiquitination activity. A complete *jet* loss-of-function mutant would reveal whether JET is essential for light-dependent TIM degradation and circadian photoresponses, or whether other E3 ligases are involved in these phenomena. The kinase SGG has recently been proposed to be involved in regulating CRY activity [58]. Flies overexpressing SGG are rhythmic in LL, like cry^b. At the molecular level, SGG overexpression increases CRY stability in S2 cells and in vivo, even in the presence of light, which usually triggers CRY degradation [65, 83]. Moreover, SGG can bind CRY, although this interaction is not affected by light. In SGG-overexpressing fly brains, TIM light-dependent degradation is impaired. SGG therefore appears to inhibit CRY's ability to trigger TIM degradation.

The CRY input pathway synchronizes the circadian clock in most tissues. However, there are a few peripheral tissues that may not require CRY for light entrainment: the Malpighian tubules (the kidney of *Drosophila*), wing, and the antennae can be efficiently synchronized in cry^b flies, even when these organs are isolated in culture media [84, 85]. Since cry^b is a severe hypomorphic allele, but not a null mutation [76], the possibility remained that in these tissues, there is enough CRY activity for entrainment in cry^b flies. However, even in flies with a complete amorphic *cry* allele (cry^0), circadian synchronization is observed in wings and antennae [76]. Thus, an unknown photopigment can mediate light entrainment in these tissues. For the circadian neurons that control locomotor behavior, it is clear that they can also be entrained by visual photoreceptors [64, 86]. However, entrainment is slower and requires long periods of light exposure [66]. The exact neural pathways by which the eyes and ocelli, an extraretinal opsin-based photoreceptor, synchronize circadian neurons are not known. However, the Hofbauer–Buchner eyelet, which is a functional remnant of the larval eye located under the retina, is most likely directly synaptically connected to the s-LN_vs [87, 88]. The molecular mechanisms synchronizing the circadian clock of these cells with visual photoreception input remains unknown.

Temperature Entrainment

Like LD cycles, warm and cold temperature (thermophase/cryophase, TC) cycles can entrain the circadian clock. Flies can be synchronized by TC cycles with an amplitude as small as 3°C [89]. Moreover, TC cycles can synchronize locomotor activity rhythms under constant light (LL) [90], even though LL disrupts circadian behavior when temperature is kept constant. Adult flies synchronized to TC cycles and released in constant conditions (darkness and constant temperature) are behaviorally rhythmic, and the phase of the rhythms is determined by the phase of the TC cycle, which demonstrates unambiguously that temperature is a circadian Zeitgeber for circadian locomotor behavior [91]. Molecular rhythms are also entrained by temperature [64, 92]. Interestingly, the phase of molecular and behavioral rhythms entrained to temperature is advanced compared to those obtained with light, if the beginning of the day (or ZT 0) is the light-on or the temperature-up transition [91, 93]. In natural conditions, however, the temperature cycle is delayed by several hours compared to the LD cycle. If this delay is taken into account, temperature and light actually generate circadian rhythms of similar phases, and these two Zeitgebers thus probably synchronize circadian rhythms synergistically [93].

Little is known about the molecular mechanisms underlying clock entrainment by temperature cycles. The antennae contain peripheral thermosensors responsible for moderate temperature sensing (around 25°C) [94]. However, ablating the antennae does not affect significantly the entrainment of peripheral circadian clocks and circadian behavior by TC cycles [92] (Busza and Emery unpublished data). Furthermore, isolated organs (legs, wings, head, etc…) exposed to TC cycles in culture show rhythmic *per-luciferase* expression, demonstrating that circadian thermoreception is tissue-autonomous, and probably cell-autonomous, like light [92].

A mutant (*nocte*) shows specific defective responses to temperature input [92]. Circadian bioluminescence rhythms in *per-luciferase* transgenic flies are severely disrupted

under a TC cycle, but LD entrainment is normal. Behavioral entrainment with TC cycle in LL is also compromised. The identification and study of the *nocte* gene should help to understand the molecular mechanisms underlying the synchronization of *Drosophila* circadian rhythms with TC cycles.

We mentioned earlier that short light pulses could phase shift the circadian clock. Thirty minutes high-temperature pulses (37°C) can do so too in wild-type flies when administered during the early night [62]. Interestingly, the photoreceptor CRY is necessary for this response (cry^b flies are insensitive to 37°C pulses), and a temperature-dependent interaction between CRY and the PER/TIM dimer is strikingly observed only during the early night, when temperature pulses can phase shift the circadian clock [95]. A mutation in the *per* gene (per^L) lowers the temperature threshold at which CRY can reset circadian behavioral rhythms and interact with PER/TIM to 30°C. It was therefore proposed that temperature affects the conformation of the PER–TIM dimer and allows it to interact with CRY, even in the absence of light. However, with temperature cycles close to those experienced by *Drosophila* in the wild, CRY does not seem to be required for temperature entrainment [64, 91], indicating the existence of other mechanisms mediating temperature responses. Interestingly, the CRY-dependent response to constant light (arrhythmicity) is influenced by ecologically relevant ambient temperature. At low temperature (15°C), wild-type flies can remain rhythmic under LL, while at 25°C they are arrhythmic [95]. Therefore, CRY light-dependent interaction with PER/TIM is probably fine-tuned by ambient temperature. This modulation might contribute to the integration of different environmental inputs.

So far, we have described the most basic forms of circadian plasticity. The clock detects a change in a physical property of its environment and adjusts its phase to be properly synchronized with the day/night cycle. These adjustments are crucial since circadian clock period does not precisely match 24 h. However, as discussed above, fruit flies are exposed to a complex environment, with seasonal changes in day length and ambient temperature, which requires refined plastic timing mechanisms. These mechanisms are found at a molecular and neural level.

Molecular Mechanisms Contributing to the Temperature-Dependent Plasticity of the Circadian Clock

Temperature-Dependent *per* Splicing

As described above, wild-type flies show a typical bimodal activity under 12:12 h LD cycles at 25°C. They anticipate the light-on and light-off transition by increasing their locomotor activity a few hours before these transitions occur (Fig. 1). The phase of the evening peak is very sensitive to temperature: it is advanced at 18°C and delayed at 29°C [96]. In constant darkness, the evening peak is also phased later with increasing temperature; but the morning peak behaves differently: it is advanced with warmer temperatures. It has been proposed that these phase adjustments are important for seasonal adaptation, but they could also be required to time precisely behavioral activity every day as a function of ambient temperature. Indeed, with cold weather, it is probably beneficial for flies to be active during the warmth of the middle of the day. In contrast, in hot weather, flies should avoid midday heat and be mostly active around dawn and dusk, when temperature is cooler.

Interestingly, the ratio of two *per* mRNA isoforms (A and B′) is regulated by temperature. *per* intron 8 is more efficiently spliced at low temperature, and this splicing advances *per* mRNA accumulation and hence PER protein synthesis [96]. Genetically manipulated flies that cannot splice intron 8, either because the intron is missing or because the splicing sites are mutated, cannot properly adjust PER cycling and circadian behavior when temperature is cold and photoperiod short (6:18 h LD cycle). Therefore, it is the action of splicing *per* intron 8 that is required for phase adjustment. Splicing is frequently coupled with recruitment of factors necessary for proper maturation of mRNAs and nuclear export [97]. This could explain how intron 8 splicing accelerates PER synthesis under cold temperature. While this model explains well the behavior of the evening peak of activity at different ambient temperatures, the causes for the delay of the morning peak of activity in DD at low temperature remain unknown. Since the morning and evening peaks are controlled by different neurons, it is possible that *per* splicing is differentially regulated in specific clock neurons.

There is a complex relationship between light input and the thermal regulation of the circadian phase. First, photoperiod contributes to the regulation of the phase of the evening peak of activity [96, 98]. It was proposed that a short photoperiod results in earlier TIM accumulation, while longer photoperiod would delay it [96]. This would affect PER accumulation similarly since TIM protects PER from degradation. Therefore, cold temperature and short photoperiod would cooperate to advance PER accumulation and hence behavior phase. There is also strong evidence that photoperiod affects the relative contribution of specific circadian neurons to the control of the phase of circadian behavior [58]. This will be discussed in detail below. Second, *per* intron 8 splicing is regulated by photoperiod length at cold temperature [99]. The efficiency of *per* splicing oscillates during the day: it is higher at night. With

a short photoperiod, the oscillation in splicing frequency is phase-advanced, and the overall splicing efficiency is increased. Moreover, light can acutely inhibit *per* intron 8 splicing [99].

The light regulation of *per* intron 8 splicing is thought to contribute to delay PER accumulation under conditions of long photoperiods and warm days. These observations have led to investigate the role of visual and CRY photoreception in the photic regulation of *per* splicing. cry^b mutant flies were found in Majercak et al. to have a reduction in splicing efficiency at both high and low temperature [99], while Collins et al. found elevated splicing efficiency in the dark phase of the LD cycle, particularly at warm temperature [100]. It is therefore difficult to speculate on a potential role of CRY in the photic regulation of *per* splicing. NORPA is a phospholipase C essential for fly vision [101]. *per* splicing was found to be elevated in blind *norpA* mutant flies [99, 100]. Since whole heads extracts were used in these studies, it is predominantly *per* splicing in the eyes that was measured. Hence, NORPA probably inhibits *per* splicing cell autonomously. Whether it does so also in the brain is not yet known, but evening behavior phase is advanced in *norpA* mutants compared to wild-type flies [99]. This would correlate with increased *per* splicing and thus suggest that NORPA either influence *per* splicing in clock neurons cell autonomously or through eye-dependent synaptic signaling. Surprisingly, however, even in DD, *norpA* mutant flies show elevated *per* splicing, and acute photic inhibition of *per* intron 8 splicing is not abolished. The conclusion is that NORPA affects *per* splicing independently of light. The light pathway that regulates *per* splicing thus remains unknown. What then is NORPA's function? It was proposed that NORPA is part of the signaling cascade determining the sensitivity of *per* splicing to temperature [99, 100]. However, in Majercak et al., the temperature dependence of *per* splicing was not attenuated in *norpA* mutants [99], while it was only partially reduced in Collins et al. [100]. What appears to be actually affected is the overall efficiency of *per* splicing—increased at all temperatures—and its circadian control under warm temperature conditions. Whether there is indeed a dedicated temperature signaling cascade regulating *per* splicing still needs to be demonstrated. It is equally possible that the mRNA conformation near the splicing sites, and thus the ability of specific splicing factors to bind these sites, is directly regulated by temperature.

Temperature-Dependent *tim* Splicing

Like *per*, the splicing of a specific *tim* intron is sensitive to temperature. *tim's* last intron is more frequently retained at cold temperature, resulting in a slightly shorter form of the TIM protein that is missing part of the cytoplasmic localization domain [93]. Whether this small truncation affects TIM activity is not yet known. In a 25/18°C TC cycle, the expression of the nonspliced isoform of *tim* is delayed relative to *per* expression, but the tim^{cold} transcript peaks in phase with *per*. At the protein level, however, PER and TIM proteins oscillate in phase in head extracts. The function of the tim^{cold} variant remains to be elucidated. It could contribute to fine-tuning PER and TIM oscillations with the daily thermal cycle or adjusting TIM cycles to colder temperature. Hence, temperature-dependent splicing of both *tim* and *per* might contribute to seasonal adaptation.

Light-Dependent Cold-Gated *tim* mRNA Induction

In addition to temperature dependent *tim* splicing, another molecular mechanism, dependent on temperature and light, might be involved in adaptation of the clock to 'winter' days. At cold temperature (18°C), but not at warm temperature (25°C), light can rapidly increase *tim* mRNA abundance [102]. Although the underlying mechanism is still unknown, transcriptional regulation is involved since light also rapidly increases *luc* mRNA in transgenic flies bearing the *luciferase* gene fused to the *tim* promoter. Moreover, CLK/CYC, but not PER/TIM, are required for *tim* photoinduction, which can happen only when the *tim* promoter is derepressed. The phototransduction cascade that results in *tim* induction at cold temperature is not yet identified. *tim* photoinduction is not affected in cry^b and *norpA* mutants, suggesting that neither CRY nor the canonical visual phototransduction cascade is required. Double *norpA*—cry^b mutants were not tested, however—and redundancy between these two pathways cannot be excluded.

What could be the role of such a mechanism? It might contribute to advance the phase of locomotor activity in short photoperiod conditions, which are usually associated with cold temperatures in natural conditions. Actually, *tim* mRNA level rises just after the light is turned on in flies entrained to a short photoperiod at 18°C, whereas *tim* mRNA accumulation is delayed by 6 h when flies are entrained to a 12:12 LD cycle.

In summary, there are multiple molecular mechanisms by which ambient temperature can adjust the phase of the *Drosophila* circadian pacemaker. Importantly, at least two of these mechanisms are not simply controlled by temperature but also by light input. Thus, they serve as mechanisms of integration of multiple inputs and should therefore contribute to both seasonal and daily adjustment of the phase of circadian rhythms. Interestingly, it is becoming increasingly clear that for circadian locomotor behavior, plastic contribution of circadian neurons also determines the adaptability of the circadian clock.

Plasticity of the *Drosophila* Circadian Neural Network

Modulation of the Circadian Neuronal Network by Light

For more than a decade, the s-LN_vs have been considered to be the circadian pacemaker cells for locomotor behavior. Indeed, they are necessary and sufficient for persistent locomotor rhythms and for the proper synchronization of PDF-negative circadian neurons under constant darkness [47, 49, 50, 56, 103]. However, recent studies have demonstrated that PDF-negative circadian neurons can also control circadian behavior when light is present.

Under constant light conditions with a specific light intensity (approximately 500 $\mu W/cm^2$), cry^b flies show a striking phenotype. Their behavioral rhythms split into two components after a few days in LL: a short rhythm with an approximately 22.5-h period and a long rhythm with a approximately 25-h period [39, 104]. This split behavior can also be observed in wild-type flies released in constant low light intensity. By monitoring PER cycling in specific groups of neurons with PER immunostaining, the short rhythm was shown to be driven by the s-LN_vs, while the long rhythm is driven by a single LN_d and the PDF-negative small LN_v [39]. This dissociation is not observed in the dark or in visually blind flies. Therefore, visual input can shorten or lengthen the period of the circadian pacemaker in specific clock neurons. This phenomenon could explain how the photoperiod influences the phase of circadian behavior: the morning oscillator (s-LN_vs) would advance its phase under long photoperiods because its period shortens, while the evening oscillator (the PDF-negative s-LN_v and one LN_d) would lengthen its period and hence delay its phase. However, this model, proposed originally by Pittendrigh and Daan based on the observation of split behavior in mammals [105], does not exactly apply to *Drosophila*. Indeed, the s-LN_vs (M-cells) appear to contribute, at least in LL, to the evening bout of activity. It is clear on behavioral recording of single flies that the evening peak of activity split after a few days in LL into the two aforementioned components. A morning component is also observed in some flies, with the characteristic short period attributed to the M-cells. Hence, it was proposed that these cells be referred to as main cells—rather than morning cells—since they can contribute to both M- and E-peak of activity [39]. A related observation was made when light intensities corresponding to moonlight levels were used instead of complete darkness in a photic cycle (LM instead of LD) [57]. Molecular oscillations were advanced in M-cells and delayed in at least one E-cell (the fifth LN_v). This plasticity in the phasing of the M- and E-cells was correlated with an advanced M-peak and a delayed E-peak. Visual photoreception was necessary for phase adjustment, as it is for modulating the period of the M- and E-cells in LL. As a result, flies show much higher nocturnal activity under an LM cycle than under an LD cycle. The intensity of light during the night might thus profoundly influence circadian behavior in the wild.

The observation that two E-cells can drive a free-running component of a different period than the M-cells strongly suggests that these cells have pacemaker properties, at least under constant light. Recently, three studies have genetically shown that PDF-negative circadian neurons can fully control circadian behavior under constant light [41, 58, 106]. In these studies, the authors analyzed the phenotype resulting from disrupting the CRY pathway in different circadian neurons.

Murad and colleagues disrupted the CRY input pathway by overexpressing the pacemaker gene *per* or *morgue* [106]. PER overexpression presumably bypasses the need for TIM to stabilize PER, and the CRY/TIM input pathway therefore becomes less effective at controlling circadian rhythms in clock neurons. The mechanism by which overexpression of MORGUE, a protein involved in proteasomal degradation [107], inhibits the CRY input pathway is not known. When a driver expressed in all circadian neurons (*tim-GAL4*; see Table 1) was used to overexpress *per* or *morgue*, flies were rhythmic in LL. To determine which neurons were responsible for the phenotype, *per* overexpression was limited to the LN_vs (with *pdf-GAL4*) or to PDF-negative circadian neurons (with *tim-GAL4* and *pdf-GAL80*). Interestingly, overexpressing *per* or *morgue* in the LN_vs did not result in rhythmicity in LL. However, *per* or *morgue* overexpression restricted to PDF-negative neurons did, suggesting that some of these neurons drive LL rhythms. To identify precisely the neurons that drive rhythmic behavior in LL, immunostainings with antibodies directed against the circadian transcription factor PDP1 [108] (for *per* overexpression) and PER (for *morgue* overexpression) were performed (PDP1 and PER levels oscillate robustly). In both cases, the M- and E-cells did not show any molecular circadian oscillations during the third day in LL. Robust molecular rhythms in PER or PDP1 abundance were limited to a subset of DN1s (approximately six cells) with a phase corresponding to that expected from the period length of *per* or *morgue* overexpressing flies in LL (26–26.5 h). This strongly suggests that the DN1s drive LL rhythms. Moreover, cry^b flies in which CRY expression is rescued specifically in the LN_vs (consequently making these neurons molecularly arrhythmic) are rhythmic in LL, and robust molecular rhythms were again observed in a subset of DN1s. Stoleru and colleagues obtained similar genetic and anatomical evidences by overexpressing *shaggy*, which also results in rhythmic behavior under constant light, since SGG inhibits CRY (see above) [58]. However, they only observed rhythmic PER nuclear entry in most DN1s and the two DN2s, but surprisingly no change in PER (or TIM) abundance.

More recently, the group of F. Rouyer used refined genetic manipulations to determine which group of cells can drive LL rhythms [41]. They first rescued *cry* expression in the cry^b background or downregulated *cry* by expressing an RNAi transgene in different groups of neurons and determined whether flies that are mosaic for wild-type CRY expression can remain rhythmic in LL. The *Mai179-GAL4* driver indicated that the E-cells (3 CRY-positive LN_ds and the PDF-negative small LN_v) play an important role in driving LL rhythms. Then, Picot et al. elegantly restricted PER expression to the E-cells of per^0;cry^b flies. These mosaic flies were rhythmic under constant light, demonstrating unambiguously that the E-cells are sufficient for LL rhythms. Then, are they strictly required? Apparently not since inactivation of these cells by restoring CRY expression in cry^b flies does not abolish rhythms: about a third of these partially rescued flies are still rhythmic in LL. Moreover, the results of the two aforementioned studies would suggest that the DN1s drive rhythms when the M- and E-cells are inactivated by CRY expression.

Interestingly, both Picot et al. and Murad et al. found that PDF-negative neurons can drive circadian rhythms in the absence of the neuropeptide PDF [41, 106], which is secreted by the LN_vs and is required for rhythms in DD [49]. The DN1s/E-cells are therefore capable of maintaining circadian behavior independently of the M-cells. However, PDF and the M-cells are not entirely without effect on LL rhythms: the period is shorter in the absence of PDF, and rhythms obtained when *morgue* is overexpressed in all circadian neurons are not as robust. In addition, with *sgg* overexpression, PDF is required for LL rhythms [58], possibly because excessive SGG not only inhibits CRY, but also alters the properties of the circadian molecular pacemaker, which might make it more susceptible to disruption.

In summary, these studies and previous work demonstrate that the M-cells are required for circadian rhythms in the dark and that PDF-negative neurons can drive circadian behavior when light is constantly present. However, to do so, the CRY input pathway needs to be disrupted in the E-cells or the DN1s. In the wild, this inhibition is most likely not required for these cells to influence circadian behavior. At most latitude, the day to night cycle will synchronize the circadian pacemaker of the E-cells/DN1s, even under short summer night. Even during the Arctic summer, sun elevation changes and thus temperature varies, and as we have seen, temperature cycles can override the disruptive effect of constant light [90].

That the E-cells or DN1s drive circadian behavior in LL while the M-cells drive circadian behavior in DD suggests that their respective contribution to circadian behavior might be regulated by the photoperiod. Stoleru et al. explored this hypothesis by overexpressing *sgg* in the M-cells or in the PDF-negative circadian neurons [58]. Excessive SGG shortens the period of the circadian molecular pacemaker to approximately 22 h, in addition to inhibit CRY. In conditions that mimic summer (14:10 h LD cycle), accelerating the pace of the circadian clock in PDF-negative neurons only affects the phase of both morning and evening anticipatory behavior, whereas speeding up the clock in the PDF-positive cells results in an early M-peak, but does not affect the E-peak. These results suggest that under long photoperiod conditions, PDF-negative neurons dominate the circadian circuitry since they determine the phase of both the M- and E-peaks. On the contrary, in 'winter' conditions (10:14 h LD cycle), the M-cells seem to be the dominant cells. Acceleration of the pacemaker in the M-cells results in advance of both the M- and the E-peak whereas acceleration of the pacemaker of the PDF-negative neurons only affects the E-peak. Therefore, photoperiod influences the relative contribution of the M- and E-cells to the overall control of circadian behavior.

How might the balance between M-cells and E-cells/DN1s neurons be regulated by the photoperiod? It was initially proposed that light might reinforce the pacemaker of the DN1s, since in DD, these cells cannot drive circadian behavior in the absence of M-cells and appear to lose molecular rhythm amplitude, while in LL they remain rhythmic. On the other hand, darkness would strengthen oscillations in the M-cells [58, 106]. However, Picot et al. have obtained solid evidences indicating that light regulates output of M- and E-cells [41] (Fig. 4). Indeed, when CRY expression is inhibited or missing in the M-cells (by expression of an RNAi transgene in wild-type flies or cell-specific rescue in cry^b flies), these cells are molecularly rhythmic in LL while behavior is arrhythmic. However, if in addition the visual photoreceptors are completely eliminated, the flies are rhythmic. This demonstrates that visual input somehow inhibits output from the M-cells. On the other hand, light promotes output from the E-cells. Indeed, in mosaic per^0;cry^b flies with *per* expression restricted to the E-cells, flies are rhythmic in LL, but not in DD, even though the circadian molecular pacemaker is functional in these cells under both conditions. It is likely that output from DN1 cells is also regulated by light.

Many questions remain. For example, why can different groups of circadian neurons (LN_ds, DN1s) apparently control circadian behavior under constant light, depending on the genetic manipulations performed, and possibly also the precise light conditions (each lab uses slightly different conditions). The LN_ds that can drive circadian behavior in LL are CRY positive [41]. Whether the DN1s driving LL rhythms are also CRY-positive is not known with certainty, but CRY needs to be inactivated for these rhythms to occur [58, 106]. Interestingly, Murad and colleagues observed circadian molecular rhythms in approximately six DN1s, and it appears that only six–eight DN1s are CRY-positive [42, 106, 109]. Hence, the CRY-positive LN_ds and DN1s

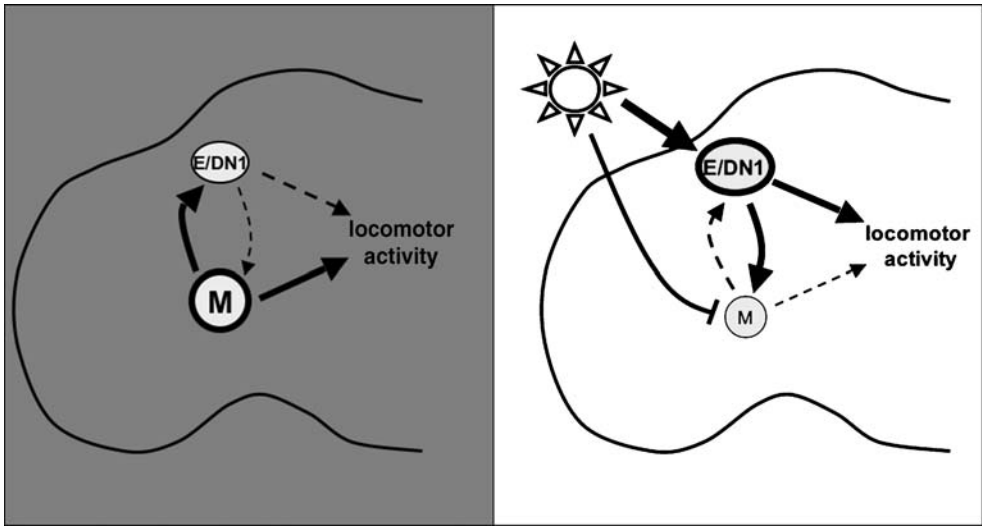

Fig. 4 Model for the regulation of the circadian neural circuit by seasonal changes in photoperiod. In the dark, the M-cells influence the phase of the E-cells and other clock neurons and determine the timing of locomotor activity (default state). When light is present, output signals from the M-cells are inhibited by visual inputs, while output from the E-cells and the DN1s is promoted. The E/DN1-cells thus take control of the circadian system. The respective contribution of the M- and E/DN1-cells to the control of circadian behavior is therefore determined by the photoperiod: long days result in a greater involvement of the E/DN1-cells in the control of the timing of locomotor behavior, and short days promote the role of the M-cells

might actually be a single functional entity, controlling circadian rhythms when photoperiod is long. Differences in CRY levels among different PDF-negative neurons [42, 109] could explain, at least in part, why specific groups of neurons can remain rhythmic in precise light conditions and genetic backgrounds. It is interesting that both the DN1s and the LN_ds send projections in the vicinity of the M-cells, while the M-cells send projections near the DN1s [37, 38, 48]. In addition, the LN_ds and DN1s respond acutely to PDF [55]. This raises the possibility that the M-cells and the LN_ds/DN1s directly and mutually influence each other. Future work will be needed to demonstrate that there is indeed a direct functional connection between these different groups of cells. The development of novel methods to manipulate neuronal activity and the recent advances in electrophysiological recording from circadian neurons [110–115] will hopefully help defining which groups of circadian neurons are functionally coupled and how.

Another question is why under specific conditions of light, the output from the M-cells is not inhibited [39]. As mentioned above, in LL, cry^b flies can show a split behavior generated by both M- and E-cells. Interestingly, it appears that this split behavior requires several days of LL before appearing, suggesting that a rearrangement of the neural circuit occurs after prolonged exposure to light. Such neural plasticity, leading to complex behavior, is also observed when electrical activity is artificially increased in the M-cells by the expression of the bacterial sodium channel NachBac [110, 112]. Strikingly, very similar periodicities as those observed with split cry^b flies are observed, and it is interesting to note that the LN_ds and a subset of DN1s stay in phase when the M-cells are manipulated, suggesting again that these cells might be a functional entity. Thus, neural activity in M-cells, induced by light, might result in a complex rearrangement of the functional interactions between elements of the circuit connecting different circadian neurons. It will be interesting to determine whether these rearrangements are associated with structural reorganization of the circuits. Structural plasticity has recently been identified in M-cells. In constant darkness, as well as under an LD cycle, the complexity of the arborization of the M-cell projections located near the DN1s/DN2s changes over time [116]. These modifications are circadianly regulated. This raises the intriguing possibility that structural changes might also contribute to the responses of circadian circuits to environmental inputs.

There are additional basic behavioral observations that are not well accounted for by the model of photic regulation of M- or E-cell output. In DD at 25°C, wild-type flies are mostly active during the subjective evening, with the morning peak progressively disappearing or maybe merging with the relatively broad evening peak (Fig. 1). If the E-cells require light for their behavioral output, why this evening peak in DD? There are several possible explanations. For example, output of additional circadian neurons—presumably in the LN_d and DN1 groups—is not inhibited in the dark and can produce the evening peak in DD. Alternatively, the E-cells could need the collaboration

of other circadian neurons in DD, but not in LL, that are not rescued in the experiments of Picot et al. [41]. It is also surprising that the M-peak tends to disappear in DD, although the M-cells are dominant in determining the phase of circadian behavior. Moreover, when these cells are the only functional circadian neurons, they generate activity concentrated in the subjective morning in DD [47]. This suggests that in wild-type flies, the M-cell output pathway that regulates morning locomotor activity can be inhibited by other clock neurons after a few days in DD.

How photic modulation of the influence of the LN_vs and the PDF-negative neurons on the circadian network permits seasonal behavioral phase adjustment still need to be clearly defined. However, it appears that the M-cells and PDF-negative circadian neurons differentially contribute to advancing and delaying circadian behavior. When CRY is only expressed in the LN_vs, flies have normal phase-advance responses with a late-night light pulse, but phase delays in response to early night light pulses are reduced [66]. Thus, the M-cells appear to be particularly efficient at advancing behavioral phase, which is expected if they determine behavior during the winter [58]. Disruption of the CRY response by *sgg* overexpression in PDF-negative neurons affects phase delays much more profoundly than phase advances [58]. This would be consistent with a summer role for PDF-negative circadian neurons, when phase should be delayed. However, the prediction would then be that both morning and evening peaks would be delayed if the PDF-negative circadian neurons determine behavioral phase during the summer, and during the winter, both peaks would move earlier under the control of the M-cells. This is not what has been observed under various photoperiods [96, 98]. The morning peak of activity appears much less sensitive to photoperiod than the evening peak, particularly when photoperiod is long. The period shortening induced by light in M-cells [39] might explain why the M-peak is relatively resistant to long photoperiod: the short period induced by light exposure could prevent the morning peak from being excessively delayed by the E/DN1-cells during the summer.

Circadian Neural Network Under Temperature Cycles

The M- and E-cells play a similar role during and after entrainment to light/dark and to thermophase/cryophase cycles [90, 91]. The E-cells control evening activity under TC cycles, but cannot maintain circadian rhythms in the absence of the M-cells, while the M-cells are necessary and sufficient for circadian behavior entrainment to TC cycles performed in DD and for free running rhythms after return to constant conditions. The role of the M-cells in morning anticipation was difficult to assay because cold temperature suppresses locomotor activity (a phenomenon called negative masking). However, during the first day after return to constant temperature, flies with only functional M-cells are mostly active during the subjective morning.

Thus, the hierarchy and the function of the M- and E-cells appear conserved for light and temperature entrainment. Surprisingly, however, when PER expression is restored specifically in the M- and E-cells of per^0 flies, circadian behavior under TC cycle is not fully rescued, while it is indistinguishable from wild-type under LD cycle [91]. Under TC cycle, the evening peak of activity occurs much too early and lasts longer, suggesting that other circadian neurons are necessary for repressing midday activity under TC. In addition, when the M- and E-cells are ablated using the *cry-GAL4* driver to express HID, a late-day peak of activity is detected under TC cycle, but not LD cycle. The phase of this peak is controlled by the circadian clock since it is absent in per^0 flies and delayed or advanced in per^L and per^S flies, respectively. Thus, there are circadian neurons that can positively and negatively influence circadian behavior specifically under TC cycles. These experiments were all performed in a 29/20°C TC cycle. It will be interesting to determine whether these thermosensitive circadian neurons are active at a specific temperature range and might thus seasonally modulate behavior. The identity of the temperature-sensitive circadian neurons is not yet known, but the poorly characterized LPNs appear to be strong candidates. These cells are not rescued or ablated when *cry-GAL4* is used to restore PER expression or to ablate M- and E-cells [91], and they might be particularly sensitive to temperature. In two studies, it was reported that circadian oscillations were only detected in these cells under TC, but not under LD [90, 91]. However, a third study did observe PER and TIM cycling in these cells in an LD cycle [38]. Thus, TC cycles might be necessary for amplifying oscillations in the LPNs and promote their function. Additional evidence demonstrating that the LPNs are particularly sensitive to temperature was obtained when out-of-phase temperature and light cycles were combined for entrainment. The LPNs, as well as the DN2s, followed the temperature cycle, while the M- and E-cells were synchronized by light [117]. It therefore appears that the M- and E-cells are poorly sensitive to temperature cycles. However, the E-peak (and hence the E-cells) is entrained to an 8-h phase-advanced TC within 1–2 days when the M-cells are ablated or when PDF is missing. At least 4 days are required when the M-cells are present. Moreover, when the M-cell pacemaker is weakened by overexpressing *per*, synchronization is greatly accelerated. Thus, the E-cells are very sensitive to temperature cycles, but the M-cells and their robust pacemaker slow down temperature synchronization of circadian behavior [91].

Taken together, these results suggest a specialization of the role of different circadian neurons in the response to

temperature cycles. The M-cells respond slowly to temperature and thus set the pace at which circadian behavior will be synchronized. The E-cells are very sensitive to temperature, which probably helps fine tuning the phase of the evening peak. In addition, under long photoperiods, the temperature sensitivity of the E-cells should influence the phasing of the whole circadian neural network. Finally, neurons specifically sensitive to temperature cycles modulate circadian behavior to ensure its proper phasing. The balance between cells that are weakly and highly sensitive to temperature might be necessary for *Drosophila* to discriminate between random temperature variations that occur when weather changes abruptly and regular oscillations in temperature.

In summary, specific groups of circadian neurons have dedicated function in the control of circadian behavior and its responses to temperature and light inputs. However, these neurons do not function cell autonomously but form an intricate and complex neural network that is remarkably adaptable to various environmental conditions. This plasticity is at least in part attributable to differential sensitivity of specific neurons to specific inputs and to modulation of their output pathways. It is remarkable that of the approximately 75 circadian neurons present in each *Drosophila* brain hemisphere, we may understand reasonably well the function of only 1/5th of them (the s-LN_vs, three LN_ds, and a few DN1s). Isolation and generation of new GAL4 and GAL80 lines with specific patterns of expression and the use of combinatorial genetic approaches (e.g., split GAL4, combination of GAL4/UAS and FLP/FRT system, reviewed in [118]) to genetically manipulate and ablate poorly studied neurons will be essential to define their function. A potential caveat or limitation might be that the effects on locomotor behavior will be more and more subtle. However, it is expected that specific populations of circadian neurons play a crucial role in other specific behaviors under circadian control, such as sleep and courtship.

Social Interactions

Drosophila are not social insects like bees and ants, which are organized in complex hierarchical colonies. However, social interactions between fruit flies occur during courtship, aggressive behavior, and around sources of food. Surprisingly, a study by Levine et al. reported that social interactions can influence the circadian clock [119]. When a small group of flies (visitors) is placed with a larger group (hosts) entrained to a different LD cycle, the flies from the larger group shift their phase toward the phase of the smaller group. Interestingly, olfactory cues are responsible for the phase shifting. The function of social/olfactory inputs needs to be defined, but a possibility exists that these cues could synchronize the activity of potential mates.

Interestingly, a recent study indicates that courtship profoundly modulates circadian behavior [1]. Isolated males show the typical bimodal activity we described, while isolated females rest much less during the day. However, when together, a female and a male change their patterns of activity. Heterosexual couples are more active during the night and in the morning and are the least active around dusk, a time when both single males and single females are the most active. The locomotor activity rhythm is directly correlated with close proximity and copulation and is driven by the male. Whether these male–female social interactions affect circadian rhythms durably (i.e., after the end of the interaction) and whether it shifts molecular oscillation of the clock has not been determined, it is therefore possible that male–female interactions only affect the behavioral output of clock neurons. Nevertheless, this study and that of Levine et al. clearly show that social context influences locomotor activity, adding an additional layer of complexity to the plastic nature of circadian behavior.

Conclusions

Studies of the last decade reveal the complexity of the mechanisms responsible for the responses of the *Drosophila* circadian clock to environmental inputs. Both neural and molecular plasticity of the circadian system have been demonstrated. This complexity is probably needed for dealing with an environment in which multiple variables can change, predictably in many cases, but also erratically. Most of the temperature-dependent molecular mechanisms we described (*per* and *tim* splicing, light-dependent *tim* induction, effects of temperature-sensitive neurons) have immediate effects on the phase of molecular oscillations and circadian behavior. They must therefore be particularly important for dealing with daily, somewhat unpredictable environmental variations in temperature. The photic regulation of circadian neurons would be predicted to have more durable effects, preparing the flies for the next days, advancing or delaying behavior as the light period of the day shortens or lengthens.

The molecular mechanisms that generate circadian rhythms are remarkably conserved in the animal kingdom [35, 120]. What about the mechanisms underlying the synchronization of circadian clocks? They appear to vary considerably between species. This is not surprising: input pathways and mechanisms of synchronization need to be adjusted to the environment in which an animal lives and also need to be adapted to the animal itself. For example, cell-autonomous circadian photoreception—as found in

Drosophila—would not be useful in mammals that are large opaque animals. In fact, the primary mechanisms by which light resets the molecular circadian pacemaker are different in flies and mammals. TIM is acutely degraded by the proteasome in *Drosophila*, while in mammals it is the induction of *per1–2* transcription that resets the circadian clock [121, 122]. Nevertheless, many basic principles governing the synchronization of circadian rhythms are similar in mammals and insects. For example, flies and mammals both rely on dedicated circadian photoreceptors (CRY, melanopsin) and on canonical visual photoreception to synchronize circadian rhythms with the LD cycle [86, 123, 124]. At a neural network level, subsets of circadian neurons are differentially regulated by light in both mammals and *Drosophila*, and this regulation is believed to be critical for the adjustment of circadian rhythms with photoperiod. Indeed, it was recently found that neurons located in the posterior and in the anterior region of the suprachiasmatic nucleus (SCN)—the primary pacemaker neuronal structure in mammals—specifically advance and delay the phase of their molecular oscillations depending on the photoperiod length [125]. This result is reminiscent of the differential effects of constant light on M- and E-cells in flies [39]. Interestingly, communication between circadian neurons is at least in part conserved at the molecular level: the PDF receptor is homologous to the vasoactive intestinal polypeptide (VIP) receptor, VPAC2, and VIP apparently serves a similar function as PDF: synchronizing circadian neurons within the SCN [52–54, 126, 127]. Future work will probably reveal additional similarities between the synchronization mechanisms of flies and mammals. For example, it will be interesting to determine whether microRNAs contribute to the plasticity of the *Drosophila* circadian clock, since recent evidence implicates a mouse miRNA (miR-132) in the modulation of light responses in mammals [128].

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