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Regulation of gustatory physiology and appetitive behavior by the circadian clock in *Drosophila melanogaster*

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Summary

Background—Circadian regulation of chemosensory processes is common in animals, but little is known about how circadian clocks control chemosensory systems or the consequences of rhythms in chemosensory system function. Taste is a major chemosensory gate used to decide whether or not an animal will eat, and the main taste organ in *Drosophila*, the proboscis, harbors autonomous circadian oscillators. Here we examine gustatory physiology, tastant-evoked appetitive behavior, and food ingestion to understand clock-dependent regulation of the *Drosophila* gustatory system.

Results—Here we report that single-unit responses from labellar gustatory receptor neurons (GRNs) to attractive and aversive tastants show diurnal and circadian rhythms in spike amplitude, frequency and duration across different classes of gustatory sensilla. Rhythms in electrophysiological responses parallel behavioral rhythms in proboscis extension reflex (PrER). Molecular oscillators in GRNs are necessary and sufficient for rhythms in gustatory responses, and drive rhythms in G protein coupled receptor kinase 2 (GPRK2) expression that mediate rhythms in taste-sensitivity. Eliminating clock function in certain GRNs increases feeding and locomotor activity, mimicking a starvation response.

Conclusions—Circadian clocks in GRNs control neuronal output and drive behavioral rhythms in taste responses that peak at a time of day when feeding is maximal in flies. Our results argue that oscillations in GPRK2 levels drive rhythms in gustatory physiology and behavior, and that GRN clocks repress feeding. The similarity in gustatory system organization and feeding behavior in flies and mammals, and diurnal changes in taste sensitivity in humans, suggest that our results are relevant to the situation in humans.

Introduction

In animals, plants, fungi and some prokaryotes endogenous circadian clocks drive daily rhythms in gene expression, physiology, metabolism and behavior, thus enabling organisms to anticipate daily environmental changes. At the molecular level, the circadian timekeeping mechanism in eukaryotes is comprised of core and interlocked transcriptional feedback loops [1]. In *Drosophila*, CLOCK-CYCLE (CLK-CYC) heterodimers bind E-boxes to activate transcription of *period* (*per*) and *timeless* (*tim*), then PER and TIM proteins nucleate

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the formation of protein complexes that feed back to repress transcription of *per*, *tim* and other CLK-CYC activated genes within these feedback loops [2]. Feedback repression is released when PER and TIM are degraded, thus initiating the next cycle of transcription [2].

Circadian clocks are present in both the central nervous system and peripheral tissues [1]. A circuit of ~ 150 brain neurons control locomotor activity rhythms in *Drosophila* [3, 4], while peripheral clocks in antenna, epidermis, oenocytes, and testis regulate local physiology [5–8]. In contrast to mammals, peripheral oscillators in *Drosophila* maintain synchrony in the absence of rhythmic input from the brain [9–12]. Although autonomous, light entrainable oscillators are known to be present in many *Drosophila* tissues including Malpighian tubules, proboscis, leg and wing [12, 13], relatively little is known about the rhythms that they control. Perhaps the best-understood peripheral oscillators in *Drosophila* reside in the antenna. These oscillators drive rhythms in spontaneous and odor-induced physiological responses in olfactory receptor neurons (ORNs), and are thought to control odor-driven chemotactic behavior in adult flies [11, 14, 15]. Circadian rhythms in odor-evoked physiological responses have also been described in humans, mice, cockroaches and moths, which implies a conserved and important function for circadian regulation of smell [16–19].

Drosophila senses taste via gustatory receptors (GRs) expressed in gustatory receptor neurons (GRNs) on the proboscis, leg, wing margins, and ovipositor [20]. At the tip of the main gustatory organ in *Drosophila*, the proboscis, is the labellum, which contains 31 pairs of taste-hairs, each housing 2 or 4 GRNs [21]. In insects, feeding is regulated by external signals such as gustatory stimuli and olfactory cues [22, 23], and internal signals such as feeding status and metabolic needs [23]. A set of conserved peptide hormones, *Drosophila* insulin-like peptides (DILPs) and the glucagon analog adipokinetic hormone (AKH), function reciprocally to control energy homeostasis in fruit flies and other animals [24]. *Drosophila* display daily rhythms in feeding that are regulated in part by circadian clocks in ORNs and the fat body [25]. Food intake is increased in *Clock* mutant mice [26, 27], demonstrating a conserved role for the circadian clock in the control of feeding.

Given the remarkable mechanistic and structural similarities between the *Drosophila* gustatory and olfactory systems and experiments demonstrating that the proboscis contains a self-sustaining oscillator [12], we reasoned that the proboscis clock controls rhythms in gustatory physiology and behavior. Here we show that GRN clocks control sugar and caffeine induced physiological and behavioral responses in the proboscis, and identify G-protein-coupled receptor kinase 2 (*Gprk2*) as a key signal transduction molecule that underlies these rhythms. Disrupting clock function in GRNs increases feeding, implying that GRN oscillators restrict food consumption.

Results

The amplitude, frequency and duration of GRN spikes are controlled by the circadian clock

Based on their size and location, labellar taste hairs are divided into large (l-type), intermediate (i-type) and small (s-type) sensilla [20, 28]. GRNs housed in l-type and s-type sensilla are classified as S neurons (responsive to sugar), W neurons (responsive to water and low osmolarity), L1 neurons (responsive to low salt concentration), and L2 neurons

(responsive to bitter compounds and high salt concentration) based on their electrophysiological response spectra [20, 29]. Recordings from single l-type sensillae were made in wild-type flies collected during 12h light:12h dark (LD) cycles. A different population of flies ($n = 6$) was recorded at each timepoint. The sweet-sensitive S neuron was stimulated by application of 100 mM sucrose [30]. A ~3.5 fold rhythm in S spike amplitude was detected with a peak at Zeitgeber Time 1 (ZT1) and a trough at ZT17 (Fig. 1a; Fig. S1). The extent of diurnal influence on spiking activity of S neurons was determined by recording the rate of firing in response to 100 mM sucrose. A ~1.5 fold rhythm in spike frequency was detected, which showed a sharp trough at ZT17 (Fig. 1b). Since the waveforms of action potentials can encode biological information [31], we investigated changes in spike duration as a function of time of day. A ~2 fold rhythm in S spike duration was found, with a peak at ZT1 and a trough at ZT17 (Fig. 1c). These rhythms in spike amplitude, frequency, and duration persisted in constant darkness (DD) (Fig. 1d–f), thereby demonstrating that the rhythms are not a passive response to LD cycles but are driven by circadian clocks. These electrophysiological responses are constantly low in *per*⁰¹ and *cyc*⁰¹ null mutants even in LD cycles (Fig. 1g–i; Fig. S1), thus demonstrating that the clock is required for the daily increase in responses from S neurons.

To determine whether other classes of GRNs and other types of sensillae exhibit circadian rhythms in spike activity, single-unit responses to the bitter compound caffeine (10 mM) were measured in L2 neurons from s-type sensilla during DD. Rhythms in spike amplitude, frequency and duration were detected that peaked at circadian time 1 (CT1), where CT0 is subjective lights-on and CT12 is subjective lights-off (Fig. 2a, c, e). These rhythms were abolished in *cyc*⁰¹ mutants in DD (Fig. 2b, d, f), where spike amplitude and frequency were near the wild-type trough and spike duration was between the wild-type peak and trough values. These results demonstrate that circadian control of spike activity is broad, encompassing bitter sensitive L2 neurons and sweet sensitive S neurons in s-type and l-type sensillae, respectively.

Tastant-induced behavior is under clock control

In response to contact-chemoreception with a phagostimulatory chemical, flies elicit a reflex-like appetitive behavior wherein they extend the proboscis to attempt feeding [32]. To determine whether the circadian clock controls tastant-driven behavior, we measured proboscis extension reflex (PrER) responses at different times of day in wild-type and clock mutant flies during LD and DD conditions. PrER responses to 100 mM sucrose in wild-type flies showed a diurnal fluctuation that peaks at dawn (ZT1) and falls to trough levels by mid-night (ZT17) in LD (Fig. 3a). These trough level PrER responses increase to near the peak level when stimulated with 500mM sucrose (Fig. 3a), indicating a clock-modulated change in sensitivity to sucrose. PrER rhythms persisted in wild-type flies during DD, demonstrating that these rhythms are under circadian control (Fig. 3b). Rhythms in PrER responses remained at constant low levels in *per*⁰¹ and *cyc*⁰¹ mutants in LD (Fig. 3c, d), showing that the clock is necessary for increased PrER responses and that light does not have a strong masking effect on PrER rhythms. PrER responses to the sugar trehalose (100mM), which also induces appetitive behavior, exhibited diurnal changes (Fig. 3e). Daily changes in responsiveness to a compound that deters appetitive behavior were measured by

quantifying the reduction in PrER responses to a sucrose solution containing caffeine [28]. The presence of caffeine decreased the probability of PrER strongly at ZT1 and only weakly at ZT17 (Fig. 3f). These results demonstrate that gustatory behavior to attractive and repulsive stimuli is under clock control.

Clocks within GRNs are necessary and sufficient for PrER rhythms

In the *Drosophila* olfactory system, peripheral clocks in OSNs drive rhythms in odor-induced physiological responses [8]. Given that the proboscis contains autonomous circadian oscillators and the PrER is initiated by GRNs [12, 33, 34], we hypothesized that peripheral oscillators in GRNs drive PrER rhythms. The presence of peripheral clocks in GRNs on the proboscis was first confirmed via immunocytochemistry. The constitutively expressed pan-neural nuclear antigen ELAV detects clusters of GRNs at the base of each sensillum (Fig. 4a). Coimmunostaining with anti-PDP1 revealed more intense PDP1 immunofluorescence in ELAV positive cells at ZT17 than at ZT5, but relatively constant immunofluorescence intensity in ELAV negative cells (Fig. 4a). All PDP1 immunostaining was eliminated in the PDP1 ϵ -specific mutant *Pdp1*³¹³⁵ [35], indicating that only PDP1 ϵ is expressed in these ELAV positive and negative cells. Rhythmic PDP1 ϵ staining in ELAV positive cells is consistent with PDP1 ϵ cycling in brain and peripheral oscillator cells [35–37], and demonstrates that the GRNs within gustatory sensilla contain circadian oscillators.

To test the idea that local oscillators within GRNs are necessary for PrER rhythms, we expressed a dominant negative form of CYC (CYC^{DN}) to abolish clock function in the sweet-sensitive S neurons that elicit PrER behavior in response to sucrose [33]. Under LD conditions, PrER responses were abolished in flies containing both the *Gr5a*-Gal4 driver, which is expressed in S neurons [34], and UAS-*cyc*^{DN} responder, but not in control flies containing the *Gr5a*-Gal4 driver or UAS-*cyc*^{DN} responder alone (Fig. 4b). This result demonstrates that circadian oscillators in GRNs are required for PrER rhythms.

We then sought to determine whether local clocks in GRNs are sufficient for PrER rhythms by generating flies with circadian oscillators only in S neurons. For this, oscillator function was rescued exclusively in S neurons by using *Gr5a*-Gal4 to drive UAS-*cyc* expression in *cyc*⁰¹ flies. PrER behavior in *cyc*⁰¹ flies containing both *Gr5a*-Gal4 and UAS-*cyc* was rhythmic, whereas *cyc*⁰¹ flies containing *Gr5a*-Gal4 or UAS-*cyc* alone were arrhythmic (Fig. 4c). These data demonstrate that clocks in GRNs are sufficient for PrER rhythms. Since clocks are not present elsewhere in *cyc*⁰¹ flies containing *Gr5a*-Gal4 and UAS-*cyc*, these data also show that central clocks in the brain are not necessary for PrER rhythms. Taken together, these results demonstrate that GRN clocks are necessary and sufficient to control rhythms in gustatory behavior.

Cycling GPRK2 levels drive PrER behavior rhythms

Since circadian oscillators in GRNs are sufficient for PrER rhythms, the clock output pathway that controls this rhythm must also reside in GRNs. To identify a clock-controlled molecule involved in gustatory signal transduction, we focused our attention on GPRK2, which is required for rhythms in olfactory responses in *Drosophila* [14, 38]. Western blot analysis shows that GPRK2 protein is expressed in the proboscis of wild-type flies, but that

GPRK2 levels are reduced in the *Gprk2*⁰⁶⁹³⁶ mutant (Fig. 5a) [39]. In contrast to the two GPRK2 isoforms that are detected in antennae [38], only one GPRK2 band is seen in the proboscis of wild-type and *Gprk2*⁰⁶⁹³⁶ flies (Fig. 5a). The levels of GPRK2 cycled ~2-fold in wild-type proboscises with a peak at ZT17 and a trough at ZT1 (Fig. 5b). GPRK2 cycling was abolished in *per*⁰¹ and *cyc*⁰¹ flies (**data not shown**), indicating circadian clock control. GPRK2 immunostaining was detected in the cell body of GRNs at the base of taste-hairs that were co-immunostained with ELAV (Fig. 5c). GPRK2 was also detected in the shaft of the sensillar hair, which contains GRN dendritic projections, and possibly support cells closely associated with GRNs (Fig. 5c).

The levels of GPRK2 in the proboscis are lowest when PrER responses peak, and peak when PrER responses are lowest. This antiphase relationship suggests that GPRK2 levels may control rhythmic PrER behavior. Consistent with this possibility, PrER responses to sucrose and trehalose were constantly repressed when GPRK2 was overexpressed, but were always high in the *Gprk2*⁰⁶⁹³⁶ mutant (Fig. 5d, e). Thus, these experiments argue that cycling GPRK2 levels drive rhythms in PrER behavior. Given that PrER responses are constantly high in *Gprk2*⁰⁶⁹³⁶ flies and that spike amplitude, frequency and duration in GRNs cycle in parallel to PrER responses, we reasoned that these spike activity parameters should be constant and relatively high in the GRNs of *Gprk2*⁰⁶⁹³⁶ flies. Rhythms in spike amplitude, frequency and duration were all abolished in *Gprk2*⁰⁶⁹³⁶ flies, where spike frequency was close to the wild-type peak, but spike amplitude was midway between the wild-type peak and trough and spike duration was only modestly higher than the wild-type trough (Fig. S2). These results suggest that certain aspects of GRN cell activity, particularly spike frequency and to a lesser extent spike amplitude, correlate with PrER behavior.

GPRK2 mediates circadian rhythms in the subcellular localization of *Drosophila* odorant receptors (ORs) [38]. Since *Drosophila* ORs and GRs belong to the same family of insect chemoreceptor proteins, we wished to determine whether GPRK2-dependent regulation of rhythmic PrER responses relies on GRs. A mutant that removes all six *Drosophila Gr64* genes (*Gr64*) shows drastically reduced PrER responses to most sugars [33]. When *Gr64* flies were stimulated with 100mM sucrose at ZT1 and ZT17 their PrER responses were not rhythmic, but *Gr64* mutants rescued by a transgene containing the entire *Gr64* gene cluster [33] recovered PrER rhythms (Fig. 5f). Overexpression of the sucrose receptor Gr64a resulted in arrhythmic PrER responses that were near the circadian peak value (Fig. 5f). Likewise, deletion of *Gr5a*, which is required for responses to trehalose [40, 41], resulted in constant low PrER responses to trehalose whereas *Gr5a* overexpression resulted in constant high responses to trehalose (**data not shown**). These results imply that GRs are not only required to detect tastants, but are necessary for sustaining rhythms in tastant-evoked appetitive behavior.

GRN clocks regulate feeding

Both external sensory cues and internal metabolic state contribute to the regulation of feeding [23]. Recent work in *Drosophila* has shown that loss of clock function in fat body increases feeding by altering metabolic state [25]. We sought to determine whether GRN oscillators also regulate feeding since they modulate taste sensitivity. Food ingestion was

measured using a blue food dye that can be quantified spectrophotometrically and the Capillary Feeder (CAFE) assay [25, 42]. Under LD conditions, flies that express CYC^{DN} in sweet-sensitive Gr5a neurons consume significantly more food over 24 h than controls carrying the driver or responder transgenes (Fig. 6a, b). Moreover, food intake is higher in the morning (ZT0–4) than in the evening (ZT12–16), demonstrating that increased consumption is not uniform during a diurnal cycle (Table S1). This result shows that circadian clocks in a subset of GRNs act to limit the amount of food intake.

Although flies that lack clocks in Gr5a neurons eat more, they do not gain weight compared to controls carrying the driver or responder transgenes alone (Fig. 6c). Nevertheless, loss of clock function in Gr5a neurons led to a considerable increase in triglyceride and glycogen content (Fig. 6d, e). Increased triglyceride and glycogen content in flies lacking clocks in Gr5a neurons is associated with higher levels of locomotor activity over a 24 h period (Fig. 6f), where increased activity levels coincide with increased feeding (Table S1). Thus, flies lacking clocks in Gr5a neurons eat more and store triglycerides and glycogen even though they expend more energy to fuel increased locomotor activity.

Discussion

Circadian rhythms in gustatory physiology and behavior

The ability to detect and discriminate tastants provides a survival advantage to animals ranging from flies to humans because chemosensation is universally employed to identify food sources and reject harmful substances [34]. Our results demonstrate for the first time that this fundamental sensory process is controlled by the circadian clock. The amplitude, frequency and duration of voltage spikes evoked by attractive and aversive tastants peak around dawn in multiple classes of GRNs and different types of taste-sensilla (Figs. 1 and 2). Ventrolateral clock neurons (LNvs) in the fly brain show rhythms in firing rate that also peak during the day [43–45], but whether a common mechanism controls rhythms in the electrical properties of GRNs, LNvs and OSNs is not known.

The PrER is a direct, robust, and all-or-none indicator of a fly's attraction and motivation to ingest a substance [32]. PrER response levels change as a function of time of day (Fig. 3), where the phase of this taste-behavior rhythm mirrors rhythms in the rate, amplitude, and duration of GRN impulses in wild-type flies under LD and DD conditions. These results suggest that spike amplitude and duration, in addition to spike frequency, are dynamic neuronal response properties capable of influencing sensitivity to chemical cues. Circadian rhythms in spike amplitude are also seen in the olfactory system of flies [14], where the phases of these electrophysiological rhythms coincide with rhythms in odor-dependent chemotactic behavior that peak during mid-night [15]. Our data suggest that rhythms in spike properties of GRNs tune the activity of downstream neurons in such a way that behavioral responses to the same stimulus show clock-regulated plasticity. Given that PrER behavior likely involves local circuitry with limited processing [20], it is surprising that this 'hardwired' behavior is subjected to daily functional remodeling by the clock, and that a straightforward predictive relationship emerges between rhythms in GRN responses and rhythms in tastant-driven appetitive behavior.

Control of PrER rhythms

We show that circadian oscillators in GRNs are necessary and sufficient for PrER rhythms (Fig. 4). To our knowledge, this is the first example where a single population of peripheral oscillator neurons is shown to generate behavioral rhythms. GRNs from the proboscis project primarily into the central portion of the suboesophageal ganglion (SOG) [46]. A number of SOG motor neurons are known to innervate muscles in proboscis and pharynx [47], which may be indirectly controlled by the GRN clock on a daily basis.

In *Drosophila*, GRNs express GPRK2, and rhythms in GPRK2 abundance are antiphase relative to PrER rhythms (Fig. 5a–f). Analyses of *Gprk2* mutant and GPRK2 overexpression flies suggest that GPRK2 levels drive rhythms in PrER responses, and correspond to GRN spike frequency and to a lesser extent spike amplitude (Fig. 5d, e; Fig. S2). Rhythms in PrER responses are also abolished by altering GR levels; increasing or decreasing *Gr64* or *Gr5a* levels results in constant high or low PrER responses, respectively (Fig. 5f; **data not shown**). PrER responses are constantly low in *Gr64* and *Gr5a* deletion mutants and GPRK2 overexpression flies, but are constantly high in *Gr64* and *Gr5a* overexpression flies and *Gprk2* mutants, which argues that the balance between GR and GPRK2 abundance determine PrER response levels. Although the phase of GPRK2 cycling is the same in OSNs and GRNs, olfactory responses (e.g. spike amplitude) peak when gustatory responses are low, and gustatory responses peak when olfactory responses are low [14] (Fig. 1, 2). The difference in gustatory and olfactory response phases implies that GPRK2 has distinctly different activities in the olfactory and gustatory systems.

In OSNs, GPRK2 rhythmically promotes dendritic localization of ORs [38]. ORs and GRs are both seven transmembrane domain proteins that belong to the same superfamily of insect chemoreceptor proteins [20]. It is tempting to speculate that GPRK2 directly phosphorylates GRs, thereby controlling the abundance or activity of GR-dependent channels or ligand gated GR-channels in GRNs. This rhythmic regulation of neuronal excitability may be translated into rhythms in spike amplitude, frequency and duration. Thus, the PrER rhythm is likely a behavioral correlate of certain features of electrophysiological rhythms such as spike frequency and perhaps spike amplitude.

Functional significance of gustatory rhythms

Peripheral oscillators may play widespread roles in sensory processing, such that the perceived meaning of a sensory input is determined not just by the modality of the signal or its intensity, but also by the circadian time when the signal is registered. Our results indicate that the clock tunes the gustatory system to a higher gain level in the morning. This may allow the fly to temporally couple the morning bout of activity with food-detection machinery that works better at dawn, leading to increased feeding. This strategy can minimize energy expenditure by shutting down hardwired taste responses to weak stimuli (behavioral noise) at times when flies are resting, and selectively boost acuity at times when they are wakeful. Interestingly, the acrophase of feeding rhythms coincides with the early morning peak in gustatory response rhythms [25].

Social experience, which can influence behavior in *Drosophila* [48], is communicated by chemosensory cues such as pheromones. Moreover, circadian clocks in oenocytes regulate rhythms in the abundance of male pheromones including 7-tricosene [7] that are detected by GRNs [29, 49]. Thus, local clocks in oenocytes temporally gate the production of male pheromones and local clocks in GRNs may temporally gate pheromone reception and signaling. Such a system could function to define a time window for social interactions and mating, and the resulting social experience may in turn influence clocks that control pheromone production and/or gustatory sensitivity.

Feeding is modulated by the gustatory clock

Increased feeding in flies that lack circadian clock function in Gr5a neurons suggests that clocks in these cells act to restrict food consumption (Fig. 6). Given that clock function was compromised in GRNs that detect sugars [33, 50], increased food consumption may be due to a change in taste sensitivity. However, loss of clock function in Gr5a neurons decreases PrER responses at ZT1 and increases PrER responses at ZT17 (Fig. 4), indicating that the clock increases taste sensitivity in the morning and decreases it at night. When feeding was measured under the same conditions as PrER responses, food intake increased as PrER responses decreased in the morning and food intake decreased as PrER responses increased in the evening (Table S1). In control genotypes having clocks in Gr5a neurons, CAFE assays show that food intake is similar in the morning and the evening, consistent with estimates of food intake at different times of day in single flies using a novel assay that measures the proportion of time flies were observed extending their proboscis to feed [51]. Our results suggest that increased feeding is not due to altered taste sensitivity in flies lacking clocks in Gr5a neurons. It is possible that the increased food intake at ZT0–4 and the decreased food intake at ZT16–20 in flies lacking Gr5a neuron oscillators is due to metabolic feedback on tissues with functional clocks. Consistent with this possibility, food intake in *Clk^{Jrk}* and *cyc⁰¹* mutant flies is the same as that in wild-type flies [25].

Flies lacking clocks in Gr5a neurons do not show a measurable gain in weight (Fig. 6c), presumably due to a high fixed level of cuticle, protein and water weight. However, their triglyceride and glycogen content increases substantially (Fig. 6d, e), indicating that the higher amounts of food consumed are being stored. Given this increase in food storage it was surprising that flies lacking clocks in Gr5a neurons were also more active (Figure 6f). Increased activity is typically observed when starved flies are searching for food [52], yet flies lacking clocks in Gr5a neurons consume more food than wild-type flies (Fig. 6a, b). The loss of clocks in Gr5a neurons may mimic starvation conditions, particularly during the day when feeding is increased and PrER responses are relatively low compared to wild-type flies (Table S1; Fig. 4). At night, even though PrER responses are higher in flies lacking clocks in Gr5a neurons than in wild-type flies, activity is already low and feeding is even lower than in wild-type flies. Thus, loss of clock function in Gr5a neurons may produce starvation signals during the day, thereby increasing activity and feeding, but not at night since feeding is decreased and flies are already inactive.

Experimental Procedures

Fly strains

0–7 day old flies reared on standard cornmeal media were entrained for 3 days in 12h light: 12h dark (LD) cycles at 25°C. Lights were turned on at ZT0 and off at ZT12. Canton-S was used as our wild-type (WT) strain. The *Gr5a*-Gal4 driver [34], and the UAS-*Gr64a* [53], UAS-*Gr5a* [40], UAS-*cyc*^{DN} [8], UAS-*cyc* [8], and UAS-*Gprk2* [38] responders were described previously. These experiments also employed the P-element insertion mutant *Gprk2*⁰⁶⁹³⁶ [39], the *Pdp1ε*-specific deletion mutant *Pdp1*³¹³⁵ [35], the *Gr5a* deletion mutant *EP(X)-5* [40], the *Gr64* mutant (R1/+;R2/+; *Gr64/ Gr64*) that lacks all six *Gr64* genes [33], and the transgenically rescued *Gr64* mutant (R1/+;R2/+; *Gr64/ Gr64* flies carrying one copy of the UAS-*Gr64abcd_GFP_f* reporter) [33].

Western blotting and Immunostaining

30–35 proboscises were dissected from flies entrained for at least 3 LD cycles. Western blots were processed as described [38]. Blots were probed with anti-GPRK2 antibody (1:1000 dilution) and anti-Actin antibody (Sigma-Aldrich, 1:10000 dilution) and visualized via ECL (Amersham). Immunostaining was carried out on cryosectioned proboscises as detailed in **Supplemental Data**.

Single-sensillum recording

Male flies (3–10 days old) entrained to LD cycles for 3 days were collected during LD or the second day of DD, mounted, and the proboscis was immobilized. Individual labellar sensillae were observed under 1200X magnification. Recordings in the dark were made using a <600nm filter. The indifferent electrode was inserted into the eye. The recording electrode contained tastant dissolved in 1 mM KCl, and was used to stimulate a sensillum by physical contact with the tip of that sensillum. All recordings using a given genotype and tastant were performed at least six times per time point from 6 flies. A new group of flies were recorded at each timepoint. 100 mM sucrose was used to stimulate S cells in accessible I-type sensilla, which respond to sugars in an identical manner [30]. 10 mM caffeine was used to stimulate s6 and s2 sensilla whose L2 neurons are responsive to bitter compounds [30]. The number of spikes initiated by the tastant was counted manually over 500 ms duration beginning 50 ms after the onset of stimulation. Spike traces were analyzed using Axoscope (Axon) software in offline mode, where the peak and trough values of individual spikes were used to compute amplitude. The time elapsed between the peak and trough values for an activity spike was used as a measure of spike duration [54].

Proboscis extension reflex (PrER) assay

3–7 day old male flies that had been entrained to LD cycles for 3 days were starved for 24 h, collected at different times during LD or the first day of DD, mounted on a slide, and allowed to recover for 30 minutes. Proboscis extension in response to 100 mM sucrose and 100 mM trehalose was recorded as described [33], with minor modifications detailed in **Supplemental Data**.

Feeding assays

3–10 day old male flies entrained for at least 3 LD cycles were given food containing 5% sucrose, 1% low melting point agarose and 0.5% brilliant blue FCF (Wako) for 24 h starting at ZT12. Flies were then collected and prepared for quantification of blue dye ingestion as described [25]. For a given genotype, at least 6 independent experiments, each set consisting of 10 flies, were carried out. CAFE (Capillary Feeding) assays were used to measure feeding behavior of grouped fruit flies [25, 42]. For each genotype, CAFE assays were conducted as described [25] except that flies were habituated to feeding from glass capillaries for 24 h and feeding was measured over 4 h. CAFE assays were repeated at least 5 times for each data point. Levels of glycogen and triglycerides were measured as previously described [25].

Activity measurement

For each line, 7–10 day old male flies were entrained for at least 3 days in LD cycles and placed in *Drosophila* Activity Monitors (Trikinetics Inc.). Activity was measured by counting the number of infrared beam breaks every 10 minutes and analyzed using Clocklab software.

Statistical analysis

Statistical analysis was done using Statistica (Statsoft). Analysis of the effects of time of day was examined by one-way analysis of variance (ANOVA). Welch's ANOVA was used for heteroscedastic data set, provided Levene's test indicated unequal variances. Posthoc comparisons were done using Scheffe's test ($\alpha = 0.05$). Unpaired Student's *t*-test (two-tailed) was used to compare values at peak and trough time points.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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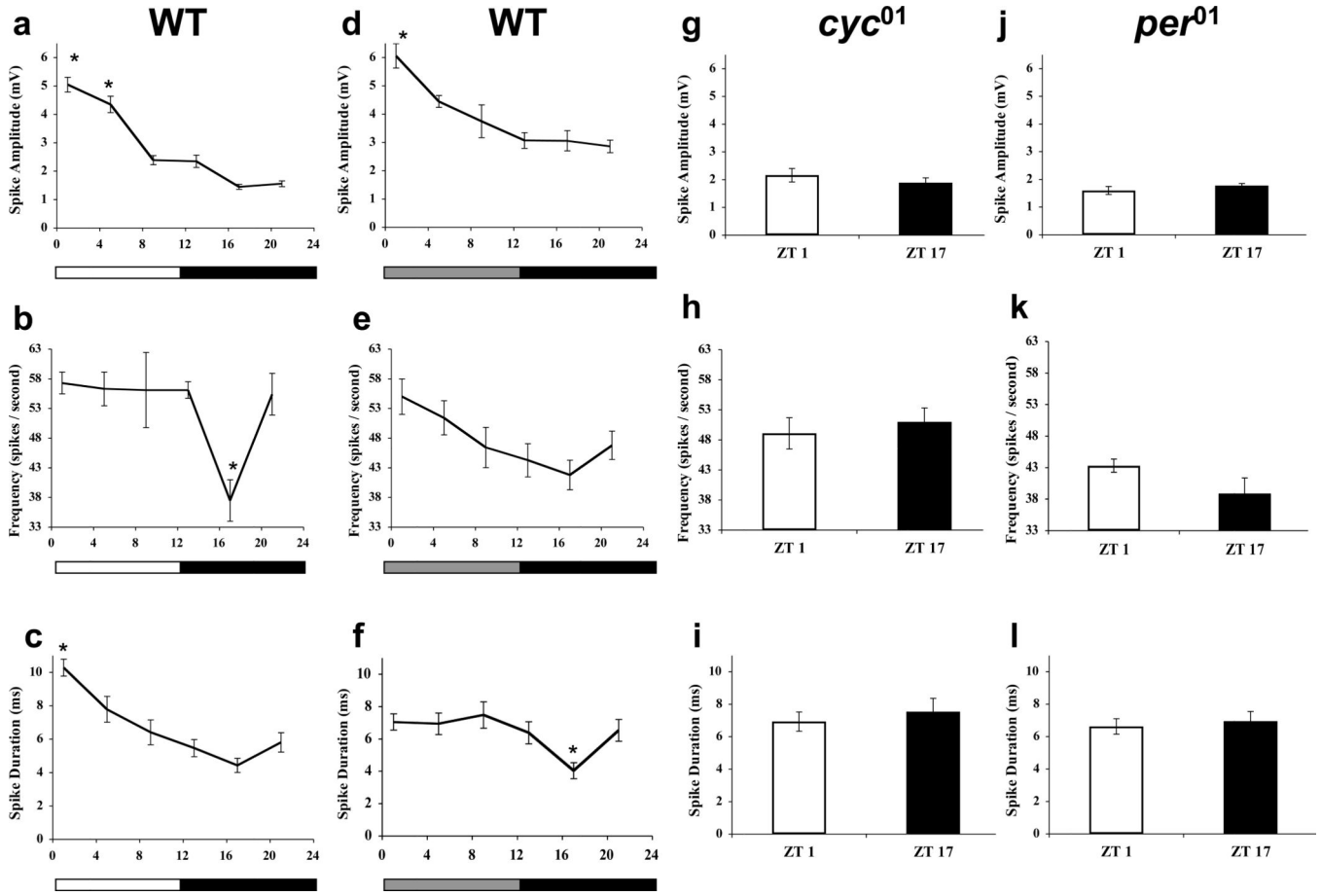


Figure 1.

S-spikes are under circadian-clock control in the I-type sensilla. Spike amplitudes (**a, d**), frequencies (**b, e**), and durations (**c, f**) were measured in WT flies collected at the indicated time points during LD cycles (**a–c**) or on the second day of constant darkness (**d–f**). The overall effects of time of day are significant ($p < 0.02$) by one-way ANOVA (**a, c–f**), and also significant ($p = 0.002$; heteroscedastic data set) by one-way Welch ANOVA (**b**). Asterisks indicate significant ($p < 0.05$) changes in spike parameters at a given time point compared to all other times of day. Spike amplitudes (**g, j**), frequencies (**h, k**), and durations (**i, l**) were measured in *cyc*⁰¹ (**g, h, i**) and *per*⁰¹ (**j, k, l**) flies collected at ZT1 and ZT17. The differences in mean amplitudes, frequencies, and durations of spikes at ZT1 and ZT17 are not significant ($p > 0.18$). Each time point represents amplitudes calculated from a minimum of 30 individual spikes in (**a, d, g, j**), frequencies calculated from a minimum of 10 individual GRNs in (**b, e, h, k**), and spike durations calculated from a minimum of 20 individual spikes in (**c, f, i, l**). All values are mean \pm S.E.M. Representative traces of single unit recordings are shown in Fig. S1.

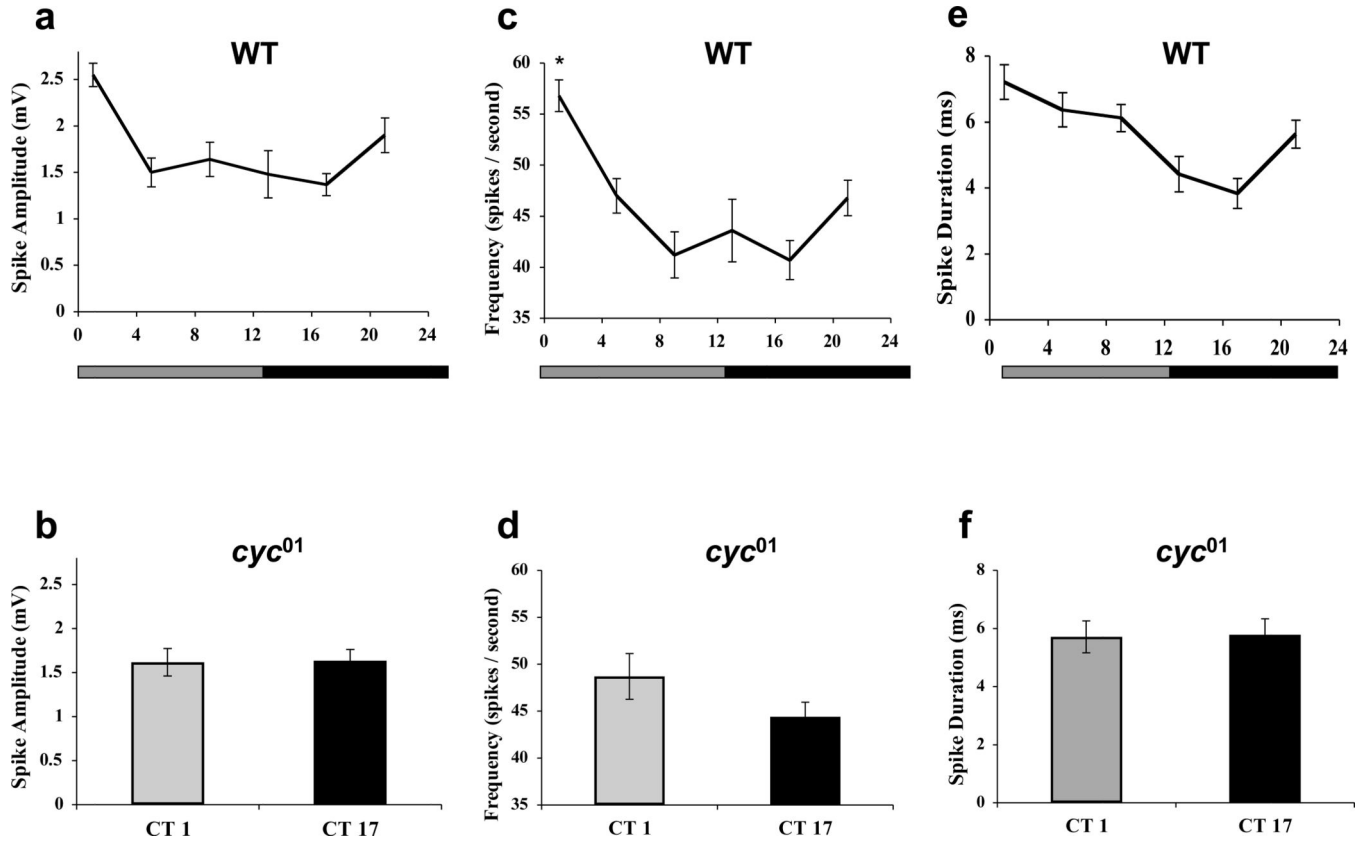
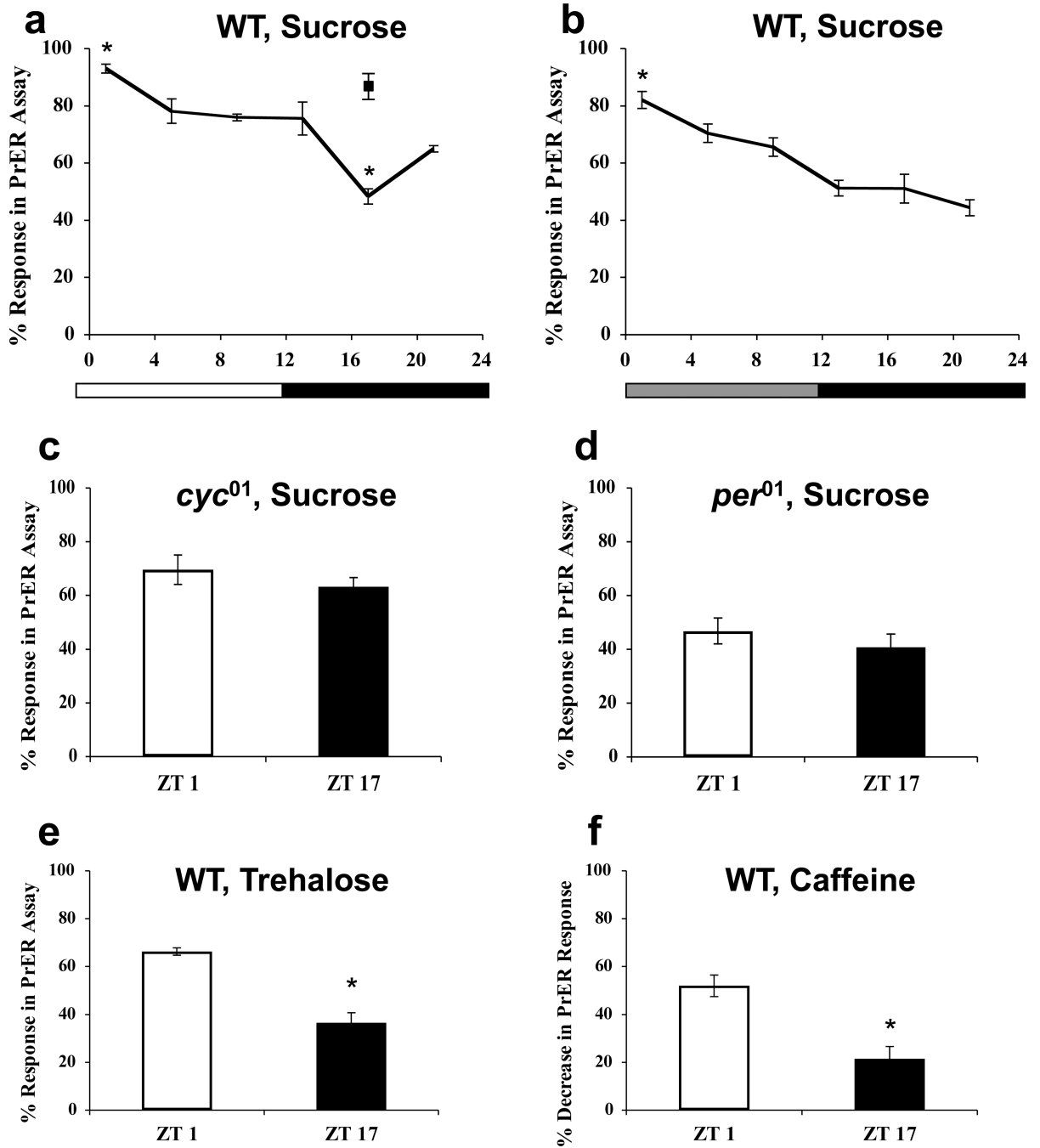


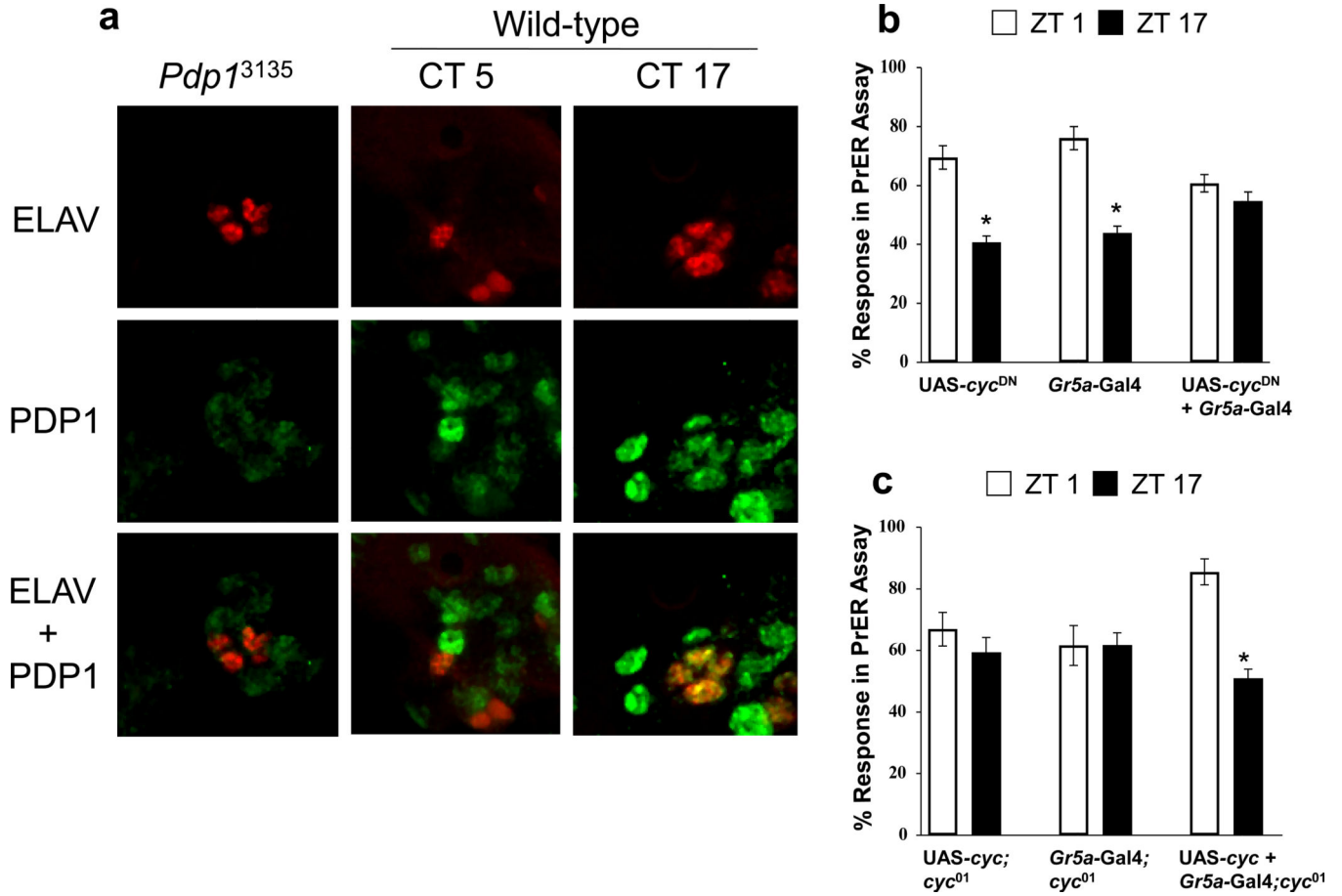
Figure 2.

L2-spikes are under circadian-clock control in s-type sensilla. Caffeine-induced L2 neuron spiking activity was measured from s-sensillae during the second day of constant darkness. (a) Spike amplitude was measured from 30 spikes in WT flies at each of the indicated time points. The overall effect of time of day is significant ($p < 0.005$) by one-way ANOVA. (b) Spike amplitude was measured from 30 spikes in *cyc*⁰¹ flies at CT1 and CT17. The difference in mean amplitudes of spikes at CT1 and CT17 is not significant ($p > 0.92$). (c) Spike frequency was measured from 10 individual GRNs in WT flies at each of the indicated time points. The overall effect of time of day is significant ($p < 0.001$) by one-way ANOVA. Asterisk indicates significant ($p < 0.05$) changes in firing frequency at CT1 compared to all other times of day. (d) Spike frequency was measured from 10 individual GRNs in *cyc*⁰¹ flies at CT1 and CT17. The difference in mean frequencies of spikes at CT1 and CT17 is not significant ($p > 0.13$). (e) Spike duration was measured from 20 individual spikes in WT flies at each of the indicated time points. The overall effect of time of day is significant ($p < 0.001$) by one-way ANOVA. (f) Spike duration was measured from 20 individual spikes in *cyc*⁰¹ flies at CT1 and CT17. The difference in the mean duration of spikes at CT1 and CT17 is not significant ($p > 0.92$). All values are mean \pm S.E.M.

**Figure 3.**

Drosophila display circadian rhythms in gustatory behavioral responses. (a, b) PrER responses to 100 mM sucrose (black line) or 500mM sucrose (filled square) were measured in WT flies during LD cycles (a) or the first day of constant darkness (b). The overall effects of time of day in LD (a) and constant darkness (b) are significant ($p < 0.001$) by one-way ANOVA. Asterisks indicate significant ($p < 0.05$) changes in PrER behavior at ZT1 and ZT17 (a) or CT1 (b) compared to all other times of day. (c, d) PrER responses to 100 mM sucrose were measured in *cyc*⁰¹(c), and *per*⁰¹(d) flies at ZT1 and ZT17. The difference in

mean PrER responses at ZT1 and ZT17 are not significant ($p > 0.30$) in *cyc*⁰¹ or *per*⁰¹ flies. **(e)** PrER responses to 100 mM trehalose were measured in WT flies at ZT1 and ZT17. Asterisks indicate a significant ($p < 0.001$) reduction in PrER responses at ZT17 compared to ZT1. **(f)** Decrease in PrER responses to a 100 mM sucrose solution containing 10 mM caffeine versus 100mM sucrose alone in WT flies at ZT1 and ZT17. Asterisk indicates significant ($p = 0.025$) decrease in PrER inhibition by caffeine at ZT17 compared to ZT1. All values are mean \pm S.E.M.

**Figure 4.**

Oscillators within GRNs are necessary and sufficient for PrER rhythms. **(a)** PDP1 and ELAV immunostaining in GRNs of *Pdp1*³¹³⁵ mutant flies collected at ZT17 and wild-type flies collected at CT5 and CT17. Anti-ELAV immunostaining (ELAV) is shown in red, anti-PDP1 immunostaining (PDP1) is shown in green, and co-localized PDP1 and ELAV immunostaining (ELAV + PDP1) is shown in yellow. **(b)** PrER responses were measured at ZT1 and ZT17 in wild-type flies bearing the *Gr5a*-Gal4, UAS-*cyc*^{DN}, or *Gr5a*-Gal4 + UAS-*cyc*^{DN} transgenes. The differences in mean PrER responses at ZT1 and ZT17 are significant ($p < 0.001$) in flies containing *Gr5a*-Gal4 and UAS-*cyc*^{DN} alone, but are not significant ($p < 0.30$) in flies carrying *Gr5a*-Gal4 + UAS-*cyc*^{DN}. **(c)** PrER responses were measured at ZT1 and ZT17 in *cyc*⁰¹ flies carrying the *Gr5a*-Gal4, UAS-*cyc*, or UAS-*cyc* + *Gr5a*-Gal4 transgenes. There are no significant ($p > 0.30$) differences in PrER responses at ZT17 and ZT1 in *cyc*⁰¹ flies carrying either UAS-*cyc* or *Gr5a*-Gal4. The differences in mean PrER responses at ZT1 and ZT17 are significant ($p < 0.001$) in *cyc*⁰¹ flies carrying UAS-*cyc* + *Gr5a*-Gal4. Asterisks denote a significant ($p < 0.05$) change in PrER responses between ZT17 and ZT1. All values are mean \pm S.E.M.

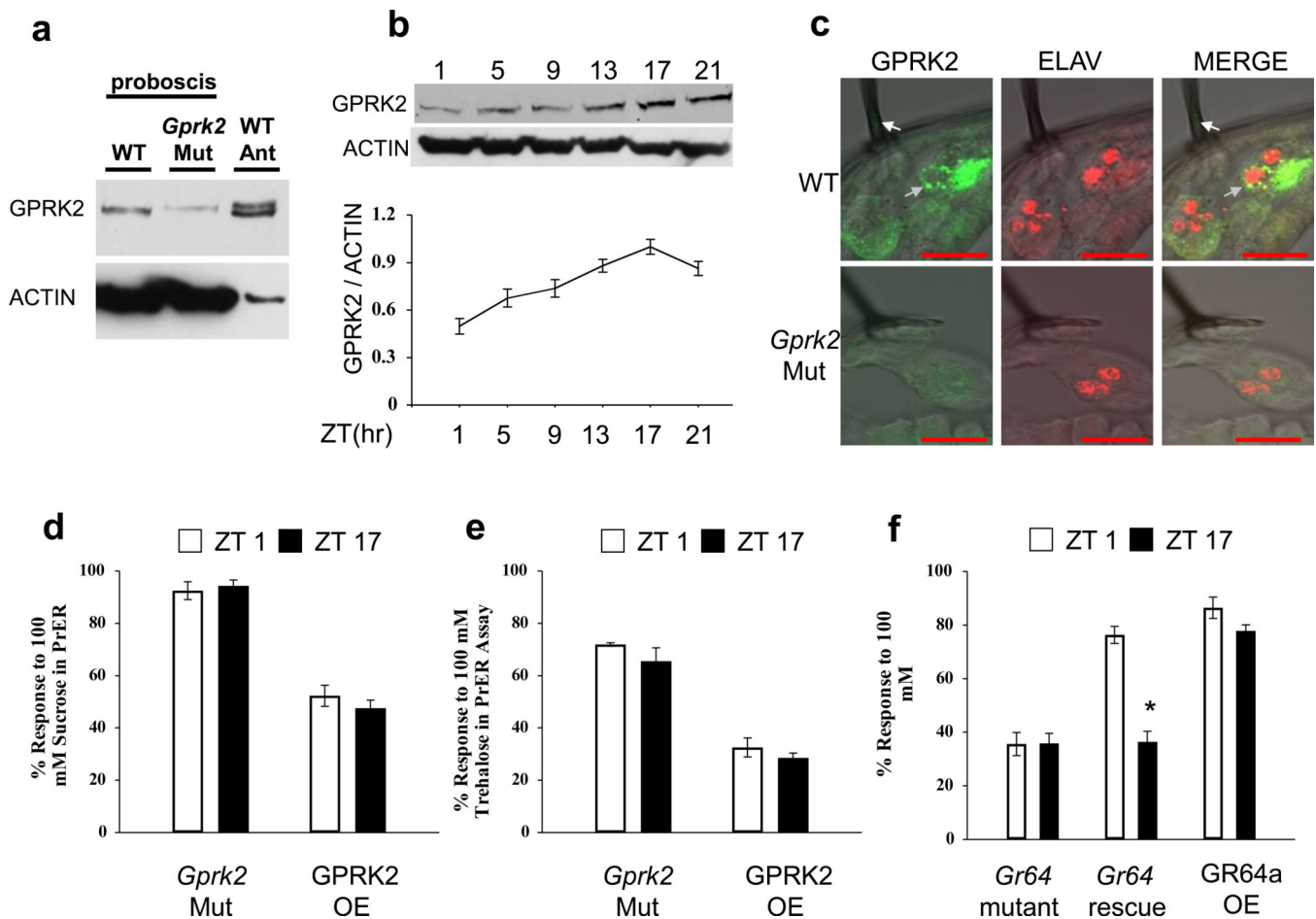


Figure 5.

GPRK2 and GR expression levels control rhythms in PrER behavior. **(a)** Western blot showing GPRK2 expression in proboscises from WT and *Gprk2*⁰⁶⁹³⁶ mutant (*Gprk2* Mut) flies and antennae (Ant) from WT flies. GPRK2 runs as two isoforms in antennae and one isoform in proboscises. ACTIN was used as a loading control **(b)** Western blot showing GPRK2 levels in proboscises of WT flies collected at the indicated times during an LD cycle. The GPRK2:ACTIN values at ZT1, ZT5, ZT9, ZT13, and ZT21 are relative to the value at ZT17, which was set to 1.0. Each timepoint represents the mean of three independent experiments. The overall effect of time of day is significant ($p < 0.005$) by one-way ANOVA. **(c)** GPRK2 and ELAV immunostaining in labellar GRNs from WT and *Gprk2* mutant flies. Anti-GPRK2 immunoreactivity is shown in green and anti-ELAV signal is shown in red. Scale bars represent 10 μ m. Grey arrows, GPRK2 localization in the cytosol; white arrows, GPRK2 immunostaining in the shaft of a sensillar hair. **(d, e)** PrER responses to sucrose and trehalose were measured at ZT1 and ZT17 in WT flies carrying *Gr5a*-Gal4 and UAS-*Gprk2*, which overexpress GPRK2 in S neurons (GPRK2 OE), and in *Gprk2*⁰⁶⁹³⁶ mutants (*Gprk2* Mut). Mean PrER responses to sucrose **(d)** and trehalose **(e)** at ZT1 and ZT17 were not significant ($p > 0.16$), and remained at constant low levels in *Gprk2* Mut flies and constant high levels in GPRK2 OX flies. For each genotype, three or more groups of 10 flies were tested for PrER responses to sucrose and trehalose at each

timepoint. Asterisks denote a significant ($p < 0.05$) change in PrER responses between ZT17 and ZT1. (f) PrER responses to 100 mM sucrose in *Gr64* mutant (*R1/+;R2/+; Gr64/Gr64*), *Gr64* rescue (*R1/+;R2/+; Gr64/Gr64* carrying one copy of the UAS-*Gr64abcd_GFP_f* reporter), and *Gr64a* overexpressing flies at ZT1 and ZT17. The differences in mean responses at ZT1 and ZT17 are not significant in *Gr64* mutants ($p > 0.90$) or *Gr64a* overexpressing flies ($p > 0.05$), but significant ($p < 0.001$) in *Gr64* rescue flies. All values are mean \pm S.E.M. As with PrER responses, lower GPRK2 expression in *Gprk2* Mut flies disrupts rhythms in GRN spike activity (Fig. S2).

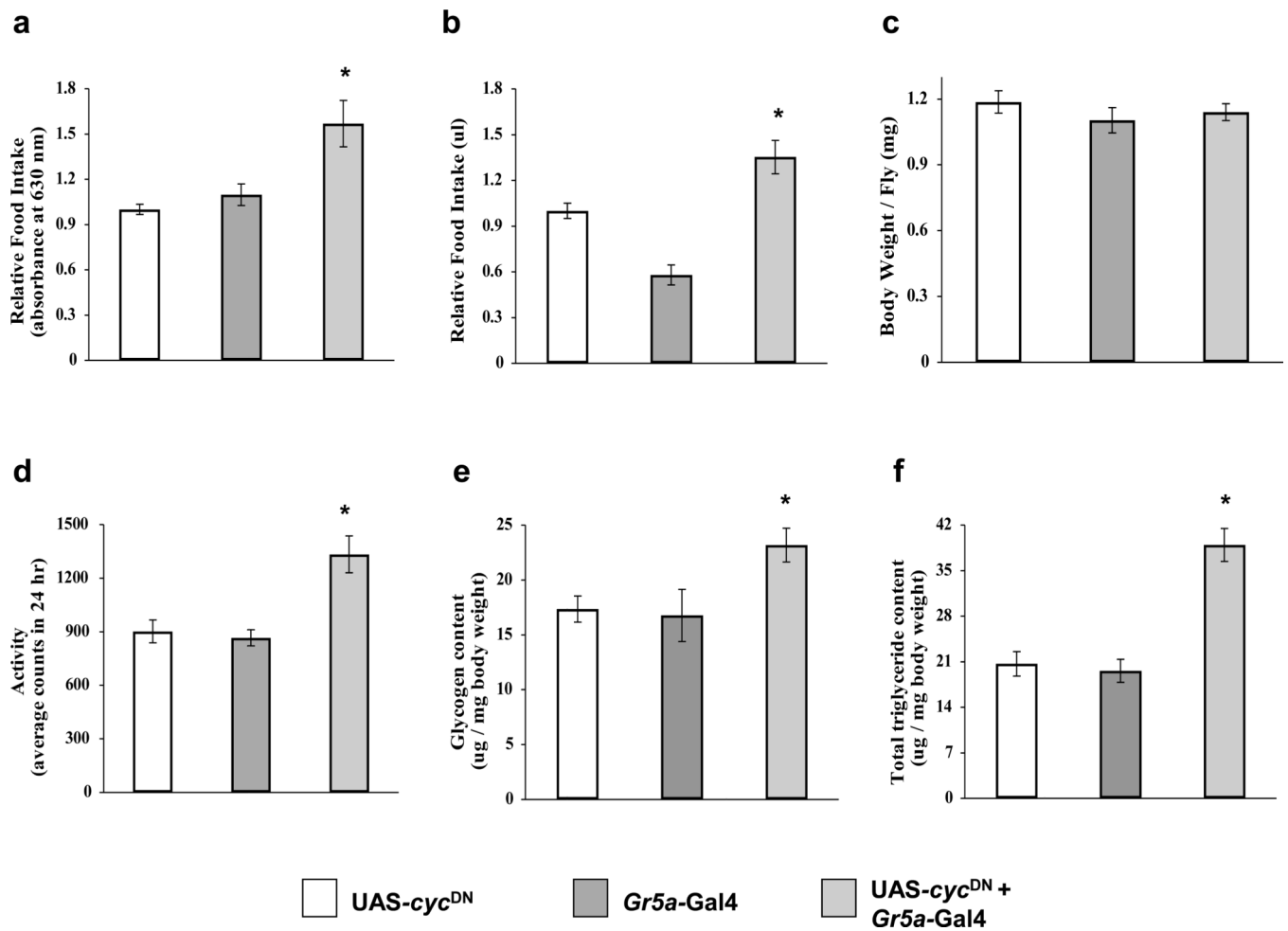


Figure 6.

Circadian clocks in Gr5a neurons regulate feeding, food storage and activity. **(a, b)** Relative food intake was measured after 24 h of feeding by quantifying the amount of blue food dye ingested **(a)** or by measuring the food consumed using the CAFE assay **(b)** (see Experimental Procedures). Flies carrying both the *Gr5a-Gal4* and *UAS-cyc^{DN}* transgenes show significantly ($p < 0.02$) increased feeding compared to control flies containing either the *UAS-cyc^{DN}* or the *Gr5a-Gal4* transgene. **(c)** The body weight of flies carrying both the *Gr5a-Gal4* and *UAS-cyc^{DN}* transgenes was not different ($p > 0.50$) than control flies bearing the *UAS-cyc^{DN}* or *Gr5a-Gal4* transgenes. **(d)** Overall activity was measured as the number of times flies crossed an infrared light beam during a 24 h period. Flies carrying both the *Gr5a-Gal4* and *UAS-cyc^{DN}* transgenes show significantly ($p < 0.001$) increased activity compared to control flies containing either the *UAS-cyc^{DN}* or the *Gr5a-Gal4* transgene. **(e)** Glycogen levels are significantly ($p < 0.04$) higher in flies carrying both the *Gr5a-Gal4* and *UAS-cyc^{DN}* transgenes than control flies bearing the *UAS-cyc^{DN}* or *Gr5a-Gal4* transgenes. **(f)** Triglyceride levels are significantly ($p < 0.001$) higher in flies carrying both the *Gr5a-Gal4* and *UAS-cyc^{DN}* transgenes than control flies bearing the *UAS-cyc^{DN}* or *Gr5a-Gal4*

transgenes. Error bars represent \pm S.E.M. Increased feeding and activity in flies lacking clocks in Gr5a neurons is not uniform over the circadian cycle (Table S1).