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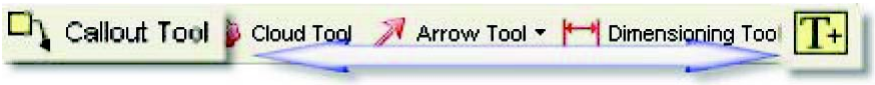

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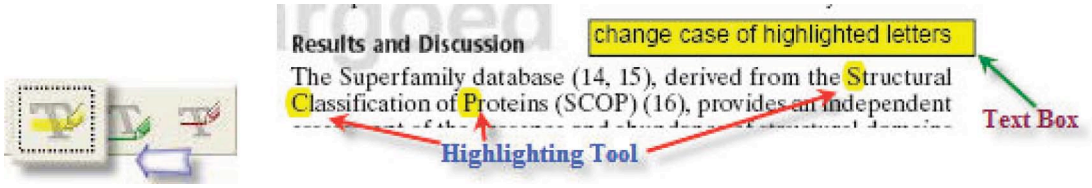

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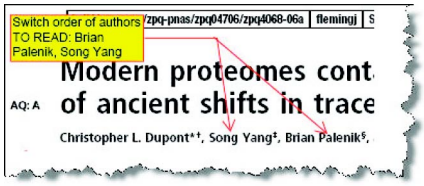

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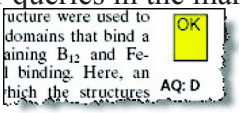

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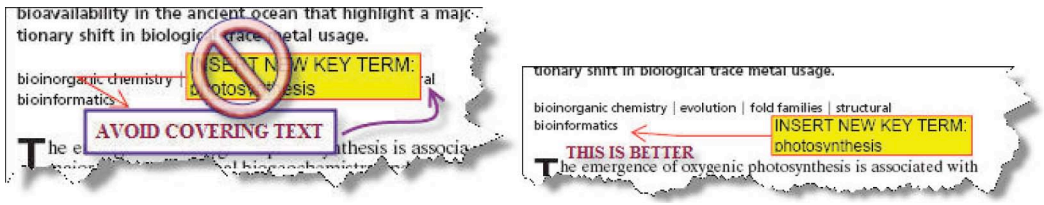

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The Underlying Genetics of *Drosophila* Circadian Behaviors

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Life is shaped by circadian clocks. This review focuses on how behavioral genetics in the fruit fly unveiled what is known today about circadian physiology. We will briefly summarize basic properties of the clock and focus on some clock-controlled behaviors to highlight how communication between central and peripheral oscillators defines their properties.

Introduction

Life on earth imposes dramatic changes to the environment; as a result, endogenous timekeeping mechanisms have evolved to help organisms predict and rapidly adjust to those changes, timing most aspects of their physiology accordingly. It is not surprising, then, that circadian rhythms are pervasive in nature and that tight molecular mechanisms were selected to sustain and adjust the internal conditions to the external milieu. Such cell-autonomous molecular clocks rely on transcriptional/translational autoregulatory feedback loops that recruit transcription factors, kinases, and phosphatases to ensure biochemical oscillations of ~24 h (for recent views on this subject, see Refs. 106, 145).

In *Drosophila melanogaster*, the molecular clock requires the basic helix-loop-helix (bHLH) PAS transcription factors, dCLOCK (dCLK) and CYCLE (CYC). This heterodimer controls the expression of *period* (*per*) and *timeless* (*tim*), whose protein products accumulate in the cytoplasm during the early night and later on move to the nucleus and repress their own transcription (27). The pace of this loop is tightly controlled by the activity of kinases [such as DOUBLETIME (83, 121), SHAGGY (102) and CASEIN KINASE 2A (97)] and phosphatases [PP1 (38) and PP2A (131)] that ultimately regulate PER/TIM stability and subcellular localization (for an in depth review, see Ref. 117). A second, interconnected, negative feedback loop controls *dClk* expression through the action of *vrille* and *Par domain protein 1e* (*Pdp1ε*), two clock-controlled genes (ccgs) that repress (VRI) and activate (PDP1ε) *dClk* transcription (26, 163). More recently, a suite of posttranscriptional regulatory mechanisms have also been shown to play a role in sustaining molecular oscillations (106). Although initially it was interpreted that all cellular clocks were built indistinguishably, differential effects of modulators of CLK-CYC activity such as CREB-binding protein (CBP/NEJ) and CLOCKWORK ORANGE (CWO) suggest a degree of cell-type specificity (117). In *Drosophila* 150 "clock" neurons are grouped in clusters named as ventrolateral neurons (LNvs; encompassing the small and large LNv groups), dorsolateral neurons (LNds), lateral posterior (LPNs), and dorsal neurons [DNs; separated in DN1, 2, and 3 (60)]. All clock neurons support the expression of bona fide clock components, with the exception of *cryptochrome* (*cry*), the circadian photoreceptor in the fly brain (143), which is expressed in roughly half of them (12, 161).

Drosophila has pioneered the field of behavioral genetics, and particularly that of molecular chronobiology, since an original screen designed to test the concept that genes are relevant to behavior led to the discovery of the first behavioral (and circadian) gene *per* (84); since then, most circadian genes were isolated through genetic screens aimed to identify changes in the rhythmic pattern of locomotor behavior (see below). In addition, *Drosophila* is an ideal model system for a genetic approach to understand behavior; to begin with, the complexity of its behavioral repertoire contrasts sharply with the simplicity of its manipulation. Moreover, several decades of genetics and molecular biology and the generosity of fly laboratories have given rise to collections of thousands of engineered transgenic lines freely available for distribution. Finally, the generalized use of binary systems (i.e., the GAL4/UAS system and the *lexA*/*lexAop* system) that rely on the spatial control of the expression of transcription factor(s), which recognize specific cis regulatory sequences not originally encoded in the fruit fly genome, make possible the activation of a gene of interest only when both transgenes are combined (16, 90, 151). Thus binary systems enable the most sophisticated combination to address the role of a given gene in a defined cell type/stage. Inducible versions of these systems (through temperature-sensitive repressors or ligand-induced activators) are available (105); these drivers, along with several collections spanning thousands of transgenic lines specifically directed to every gene in the fly genome (77, 113), enable a highly sophisticated interrogation of the role of a gene or group of cells in the process of interest in a defined time window. Throughout this

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review, we will revisit different aspects of adult physiology that are under the control of the clock, with a focus on those for which behavioral genetics have provided particular insight.

Eclosion as a "One Time Only" Output of the Clock

In insects, development and growth occur through multiple stages. Each of them ends when an insect molt produces a new cuticle. The final step is the adult ecdysis, where the shredding of the remaining cuticle occurs, and the adult emerges from the pupal case. Timing of eclosion is critical for survival and is finely controlled at different levels by steroid hormones, neuropeptides, and the circadian clock (37, 110). Among neuropeptides, the crustacean cardioactive peptide (CCAP), a neuropeptide secreted by cells localized in the ventral nerve cord, has an important role turning on the ecdysis motor program and regulating the circadian timing of adult emergence (118). As a result of such complex control, fruit flies emerge around dawn and early morning, after they have reached developmental maturity. Since eclosion occurs only once in the course of development, circadian rhythmicity stems from the analysis of the population (141), which contrasts with individual rhythmic behaviors such as locomotor activity and egg-laying. To monitor eclosion under laboratory conditions, males and females are housed together under controlled 12:12-h light-dark (LD) cycles. Adult emergence is scored at regular intervals (typically 2 h) over the course of several days. Eclosion can also be measured in an automated fashion (115). If the circadian clock is working properly, eclosion is preserved under free-running [constant darkness (DD)] conditions, with a period close to 24 h. However, if the clock is experimentally accelerated, delayed, or damaged, patterns will show a short or long period or arrhythmicity, respectively.

Although eclosion was described as a circadian output almost 50 years ago, the cellular basis for its circadian control was only recently uncovered. Through behavioral analysis and immunohistochemistry, it was established that the prothoracic gland (PG), an endocrine organ that produces the steroid hormone ecdysone (E) and assesses the growth and size of the developing pupae (112), contains a functional peripheral clock; rhythmic PER and TIM oscillations are detected just before eclosion (34, 99). The PG peripheral clock appears to be necessary for defining a window for eclosion (so-called "gating"), since targeted disruption of its oscillator through *tim* overexpression induces arrhythmic eclosion under DD, in a condition in which the central clock is fully functional (115). Moreover, a reduction in the rhythmic release of ecdysteroids from PG cells was proposed as the mechanism of initiating the endocrine cascade required for eclosion (110).

To establish whether there is a hierarchy among central and peripheral clocks in the timing of eclosion, the LNvs were ablated through expression of the proapoptotic gene *head-involution defective* (*hid*). Under these conditions, rhythmic eclosion is lost, and no differences in TIM expression over the course of the day are observed in the PG, underscoring its dependence to the central (LNv) clock (115). This relationship was further studied in organotypic cultures using a *per*-luc transcriptional reporter (142) in PGs dissected with or without the associated central nervous system; under these conditions, it was found that *per* oscillations are cell-autonomous but highly dependent on photoperception from the central clock, whereas TIM accumulation is independent of the central clock (114). These results suggest that light input and neuroendocrine signals arising from brain clocks coordinate molecular oscillators in this peripheral tissue. Recently, elegant work characterized the neuropeptide pathway involved in the control of rhythmic eclosion, connecting both central and peripheral clocks (135). Through genetic encoded calcium sensors and optogenetics, they showed that timing is transmitted by the sLNvs via the secretion of the short neuropeptide F precursor (sNPF), which silence non-circadian prothoracicotropic hormone (PTTH)+ neurons in the protocerebrum. They project to the PG and secrete PTTH (FIGURE 1). Knocking down the expression of the PTTH receptor *torso* in the PG eliminates the molecular oscillations along with the circadian rhythmicity of adult emergency. Finally, the hierarchy of central over peripheral clocks was assessed by expressing different *doubletime* kinase alleles in a tissue-specific manner to accelerate or slow down independent clocks, establishing that the central clock exerts a dominant role in the control of rhythmic eclosion. However, coupling between central and peripheral clocks is necessary (135).

Although much is known about the molecular mechanisms that produce rhythmicity within circadian pacemakers, less is known about the signals that feedback to the central clock to inform about general physiology. New findings are starting to shed some light on this issue. In adult flies, ecdysone via its cognate receptor EcR regulates the molecular brain oscillator by modulating the expression of microRNA *let-7*, which inhibits *cwo* expression that contributes to sustaining a high-amplitude circadian oscillation (22, 74, 96, 103). Additionally, ecdysone through its downstream target ecdysone-induced protein 75 (Eip75)

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modulates the brain clock by affecting *dClk/per* expression (72, 88). Both feedbacks enable the central brain to respond to endocrine signals and in turn get coupled to the peripheral clocks and fine-tune its output and adjust different physiological processes to the requirement of the moment.

Circadian Control of Rest-Activity Patterns

Drosophila is active around dawn and dusk under laboratory conditions, increasing its activity in preparation for the changes associated to the

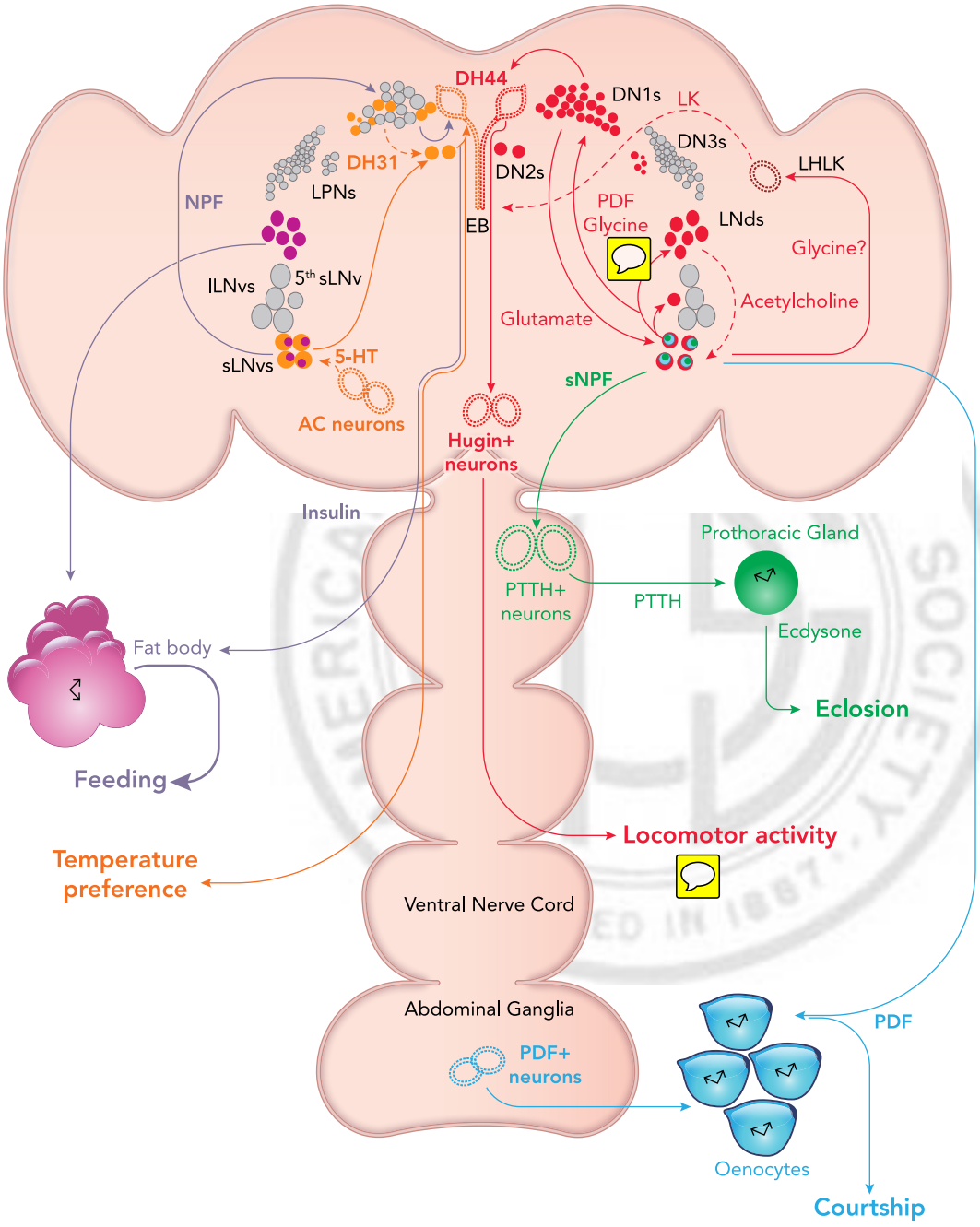

FIGURE 1. **Central to peripheral connectivity**
Schematic diagram of a central brain displaying circadian neurons (not to scale; only the somas are shown), the ventral nerve chord, and (some of the) organs that support a peripheral clock (PG, fat body, oenocytes). The emphasis is placed on central to peripheral communication underlying specific circadian behaviors (color coded). Solid lines indicate the somas of the clock neurons, and the broken lines indicate the somas of non-circadian cells. Within central clocks, communication is supported by neurotransmitters and neuropeptides; toward the periphery, direct neuronal connections as well as neuroendocrine signals are recruited. Solid arrows indicate well-established communication pathways (i.e., PDF) or synaptic contacts. Dashed arrows describe putative information flow (i.e., DH31). LHLK, lateral horn leucokinin (one soma per brain hemisphere, according to Ref. 19); LK, leucokinin neuropeptide; EB, ellipsoid body.

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day-night transitions; this behavior appears to be even more elaborated under (semi) natural conditions (49, 107). Rhythmic locomotor behavior depends primarily on the activity of the sLNvs, LNds, and DN1s, but all clusters contribute to different extents (60). Each of these clock neurons sustain a cell autonomous clock but rely on the communication among each other for entrainment (i.e., a response to a change in the environmental conditions) and phase adjustments (reviewed in Ref. 8). Rhythmic locomotor activity patterns depend on the coherence of the molecular clocks residing in both lateral and dorsal clusters (158). Furthermore, "non-clock" neurons also contribute to orchestrate the animal's activity pattern throughout the day (18, 19, 47, 82); however, the precise mechanism that dictates the shape of activity rhythms is still a work in progress.

Over four decades of genetic screens yielded most of what is known today about the control of locomotor rhythmicity (4). Initially, behavioral screens designed to identify altered activity patterns (i.e., changes in periodicity or reduced rhythmicity) involved chemically induced mutations, which in due time enabled the identification of the molecular components of the circadian clock; such is the case of *per* (84), *andante* (85), *tim* (134), *dClk* (2), *cyc* (127), and *dbt* (121); as screens went by, new alleles of the same "clock genes" were repetitively isolated (1, 54, 124, 125, 144). Soon enough, transposons became a more efficient means to generate mutants due to the simplicity to identify the affected locus; through more elaborated genetic screens, additional clock components such as *shaggy* and *cryptochrome* were identified (102, 143).

Independent molecular clocks in the brain residing in neurons and glia (116) are synchronized by external cues, most predominantly (but not restricted to) light and temperature. Light is perceived by the circadian network through external photoreceptors located in the compound eye, ocelli, and the Hofbauer Buchner eyelet (59), as well as through the internal "non-visual" photoreceptor CRY (35). Only a subset of clock neurons are in contact with photoperceptive organs or express CRY (160); thus synchronization to light cycles likely represents a circuital property (Ref. 132; reviewed in Ref. 159). A similar scenario is emerging regarding synchronization to low-amplitude temperature cycles, although the latter depends on communication from the periphery, particularly the chordotonal organs (20); interestingly, both light and temperature input converge on TIM degradation, despite the underlying molecular mechanisms appearing to be different (147).

Communication between clock neurons in the brain is essential to rhythmic locomotor behavior. The most striking example is the progressive loss of rhythmicity derived from the absence of pigment dispersing factor (PDF) (122), a neuropeptide released by the LNvs that impinges on roughly half of the circadian neurons; it synchronizes the phase of the sLNvs and DN1s, while it slows down the pace and increases the amplitude of the LNds and fifth sLNv (70, 92, 98, 162). PDF acts through the G-protein-coupled receptor PDFR (69, 91, 109) and signals through cAMP, leading to an increase in PKA that results in enhanced PER and TIM stability (95, 136, 137). Interestingly, RNAi-based genetic screens uncovered cluster-specific components of this signaling cascade, underscoring a degree of specificity that could help explain the different outcomes derived from cluster-specific PDFR activation (32, 33). Despite PDF, neurons and PDF itself are the focus of intense attention; we still lack fundamental insight about its function and regulation. Recently, it has been shown that PDF signaling reduces CLK-mediated transcription in adult brain preparations (111), as well as contributes to molecular oscillations at night through upregulation of CLK-CYC-mediated transcription and increases in PER stability in a cell-autonomous manner in larval brains (128). These seemingly divergent observations could be accounted for by some technical aspects derived from the novel circadian reporters, the timescale of the observations (acute vs. a response integrated after several hours), along with the possibility that the substrate under analysis is basically distinct, i.e., adult or larval circadian brains, respectively, and the underlying circadian network be responsible for the disparity in the outcome resulting from PDF's activity. PDF relevance to different aspects of circadian physiology has been discussed elsewhere (138).

Despite PDF's key role in the communication of the circadian ensemble, other neuropeptides [i.e., short neuropeptide F (sNPF) and ion transport peptide] contribute to rhythmic locomotor behavior (63, 64, 73). Behavioral miss-expression screens also retrieved unexpected candidates mediating communication between circadian clusters (9, 15); one of them uncovered a role for myocyte enhancer factor 2 (*Mef2*), a clock-controlled gene encoding a transcription factor that is responsive to neuronal activity and contributes to other circadian outputs, i.e., the structural remodeling of neuronal terminals (140). Another one linked the bone morphogenetic protein (BMP) retrograde signaling pathway to the central clock, providing a potential means to integrate signals from different circadian clusters (9).

Classical neurotransmitters also contribute to communication among circadian clusters; a clear example is the observation that impairing neurotransmitter release/reuptake affects rhythmic

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locomotor behavior, even if restricted to the LNvs (76, 79, 154). In addition, immunohistochemical analysis predicts that at least some LNds and the fifth sLNv are cholinergic (73), and some DN1s and DN3s are glutamatergic (52). Interestingly, there appears to be a reciprocal inhibitory modulation between the sLNvs and DN1ps through glycine and glutamate, respectively, which is essential for rhythmic patterns (23, 24, 40, 50), although the logic of the connectivity within most circadian (sub)clusters is still missing.

Despite rhythmic locomotor behavior being defined by the circadian network, other neuronal clusters also contribute to this output pathway; an emerging picture posits the sLNvs contacting a group of DN1s, which in turn contact the diuretic hormone 44 (DH44)+ neurons in the *Pars Intercerebralis* (PI), a neurosecretory structure playing a similar role to that of the hypothalamus (18). Those PI neurons are connected to hugin+ ones in the subesophageal zone, a sensorimotor control center. Hugin+ neurons have descending projections into the ventral nerve cord (VNC), where they potentially regulate motor circuits driving locomotion (FIGURE 1). Surprisingly, despite DH44 and hugin modulating rhythmic activity patterns, manipulations of this circuit barely affect feeding rhythms (see below and Ref. 82).

A Time to Eat

Feeding is an essential activity. Animals need to ingest appropriate nutrients depending on their internal state to maintain nutritional homeostasis, and thus their feeding pattern is dependent on their developmental, reproductive, or internal physiological state (152). Since the search for food is closely associated with activity periods in animals, circadian clocks play a role in metabolic control. In the wild, animals tend to eat at specific times of day that may vary from one species to another. This behavioral pattern is often maintained in animals kept under controlled laboratory conditions. To measure the amount of food consumed along the day, several methods are available; a spectrophotometric one based on the consumption of dye-labeled food at different times of the day, or CApillary FEeder (CAFE) assay (64), where flies are fed on a sucrose solution maintained in a capillary tube and food consumption is measured by assessing the volume change. New automated systems have recently been developed, e.g., the fly liquid-food interaction counter (FLIC), based on an electronic circuit that can be monitored continuously to signal when a single fly (or group of flies) interacts with food enabling continuous, simultaneous, and automated analysis of thousands of flies (123), and the fly proboscis and activity detector (flyPAD), which uses capacitive-based measurements to detect the physical interaction of individual flies with food (71). Using some of these methods, flies were shown to eat during daytime in LD cycles; this feeding rhythm is driven by the circadian clock (flies maintain a rhythmic feeding pattern in DD), and, accordingly, it is lost in clock mutants (156). Several peripheral tissues expressing clock genes are involved in nutrient sensing or regulation of energy homeostasis; however, the fat body clock is relevant for this function. The fat body plays an important role, similar to the liver in mammals, in regulating energy metabolism (133). Interestingly, expression of a dominant negative version of CLK (UAS-$dClk^{DN}$) exclusively in the fat body triggered a dampening of its molecular rhythms (both in *tim* mRNA as well as in TIM cycling), and, accordingly, the feeding pattern exhibited a phase change. In addition, flies lacking clocks in the fat body displayed opposite effects in glycogen levels and starvation sensitivity compared with those lacking a neuronal clock. Altogether, these results show that the clock present in the fat body regulates feeding behavior and suggest that the fat body and neuronal clocks are coordinated in opposite ways to provide an optimal metabolic state (FIGURE 1). Such homeostasis requires interaction between clocks, but how this occurs is only starting to unfold.

The fat body clock regulates the expression of 60% of its transcripts, among them those involved in metabolism, detoxification, reproduction, and innate immunity; out of these cycling genes, 40% are also controlled by external factors, including light, food, and even clocks present in different tissues (155), particularly, the neuropeptide F (NPF)+ LNds neurons, which drive rhythmic expression of specific fat body genes through neuroendocrine NPF signaling (36).

What other signals impinge on the fat body clock to regulate its function? One of the major inputs is the LD cycle that drives the feeding rhythm and leads to a cyclic expression of metabolic genes (156). In addition, the clock present in the sLNvs regulates the fat body clock in the absence of environmental cues (36). Finally, nutrients are known to be strong entrainment signals for peripheral clocks; particularly, the fat body clock can be driven independently of the central one by a restricted feeding (RF) paradigm, which consists of feeding flies at times when they normally do not eat (155). However, RF does not entrain brain clocks [as it appears to be the case in mammals (68)] and thus provides a venue to test the impact on internal physiology of experiencing desynchrony between timing signals released by different clocks. To address this, reproductive capacity of the female flies feeding at different day times

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was examined. Flies fed during the day laid more eggs than flies fed at night. Thus circadian desynchrony leads to defects in overall reproductive capacity, which is even more affected in flies with disrupted clocks. These results suggest that coherence between different clocks is important for fitness.

How central clocks transmit time-of-day information to the periphery involves both direct neural connections and humoral control. In that regard, a circadian clock-regulated output gene, *takeout* (*to*), modulates feeding behavior by conveying temporal information about the internal nutritional state (13, 130). This suggests a direct molecular link between the circadian clock and the feeding/starvation response. As mentioned earlier, the PI is connected to the core clock LNvs through DN1 neurons (18, 19, 39, 119). The PI regulates a number of processes that are under circadian control, including locomotion, metabolism, and sleep (10, 17, 25, 30, 39, 126). However, PI cells are not circadian clocks. PI is best known as the site of insulin-producing cells (IPCs) in *Drosophila*. IPCs are functionally connected via DN1 neurons to the central clock circuit and drive rhythmic expression of a lipase transcript [*sex-specific enzyme 2* (*sxe2*)] in the fat body. IPC regulation of *sxe2* mRNA rhythms is dependent on the presence of insulin and functional insulin receptors in the fat body, suggesting that insulin could transmit time-of-day signals from IPCs directly to the fat body. Additionally, rhythmic expression of *sxe2* is regulated by feeding. These findings indicate circuit-level regulation of metabolism by clock cells and once again support a role for the PI in integrating circadian control of behavior and physiology (6).

In addition, feeding is also regulated by neuropeptides; for example, neurons expressing allatostatin A (AstA)-related neuropeptides regulate feeding behavior (62), and a subgroup of AStA+ posterior lateral protocerebrum neurons and enteroendocrine cells are targets of the central clock output factor PDF (21). However, very little is known regarding signals released by clocks to relevant peptidergic cells and should be the focus of more intense research.

Neurobiological Substrates of Temperature Preference

Drosophila's natural environment is thermally heterogeneous. Since flies are ectotherms, they regulate their bodies' temperature by selecting an environment with their preferred temperature. As mammals, flies exhibit a circadian rhythm in body temperature originated from the circadian modulation on temperature preference. In the laboratory, temperature preference is assessed by placing a group of animals in a temperature gradient (usually 18–32°C) and letting them choose where to rest 30 min later (45). Histamine and dopaminergic signaling to, among other neuropils, the mushroom bodies is involved in temperature-dependent behavioral changes (5, 65, 149). But the core of warmth avoidance and circadian temperature preference relies on *dTrpA1*, the *Drosophila* temperature-activated transient receptor potential channel. ACs are a small group of neurons within the brain with the ability to act as thermosensors. As temperature increases, the AC neuron dTrpA1 channel opens a cationic conductance which in turn leads to depolarization; animals lacking dTrpA1 select warmer temperatures, indicative of a heat-avoidance function for ACs (51). On the other hand, cold avoidance is dependent on antennal sensors. Besides TrpA1, the *Drosophila* genome encodes 13 TRP channels, among them *painless* and *pyrexia*, which activate in the noxious range (≈40°C; reviewed in Ref. 11).

Flies prefer warmer temperatures during the day (~1–1.5°C increase) and colder at night. The warmest preference is observed in the evening (at ZT10-12) and the lowest during the early night (ZT13-15). This temperature preference rhythm (TPR) remains in DD and is abolished in per^{01} and tim^{01}, indicative of a clock-controlled function. Since TRP is not abolished by constant light and clock mutants show a masking effect under LD, light appears to have a direct effect on a fly's temperature preference. In fact, light itself modulates temperature preference through the activity of DN1s but not their clocks (58), suggesting a partial overlap between the circuits controlling temperature preference-associated behaviors. Daily fluctuations on temperature preference have been known for a long time, but the functional connection between the two emerged by chance in a screen aimed at identifying genes involved in temperature sensing, which uncovered a role for PDFR. *Pdfr* nulls not only loose daily fluctuations but also prefer slightly cooler temperatures (69). In addition, the crucial involvement of DN2s on TPR was elegantly assessed by rescuing per expression in per^{01} and restoring the warmer daytime preference (75). PDFR expression within DN2s is sufficient to restore TPR in $pdfr^{5304}$ at the day-to-night transition. Specifically, PDFR is involved in regulating colder preference at night-onset, but signaling is not triggered by PDF but through diuretic hormone 31 (DH31), which also activates PDFR (109). DH31 mutants loose TPR, but its restitution to DN2s restores this response (46). DH31 could be released by DN1s. Interestingly, loss of DH31 signaling does not affect circadian locomotor rhythm, although it increases sleep (89). Although warmer temperature preference during daytime is the most salient

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feature of this clock-controlled behavior, another property that was analyzed in more depth is the increase in preferred temperature before dawn. It depends on sLNvs and DN2s clock neurons. Surprisingly, when these neurons are silenced, the TRP is even more noticeable, probably indicating a clock-controlled buffering on TPR. ACs neurons could contact sLNvs through 5-HT, which in turn conveys temperature-related information to DN2s preferentially late at night, when synaptic contacts are increased (FIGURE 1). Activity in this circuitry would motivate the animals to choose a warmer temperature at the end of the night (146).

Circadian Entrainment to Temperature Cycles

Circadian rhythm in temperature choice should keep the animal in the optimum environment for the many activities it will perform through the day. But temperature itself is an input that can interact with the circadian circuity to change the pattern of locomotor activity, or synchronize it, as a *zeitgeber*. On one hand, the classical male bimodal locomotor rhythm was confronted by a three-peak activity pattern under summer semi-natural conditions. Although still controversial, the afternoon activity bout is dependent both on light and heat, and would be recruited by the activation of TrpA1+ cells distinct to the ACs. In turn, they would contact clock neurons to modulate the phase of this afternoon locomotor activity that is envisioned as an escape response to higher temperature and low humidity (28, 29, 48, 150). On the other hand, periphery-to-brain temperature signals through *pyrexia*—in the femur chordotonal organ—and *Drosophila* ionotropic receptor 25a (IR25a)—in leg neurons—convey information relative to temperature in the lower range or to low-amplitude temperature cycles in the higher end, respectively, to dorsal and lateral clock neurons to synchronize *tim* oscillations in constant conditions (20, 153). Notably, different clock neurons are recruited for synchronization to colder or warmer temperature cycles, and this difference may rely on CRY (44); furthermore, the neural circuitry (and its activity pattern) that underlies temperature synchronization and temperature preference is different.

The Influence of Clocks in Social Life

Flies display complex social behaviors, from the recognition of conspecifics to courtship, fighting, and mating. Temporal organization of socio-sexual interaction enables the specification of reproductive barriers in sympatry species (56, 129, 148). *Drosophila* species exhibit strong diurnal variations in courting activity. Courtship consists of a complex locomotor pattern that includes male orientation to and chasing the female, wing display, licking, and attempting copulation. In the field, courtship rarely leads to mating; courtship with individual females is fairly short spanned, since males move around courting several different ones (120). Under laboratory conditions, courtship and copulation depend on many variables such as age, body size, previous housing conditions, number of possible sex-mates, and whether the experimental design involves short- or long-lasting interactions.

The locomotor activity pattern of female/male couples is different to the activity of animals in isolation; they are active all day long, with a maximum around dawn and a minimum during the evening and early night. Males recover their solitary locomotor rhythm when isolated after the cohabitation experience, indicative of an interaction between the circadian rhythm and the presence of the female. The locomotor activity rhythm of female/male couples masks an intention of intercourse, since a "close proximity" rhythm is found. Thus, a male sex-driven rhythm appears when a female/male couple is housed together. This MSDR is driven by the male circadian clock and depends on olfactory cues received in the antennae (43). It also depends on the LNvs and DN1s clocks as well as on PDF signaling (FIGURE 1). In fact, the sLNvs appear to drive the DN1s molecular clock that regulates MSDR (42). MSDR reflects the expression of a male courtship goal. Courtship is a sexual dimorphic behavior supported by *fruitless* neurons. In males, sLNvs, LNds, and DN1s express FRU^M, suggesting that such clock neurons would provide the time cue to court females at a fruitful time. Three NPF+-expressing LNds are characteristic of male flies (FIGURE 2). Fru^M mutant males, lacking NPF in LNds, display their courtship activity arrhythmically (41). $dClk^{Jrk}$ and Cyc^{02} also lack male-specific NPF+ LNds, indicative of a double dependence, i.e., *npf* is a sex- and a clock-controlled gene. Nevertheless, NPF+ neuron involvement in courtship behavior is still a matter of debate in the field; likewise, the contribution of NPF+ LNds to its rhythmicity is even more debatable (53, 93). Courtship also implicates specific odor signaling through sex pheromones. Specific cuticular hydrocarbons synthesized in oenocytes act as female attractants. Oenocytes are ectoderm-derived tissues located in the inner cuticular surface of the abdomen that encompass peripheral clocks, since they cyclically express the core clock genes. Both male and female pheromones are rhythmically released to the outer body surface, and this pattern is supported by the clock-controlled expression of *desaturase1*, an enzyme involved in the synthesis of cuticular hydrocarbons.

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Rhythmicity depends on clock function within oenocytes (86, 87). The central to oenocyte interclock communication is mediated by PDF (FIGURE 1). In addition to the LNvs, some abdominal ganglia neurons (AbNs) release PDF. PDF downregulation on each specific group results in the lengthening of the free-running period in the oenocyte clock, but only PDF+ AbNs are responsible for controlling sex hormone production. Although PDF is not necessary for synchronization among oenocyte clocks, it is required to set the period and lock their phase to the central clock. As expected, the rhythm of sex pheromone expression is obliterated when PDF signaling is disrupted; a membrane-tethered version of PDF revealed that oenocytes directly respond to this neuropeptide (86).

Mating, on the other hand, was also reported to depend on a circadian clock, but the one in the female's brain (14, 129). Unreceptive females extrude the ovipositor and reject the male by kicking him, but the receptive ones slow down and facilitate copulation. Females mate preferentially during daytime, with a deep trough during the evening and early night. Since females lacking LNvs (*disco* mutants) do not show any rhythm, it was concluded that circadian mating activity depends on their central pacemakers (129). Clock mutants display a rhythm in LD but not in DD (an example of masking effect). Antennal peripheral clocks are not crucial for active mating, since females with impaired olfaction do not show any change in its temporal distribution (14, 78). Central PDF is also influential in social interactions, since it conveys phase information to peripheral clocks and couples the circadian rhythm of sexual hormone production within oenocytes to mating behavior (86). Interestingly, pdf^{01} males mate more, whereas females mate less than controls. Remarkably, the timespan of copula rely on some male clock neurons (sLNvs and LNds) and genes (*per* and *tim*) but does not depend on circadian clock function (7, 80, 81). Social experience influences circadian locomotor activity; for instance, aggregation increases the coherence of the circadian phase through odor signaling (94, 101). Among males, social interaction modifies the oenocyte clock, the pattern of pheromone expression, and mating (87). But the cyclic presence of conspecifics does not synchronize activity rhythms (100). However, the molecular clock of the male DN1s is slightly shifted toward the female rhythm (55), underscoring a subtle cross talk.

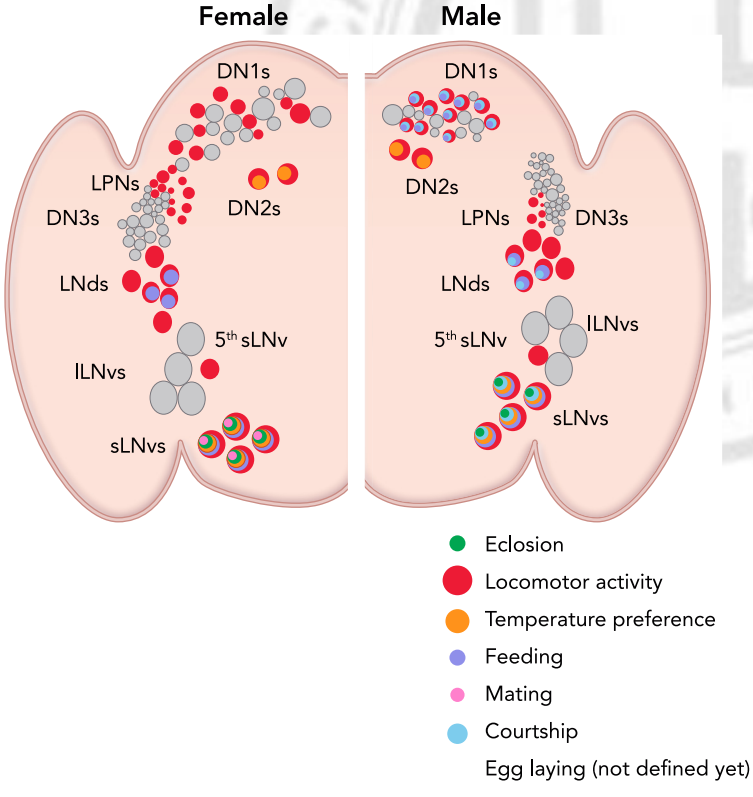

FIGURE 2. **Circadian neurons drive rhythmic behaviors**
Schematic diagram (not to scale) of an adult brain displaying female (left hemisphere) and male (right hemisphere) circadian networks, highlighting the anatomical substrates of specific behaviors (color coded); the emphasis is placed on the ones for which sexual dimorphism has been defined. Of note, the position of the somas in both hemispheres highlights individual differences that are not sex-specific. For the sake of clarity, only the somas are shown.

Circadian Control of Egg-Laying

The oviposition or egg-laying behavior is another physiological process that is under the control of the circadian clock. The periodicity in oviposition is one of the less-studied rhythms in *Drosophila*, perhaps due to the difficulties involved in monitoring and recording this behavior, possibly as a result of the discrete character of the measured variable (the number of laid eggs) that enables only a few measurements per day; collection and egg counting is done manually, making the experiments particularly demanding and labor-intensive. The periodic deposition of eggs involves a series of events ranging from the production of oocytes to the deposition of eggs in the most appropriate place (3, 157). The circadian rhythmicity of this behavior is revealed by its persistence under DD, with a period of ~24 h and a peak of egg deposition near night onset. Egg-laying rhythmicity is temperature-compensated and remains invariant, despite the nutritional state (67). Moreover, oviposition is rhythmic in virgin females as well as in mated ones, suggesting that this rhythm is not regulated by the act of mating and is endogenously driven (108). The molecular mechanism underlying rhythmic control of this behavior is not known.

Although egg-laying occurs in a circadian fashion, it appears to be inherently different to other well-characterized rhythmic behaviors, opening

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the possibility that oviposition depends on oscillators that are different from those involved in rest/activity cycles or eclosion; as an example, oviposition is rhythmic under constant light that triggers arrhythmicity in other circadian behaviors (139) and persists in per^{01} (104), although some residual clock activity has described in that mutant background (31, 61). More recently, egg-laying behavior was examined in a different clock mutant, Clk^{jrk}. These mutants indeed lose rhythmicity, suggesting that timing is CLK-dependent (155); however, the mutation also impairs the development of the fly's clock system and could potentially affect other neuronal structures related to oviposition (66). Thus further studies are required to reveal which clock genes are indeed involved in rhythmic control of this behavior.

To more specifically address the role of sLNvs and PDF in the control of the circadian oviposition, sLNvs were eliminated through *hid* expression (67). Strikingly, egg-laying is still rhythmic in sLNv-ablated flies, whereas locomotor activity and eclosion become arrhythmic. Moreover, egg-laying rhythms persist in DD in pdf^{01} mutants, indicating that PDF signaling is not required for the persistence of the rhythm under DD. However, sLNv-ablated flies show a significantly altered period, suggesting that, despite sLNvs not being critical, they may influence the circadian period of oviposition through yet unknown mechanisms.

Although oviposition exhibits a circadian component, the identity and location of the neurons that govern this rhythm have not been described. The contribution of peripheral clocks located in the fat body was examined through expression of a dominant negative CLOCK version (156). This resulted in rhythmic egg deposition along several days, although the egg number decreased with time. No differences were observed when clockless fat body males were examined. These results suggest that the fat body clock is not responsible for the control of the timing of oviposition (156).

Among circadian outputs, rhythmic oviposition is the least understood, and many aspects still remain to be elucidated: Which is the neuronal circuit responsible for the control of this behavior? Which cellular mechanism(s) are recruited? How is it regulated? Do hormones play a role? It is high time to concentrate on other rhythmic behaviors that are important for the fly's fitness.

Concluding Remarks

In *Drosophila*, central clock neurons are barely 150 units, but their connectivity is widespread, an indication of the colossal complexity achievable by a relatively small brain. We are just starting to unveil the complexity of the network that imparts temporal information to the different circuits underlying behavior (FIGURE 1); as an example, the sLNvs change synaptic contacts across the day (47) and so would be expected at least for other clock neurons. The precise coordination of amplitude and phase of all clocks is essential for the well-being of animals (57); despite us beginning to understand how multiple circadian clocks in the body are coordinated through central-peripheral interactions, much less is known regarding how peripheral clocks feedback to the central oscillators, enabling integration of different cues to finally orchestrate a coordinated physiological response. The intricacy of this regulation underscores the relevance of keeping brain and body clocks in tune. Organization of modern societies (exposure to light, food, and social activity at night) threatens human health since, among other factors, it undermines the operation of the circadian clock. Modeling in *Drosophila* the conflicting interactions between modern lifestyle and the ancestral molecular clock should provide an opportunity to explore potential venues to ameliorate their impact. ■

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

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■= Given-Name, ■= Surname