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Regulation of *Drosophila* circadian rhythms by miRNA let-7 is mediated by a regulatory cycle

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MicroRNA-mediated post-transcriptional regulations are increasingly recognized as important components of the circadian rhythm. Here we identify microRNA let-7, part of the *Drosophila* let-7-Complex, as a regulator of circadian rhythms mediated by a circadian regulatory cycle. Overexpression of let-7 in clock neurons lengthens circadian period and its deletion attenuates the morning activity peak as well as molecular oscillation. Let-7 regulates the circadian rhythm via repression of CLOCKWORK ORANGE (CWO). Conversely, upregulated *cwo* in *cwo*-expressing cells can rescue the phenotype of let-7-Complex overexpression. Moreover, circadian prothoracicotropic hormone (PTTH) and CLOCK-regulated 20-OH ecdysteroid signalling contribute to the circadian expression of let-7 through the 20-OH ecdysteroid receptor. Thus, we find a regulatory cycle involving PTTH, a direct target of CLOCK, and PTTH-driven miRNA let-7.

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Almost all animals display a wide range of circadian rhythms in behaviour and physiology, such as locomotor activity, sleep, learning and memory, mating and endocrine function[1–4]. The circadian system includes an input pathway, a core clock system and an output pathway[5]. Through the input pathway, a circadian oscillator receives external information, such as light and temperature signals, that entrain the core oscillator[6]. The core clock system is an integrative oscillator for a self-sustaining rhythm, and the output pathways transmit signals from the oscillator to observable rhythms in behaviour and physiology.

Numerous studies have focused on the molecular mechanisms and neural network properties of the circadian oscillator. The positive transcriptional regulatory factors CLOCK (CLK) and CYCLE (CYC) form heterodimers that lead to the transcription of the negative regulators PERIOD (PER) and TIMELESS (TIM) via binding to E-boxes in their promoters[7,8]. PER and TIM proteins accumulate in the cytoplasm with a delay and enter the nucleus to repress CLK-CYC-mediated transcription by collaborating with other factors like kinases[9]. CLK-CYC also activates two other feedback loops critical for the phase and amplitude of core oscillators and outputs. One feedback loop affecting *Clk* mRNA cycling is mediated by VRILLE (VRI) and PAR DOMAIN PROTEIN 1 expression[10,11], and another is mediated by CLOCKWORK ORANGE (CWO), which is activated by CLK-CYC[12–15]. The *per* and *tim* genes receive two opposing signals form DNA-binding transcription factors—a direct activation from CLK-CYC and a direct repression from CWO. In addition, CWO regulates the level of the CLK-CYC, presumably through its actions on each of three main loops[16]. So *cwo* contributes to sustaining a high-amplitude circadian oscillation *in vivo*[12–14]. The *Drosophila* rhythm strength is sensitive to the level and timing of *cwo* expression, although the amplitude of the *cwo* mRNA oscillation is much weaker than that of the other core clock genes[15].

In the adult fly brain, ~150 clock neurons are organized into six major clusters: three dorsal clusters (DN1, DNs and DN3), a dorsal–lateral cluster and two groups of ventral–lateral neurons (sLNvs and lLNvs)[17]. Under light-dark (LD) conditions, flies show a morning activity peak (M) around the time of lights-on and an evening activity peak (E) around the time of lights-off. Flies can increase their locomotor activity in advance of both dark-to-light and light-to-dark transitions—a phenomenon termed anticipation. The morning and evening anticipatory behaviour is generated in different clusters of clock neurons[18,19].

Beyond the abundant information on the transcriptional–translation feedback loops of circadian rhythms, the importance of post-transcriptional mechanisms is increasingly recognized in circadian rhythms[20,21], such as regulation by microRNAs (miRNAs)[22]. The miRNAs are endogenous small non-coding RNAs (19–25 nt) that function in post-transcriptional regulation of gene expression, and are found to target more than 30% of all protein-coding mRNAs in mammals[23,24]. Via base-pairing with specific sequences of target genes typically found in mRNA 3′ untranslated regions (3′UTRs), they usually result in gene silencing via translational repression or target mRNA degradation by an RNA-induced silencing complex consisting of Argonaute catalytic subunits (Ago1 in *Drosophila*)[25,26]. One requirement for specificity is an accurate match between the 5′-proximal 'seed' region of the miRNA and the 3′UTRs of its target mRNA[27]. Previous studies have revealed that miRNAs are involved in regulation of the core circadian oscillator and the clock outputs in *Drosophila*. By immunoprecipitating mRNAs bound to AGO1, Kadener *et al.*[28] found that core clock genes *Clk*, *Vri* and *Cwo* are associated with RNA-induced silencing complex, and that bantam-dependent regulation of *Clk* expression is required for circadian rhythm. Two miRNAs (miR-263a and -263b) were also found to exhibit robust daily changes in abundance in wild-type flies that are abolished in the cyc^{01} mutants[29]. In addition, the miR-279 influences circadian behavioural outputs via targeting *upd*, a ligand of the JAK/STAT pathway[30], and the oscillating miRNA 959–964 cluster impacts *Drosophila* feeding time and circadian outputs[31].

To further investigate the role of miRNAs in the *Drosophila* core circadian clock, we focus on the core clock gene *cwo* because it is rhythmically expressed and contributes to a high-amplitude circadian oscillation of core clock genes *in vivo*. *Drosophila* rhythm strength is sensitive to *cwo* both in expression level and timing[12–14]. Here we report that the miRNA *let-7* affects the locomotor rhythm through the circadian-relevant target gene *cwo* in the central clock. We also study the signal–transduction pathway and molecular mechanism by which *let-7* regulates the daily circadian rhythm, and show that *let-7* is involved in an ecdysteroid-signalling cycle.

Results

Overexpression of let-7 prolongs activity rhythms. Because the expression of the *let-7*-Complex (*let-7-C*) or *let-7* driven by the *tim*-GAL4 led to embryonic lethality, we used a *pdf*-Gal4 driver to overexpress *let-7-C* or *let-7* in the *pdf*-expressing cells (LNvs), which control locomotor activity rhythms. *Let-7-C* or *let-7* overexpression driven by *pdf*-GAL4 lengthened circadian period about 2 and 1 h, respectively. In flies expressing mutant *let-7* (*UAS-mut-let-7*), which was generated by site-directed mutagenesis of the *UAS-let-7* construct, with G-C transitions at seed sequence sites 3-4 of the mature *let-7* miRNA and complementary mutations on the opposing side of the hairpin[32] (Supplementary Fig. 1a), the circadian period was normal (Table 1 and Fig. 1a). These results indicate that *let-7* affects circadian period. To exclude the possibility that the behavioural phenotype was caused by ectopic induction of *let-7* in neurons where it is usually not expressed, we utilized a knock-in Gal4 in the *let-7-C* locus (Supplementary Fig. 1b)[33] to drive expression of *let-7-C*, and showed the miRNA levels of *let-7*, *miR-125* and *miR-100* were more abundant (Supplementary Fig. 2a). Overexpression of *let-7-C* driven by *let-7-C-Gal4* phenocopies the effect driven by *pdf-Gal4* (Fig. 1a and Table 1), suggesting that *let-7* regulates circadian rhythms in regions of its normal expression. To distinguish effects of *let-7* in adult and in developmental processes, we used a temperature-sensitive *tubulin-Gal80^{ts}* coupled with the *tim-Gal4* driver to overexpress *let-7-C*, *let-7* and *miR-125* in adults. Results showed that the miRNA levels of *let-7* and *miR-125* were more abundant compared to control (Supplementary Fig. 2b). Adult-specific overexpression of *let-7-C* and *let-7* in *tim* cells resulted in longer circadian periods (Fig. 1b) and lower percentages of rhythmic flies when flies were shifted from 18 to 29 °C. Circadian behavioural rhythms were present in 58.1% ($\tau = 25.2$ h, $n = 43$) and 77.8% ($\tau = 25.0$ h, $n = 45$) of the *let-7-C* and *let-7* flies, respectively, in constant darkness (DD), while control flies exhibited normal behavioural rhythms (85.1% rhythmic, $\tau = 23.8$ h, $n = 47$). By contrast, the flies with *miR-125* overexpression and control flies exhibited similar behavioural rhythm (87.8% rhythmic, $\tau = 24.3$ h, $n = 49$). The results show that *let-7* affects the central clock. We further drove *let-7-C* expression with a drug (RU486) inducible pan-neuronal driver (elavGeneSwitch; *elavGS*), which also lengthened period in DD (Fig. 1c). Taken together, all of these results demonstrate that adult-specific overexpression of *let-7* in circadian cells lengthens circadian period and reduces rhythmicity.

Table 1 | Locomotor activity of flies with altered *let-7* levels.

Genetype	Total flies	Rhythmic flies (%)	Period (h)	Power
pdf-Gal4/+; UAS-let-7-C/+	80	95.0	26.12 ± 0.24	82.2 ± 2.89
pdf-Gal4/+	61	100	24.02 ± 0.20	98.3 ± 4.24
UAS-let-7-C/+	51	95.2	23.86 ± 0.52	74.3 ± 5.13
let-7-C-Gal4/+; UAS-let-7-C/+	53	90.0	25.04 ± 0.26	79.7 ± 5.74
let-7-C-Gal4/+ (let-7-C^{GKI}/+)	60	97.0	23.89 ± 0.05	113.5 ± 3.28
pdf-Gal4/+; UAS-let-7/+	31	100	24.98 ± 0.04	80.1 ± 5.29
pdf-Gal4/+; UAS-mut-let-7/+	39	99.0	23.14 ± 0.21	85.6 ± 5.63
let-7-C$^{KO1/GKI}$	48	50.0	23.75 ± 0.18	58.5 ± 4.34
let-7-C^{KO1}/+	47	91.0	23.71 ± 0.04	97.7 ± 5.21
P{W8, let-7-C}/Y; let-7-C$^{KO1/GKI}$	56	100	24.79 ± 0.07	89.2 ± 6.27
P{W8, let-7-C}/Y; let-7-C$^{KO1/KO1}$	47	92.0	24.36 ± 0.06	75.2 ± 6.30
yw;△let-7,miR-125	42	48.0	24.20 ± 0.20	52.3 ± 5.84
yw;△let-7,miR-125/+	36	90.0	23.90 ± 0.06	94.2 ± 9.97

Let-7 overexpression in clock neurons maintain the normal rhythm. Both *let-7-C* and *let-7* mutants become largely arrhythmic in constant darkness (DD). Flies regain the normal rhythm by inserting the P element—*P{W8, let-7-C}* into *let-7-C* mutants.

Let-7 is required for normal circadian rhythms. Deletions of the *let-7-C* and *let-7,miR-125* had been generated by homologous recombination methods[32,33]. Single *let-7*, *miR-125*, *miR-100* mutant strains were generated by deletions of rescuing transgenes, in which the expression of *let-7*, *miR-100* or *miR-125* had been separately eliminated, introduced into strains with deletions of the entire endogenous *let-7-C* locus—named △*let-7* (*KO1/GKI;pP{W8,let-7-C$^{\triangle let-7}$}/+*), △*miR-100* (*KO1/GKI;pP{W8,let-7-C$^{\triangle miR-100}$}/+*) and △*miR-125* (*KO1/GKI;pP{W8,let-7-C$^{\triangle miR-125}$}/+*) (Supplementary Fig. 1b)[33]. In this study, PCR and real time quantitative reverse transcriptase PCR analysis were performed to confirm the deletion of the *let-7-C* locus and individual miRNAs (Supplementary Fig. 2c,d).

To test whether the *let-7* is required for normal circadian rhythmicity, we analysed the locomotor activity in different *let-7-C* mutants. Results showed that there was no anticipation of lights-on in *let-7-C* mutants (*KO1/GKI*) under LD, and this phenotype was conspicuously different from that of wild-type and *let-7-C* mutant heterozygous flies (*KO1/+* & *GKI/+*) (Fig. 2). The △*miR-100* mutants exhibited normal morning and evening peaks, suggesting that *miR-100* was not responsible for the circadian defect. In contrast, △*let-7,miR-125* mutants exhibited severely suppressed morning peaks before light-on. The absence of lights-on anticipation was also observed in △*let-7* mutants but not in △*miR-125* mutants (Fig. 2), indicating that *let-7* is responsible for the circadian defect in morning anticipation in *let-7-C* mutants, which could be well rescued by *UAS-let-7* in pigment dispersing factor (PDF) neurons (Fig. 2). Because the lights-on response in LD can mask some of the clock-driven morning behaviour[34], the first day of DD after LD entrainment was also assessed, and this analysis confirmed the lack of morning anticipation in the *let-7* mutants (Fig. 2). Besides, both *let-7-C* and △*let-7,miR-125* mutants became largely arrhythmic in DD (both produced about 50% rhythmic flies), indicating that *let-7* is also required for robust 24 h free-running rhythms (Table 1).

CLOCKWORK ORANGE (*cwo*) is a target of let-7. The *Drosophila Cwo* gene was identified by bioinformatics as a possible target for *let-7* (refs 35,36) (Supplementary Fig. 3). *Cwo* produces three mRNAs that encode two different proteins, in which all of the transcripts contain the same ∼1.4-kb 3′ UTR. Detection of AGO1-associated mRNAs from fly heads also showed that *Cwo* might be a miRNA-regulated target[28]. To test whether *let-7* can actually regulate the *Cwo* 3′ UTR, S2 cells were transfected to express the 3′ UTR of *Cwo* downstream of a luciferase reporter. The cells were also separately transfected to express *pri-let-7* (long *let-7* precursor). Results showed that the *let-7* reduced activity of the luciferase ∼70%, and mutation of the *let-7* target sites in the seed regions of the *Cwo* 3′UTR produced a recovery of activity compared to control (Fig. 3a). The results in *let-7*-transfected S2 cells showed that *let-7* expression directly reduced the *Cwo* mRNA and protein levels (Fig. 3b). Moreover, AGO1-associated *cwo* mRNA from fly heads of wild-type and the △*let-7* mutant using the AGO1 IP method of Kadener *et al.*[28] were also assayed. Results showed that *cwo* mRNA from AGO1 immunoprecipitates was greatly diminished in △*let-7* mutant (Fig. 3c). All these results support that *cwo* is a direct target of *let-7*.

Does downregulation of *Cwo* by *let-7* contribute to the normal circadian rhythm? To explore this question, we altered the *Cwo* level by using *UAS-let-7-C* driven by *cwo-GAL4*. Results showed that *let-7-C* overexpression driven by *cwo-GAL4* (*cwo-Gal4/UAS-let-7-C*) lengthened circadian period (Fig. 3d), which is consistent with the effect of *cwo-RNAi* (long circadian period) driven by both *cwo-Gal4* and *tim-Gal4* (Fig. 3d), and it was able to be partially rescued by *Cwo* overexpression (*UAS-cwo/+; cwo-Gal4/UAS-let-7-C*) (Fig. 3d and Supplementary Fig. 4). *Let-7* expression in *cwo* neurons (*cwo-Gal4/UAS-let-7*) resulted in complete lethality. In order to verify that the lethality is due to the reduction of *Cwo*, we also performed a genetic rescue experiment, in which the viability was normal in *UAS-cwo/+; cwo-Gal4/UAS-let-7* transgenic flies compared to controls (Supplementary Fig. 5). Taken together, all these findings demonstrate that miRNA *let-7*-mediated *Cwo* regulation contributes to the circadian rhythm.

Let-7 regulates pacemaker neurons. Because *let-7* appears to function in the central clock, we determined whether *let-7* is expressed in the central pacemaker by labelling *let-7-C*-expressing neurons (*let-7-C-GAL4 × UAS-EGFP*) with green fluorescent protein (GFP) and immunolabeling with anti-PDF antibody. Results showed that *let-7* was broadly expressed in the fly brain. The GFP signal was enriched in brain regions such as the mushroom bodies (Fig. 4a). The co-detection in brains for GFP and PDF revealed that *let-7-C* was expressed in the LNvs (Fig. 4a). The presence of a GFP signal in clock neurons in *let-7-C-GAL4:UAS-EGFP* flies further supports the action of *let-7* in the central pacemaker. To further investigate the expression of *let-7* and its target *Cwo* in the brain, we also analysed two transcriptional reporters that contain almost the entire first intron of *let-7-C* (*let-7-Cp12.5kb::lacZ*) or just the 20-OH ecdysteroid (20E)-responsive 3.3 kb fragment (*let-7-Cp3.3kb::lacZ*) with

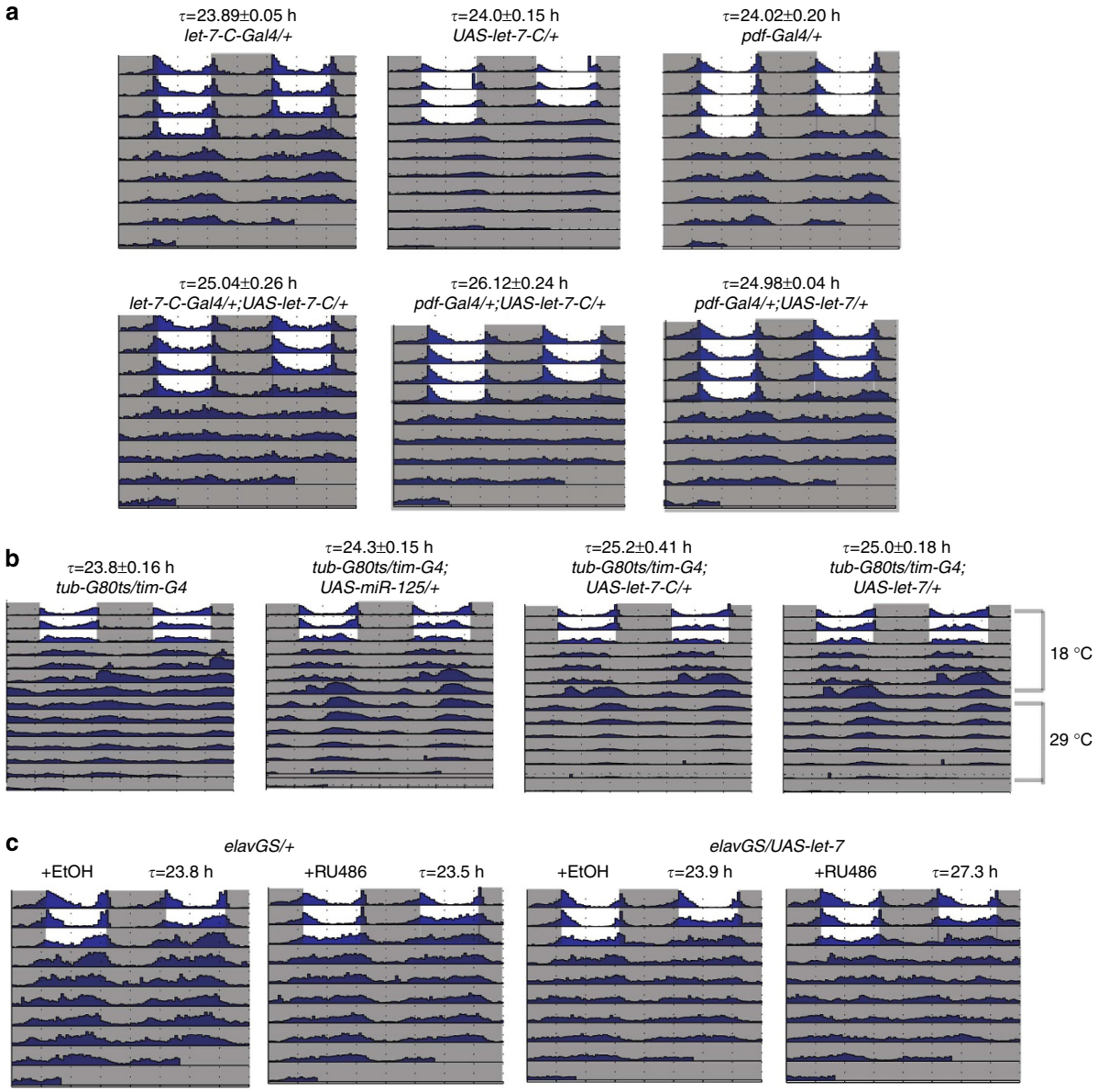

Figure 1 | Overexpression of *let-7* influences locomotor activity rhythms. (**a**) Overexpression of *let-7-C* or *let-7* with *let-7-C-Gal4 and pdf-Gal4* leads to long behavioural periods in DD. The genotypes are indicated on top of the panels. The white and grey parts indicate light and dark, respectively. (**b**) Induction of *let-7* in *tim* cells leads to long behavioural periods in DD. To restrict expression of *let-7* in adults, *tim-Gal4*-induced expression was repressed during development by a *tubulin-GAL80^{ts}*. Flies were reared and entrained for 3 days following eclosion at 18 °C, free-running activity rhythms were monitored for 3 days at 18 °C, and then the temperature was shifted to 29 °C. The genotypes are indicated on the top of the panels. The white and grey parts indicate light and dark, respectively. (**c**) Pan-neuronal induction of let-7 in adulthood leads to a long period. Files were reared and then aged for 3 days following eclosion on regular food. They were fed either 500 μM RU486 or ethanol (vehicle control) from the time of entrainment. Average periods of the DD records are shown. The white and gray parts indicate day and night, respectively. Each experiment was conducted at least three times, and 60–90 flies of each genotype were used for behavior assays.

cwo-Gal4 driving GFP[37]. The *Cwo* reporter was prominently expressed in the LNvs, the PI neurons and the SG (Fig. 4b). The spatial expression of lacZ resembled that previously reported in *let-7-C-Gal4:UAS-EGFP*. All of the reporters exhibit co-expression of *let-7-C* in the LNv neurons.

To test whether *let-7* affects the central clock, we explored its effects on the daily expression of CWO proteins. Temporal expression profiles of CWO protein in wild-type and *let-7-C* mutant and △*let-7* mutant flies under LD conditions showed that CWO was highly expressed in these two mutant fly heads compared to wild-type flies (Fig. 5). In addition, as shown in Fig. 6, *let-7-C* mutant flies and wild-type controls do not exhibit similar daily PER and PDF cycling in the central clock tissue, in which PDF is higher and nuclear PER accumulates sooner in *let-7-C* mutant flies due to a decrease of *let-7*, without an effect on the time of peak nuclear PER accumulation. These results above indicate that *let-7* has a function in the core clock through CWO.

A novel regulatory cycle in circadian rhythm. To test whether *let-7* itself is regulated by the circadian clock, we further assayed *let-7* in wild-type flies and *Clk^{Jrk}* mutants and found that the expression of mature *let-7* exhibited statistically significant (albeit

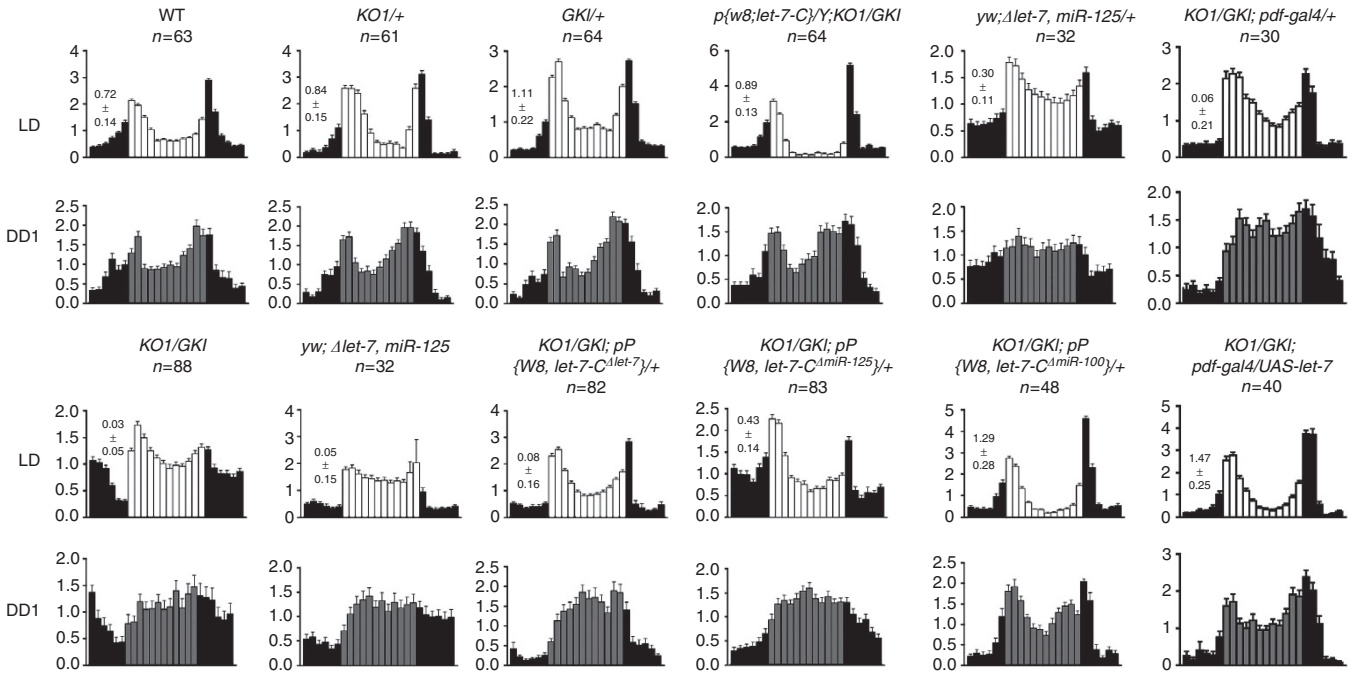

Figure 2 | Genetic ablation of *Let-7* causes flies lose morning anticipation. Comparison of the circadian locomotor activity in light/dark cycles of normal *let-7-C* expression and mutant *let-7-C* expression flies. Histograms represent the distribution of activity through 24 h, averaged for flies over three LD days and the first day of DD (DD1), and show the activity for each 1 h. Normalized activity profiles are shown. The white and black bars indicate day and night, respectively, while grey and black bars indicate subjective day and night, respectively. Numerical values indicate measures of morning anticipation. Note that genotypes devoid of anticipatory behaviour still respond to light by exhibiting high activity after the light transitions. Total numbers of each genotype are shown in the figure, and each experiment was conducted at least three times. The values were plotted with means ± s.e.m.

weak) cycling with peak levels in the late day in wild-type flies but not in Clk^{Jrk} flies under an LD condition (Fig. 7a). However, *pri-let-7-C* in wild-type flies did not exhibit a daily rhythm compared to mature *let-7* under an LD condition (Fig. 7a). This suggests that *let-7* cycling may be post-transcriptionally regulated, which is consistent with a previous report[38].

To investigate whether CLK could directly activate *let-7*, the *let-7-C* locus was examined for CLK-biding sites. Since bHLH transcription factors are known to bind to the consensus hexanucleotide sequence E-box (CANNTG) we focused on the various CLK-binding E-boxes described previously[39]. Twenty-one E-boxes were found within a 12.5 kb region surrounding *let-7-C*. Sixteen of these E-boxes are evolutionarily conserved across five *Drosophila* genomes (*D.melanogaster*, *D.simulans*, *D.sechellia*, *D.yakuba* and *D.erecta*). We therefore examined whether CLK-CYC can induce gene expression through these E-boxes in *Drosophila* S2 cell cultures. Cotransfection with CLK did not induce luciferase expression with 7 fragments from the *pri-let-7-C* promoter region (Supplementary Fig. 6), indicating that *pri-let-7-C* cannot be directly regulated by CLK-CYC through E-boxes, and suggesting that the clock regulates *let-7* via an indirect pathway.

A previous publication has shown that the ecdysteroid receptor (EcR) may effectively target the promoter of *let-7-C* and activate *let-7-C*[37]. Thus, it is important to determine if EcR signalling drives *let-7-C* expression in the adult brain. First, we found that the brain prothoracicotropic hormone (PTTH), an activating hormone for ecdysteroid release, had a daily mRNA rhythm with two peaks at ZT4 and ZT16, and these were blunted in the Clk^{Jrk} mutant in LD (Fig. 7b). In addition, the *ptth* mRNA oscillation in wild-type and Clk^{Jrk} was weakened in DD, although a relatively higher expression at CT4 persisted in wild-type (Fig. 7b). The *ptth* transcriptional periodicity has previously been shown to be correlated with ecdysteroid titre in *Drosophila*[40]. To further investigate whether CLK could directly activate *ptth*, we fused the promoter of *ptth* from the chr2L: 573711–575711 region with luciferase (ptth-luciferase), and then tested it in S2 cells with tim-luciferase as a positive control. Results showed that the ptth-luciferase was significantly activated when co-transfected with CLK/CYC-expressing plasmids (Fig. 7c), indicating PTTH is a critical target of the clock and may be involved in regulation of the *let-7* oscillation. The most prominent peak of *ptth* mRNA at ZT 4 is not typical of most CLK/CYC-dependent genes, which peak close to ZT16. However, a previous study showed that PTTH cells might be targets of the s LNv or DN cells and thus stay under control of the master clock[41]. Our results have also shown that *ptth* is a direct target of CLK/CYC. We speculate that the ZT4 and ZT16 peaks are consequences of multiple circadian inputs to the *ptth* gene (that is, direct activation by CLK/CYC and inputs resulting from LNv/DN signals).

Furthermore, we compared the levels of the 20E steroid hormone (a downstream target of PTTH) at ZT2, 8, 14, 20 between Clk^{Jrk} mutants and wild-type flies, and the results showed that 20E oscillated with a daily rhythm in the wild-type flies but lacked a daily oscillation in the Clk^{Jrk} mutant (Fig. 7d). Compared to the 20E oscillation, the oscillation of *let-7* showed a significant phase delay (Fig. 7a,d).

Moreover, a transgenic line in which each of the ecdysone response elements (EcREs) sequences in the *let-7-C* locus were deleted[37] was used to confirm the necessity of EcREs for circadian *let-7-C* expression. In contrast to the wild-type lacZ reporter, expression of the mutated *let-7-Cp12.5kb△EcRE1-3::lacZ* reporter was strongly blocked in the pacemaker neurons (Fig. 8a). The ecdysteroid signal is thought to act through a heteromeric receptor composed of the EcR and USP nuclear receptor proteins. So we also blocked ecdysteroid signalling by using the transgenic

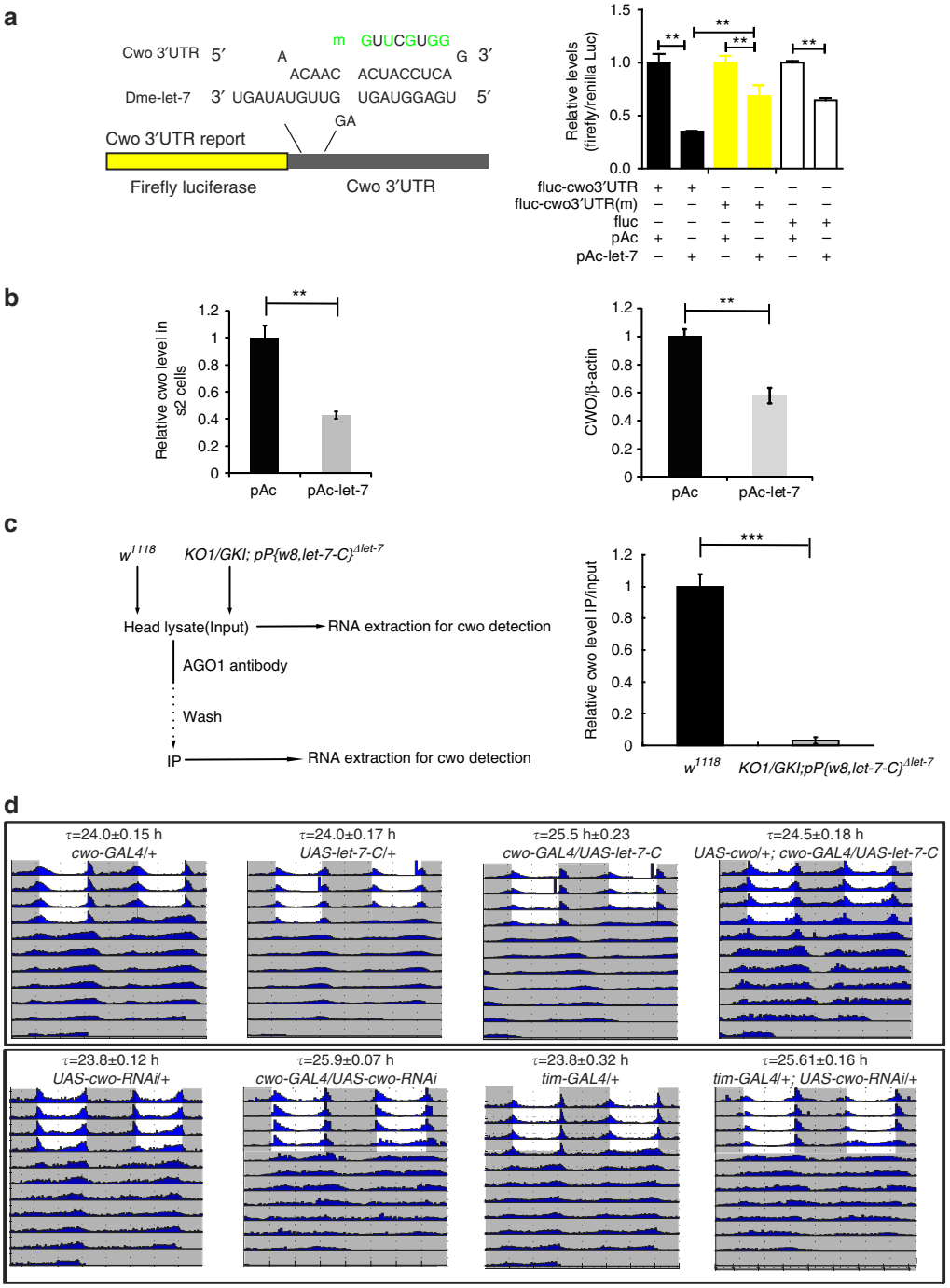

Figure 3 | *Cwo* is a target of *let-7*. (**a**) The native site of the *Cwo* 3′UTR sequence containing the *let-7* target was amplified and introduced into a firefly luciferase (fluc) reporter vector (pAc-fluc-cwo3′UTR). Sequence with selected point mutations (m) in the *Cwo* 3′UTR was also constructed as a negative control for the same reporter assays. Luciferase reporter assays were performed after cotransfection with pAc-fluc-cwo3′UTR, pAc-fluc-cwo3′UTR (m) or fluc together with an empty vector (pAc) or a *let-7* expression vector (pAc-let-7). In all cases, cotransfection with pCopia-Renilla Luciferase was performed. For each condition, a normalized firefly/renilla luciferase value was plotted with ± s.e.m. (**b**) *let-7* inhibits CWO protein level by affecting *Cwo* mRNA *in vitro*. S2 cells transfected with pAc or pAc-let-7 were lysed and analysed for both *Cwo* mRNA and CWO protein. *Cwo* mRNA was detected by quantitative PCR and normalized to *rp49* mRNA, and CWO protein was detected by immunoblot with β-actin as control. Values are reported as fold changes (means ± s.e.m.) relative to cells transfected with control expression plasmid. (**c**) Detection of AGO1-associated *cwo* mRNAs from w^{1118} and *KO1/GKI*; $p\{w8,let\text{-}7\text{-}C^{\Delta let\text{-}7}\}$ fly heads. Diagram illustrates the AGO1 IP procedure, and the enrichment of *cwo* was calculated as a ratio between the expression values in the immunoprecipitation and the input fractions, normalized to the value for w^{1118}. AGO1-associated *cwo* mRNA from w^{1118} was significantly higher than AGO1-associated *cwo* mRNA from *KO1/GKI*; $p\{w8,let\text{-}7\text{-}C^{\Delta let\text{-}7}\}$. (**d**) Overexpression of *let-7-C* or RNAi knockdown of *cwo* with *cwo-Gal4* or *tim-GAL4* leads to long behavioural periods in DD. The genotypes are indicated on top of the panels. The white and grey parts indicate light and dark, respectively. Expression of *Cwo* was able to partially rescue the phenotype of *let-7-C* overexpression in *cwo* cells (*UAS-cwo/+*; *cwo-Gal4/UAS-let-7-C*) (top panels). Knockdown of *Cwo* in either *cwo*- (bottom panels) or *tim*-expressing (bottom panels) neurons causes long periods, which mimicked the phenotype of *let-7-C* overexpression. Each experiment was conducted at least three times, and 60–90 flies of each genotype were used for behaviour assays. A significant difference by Student's *t*-test is indicated (**$P<0.01$ and ***$P<0.001$).

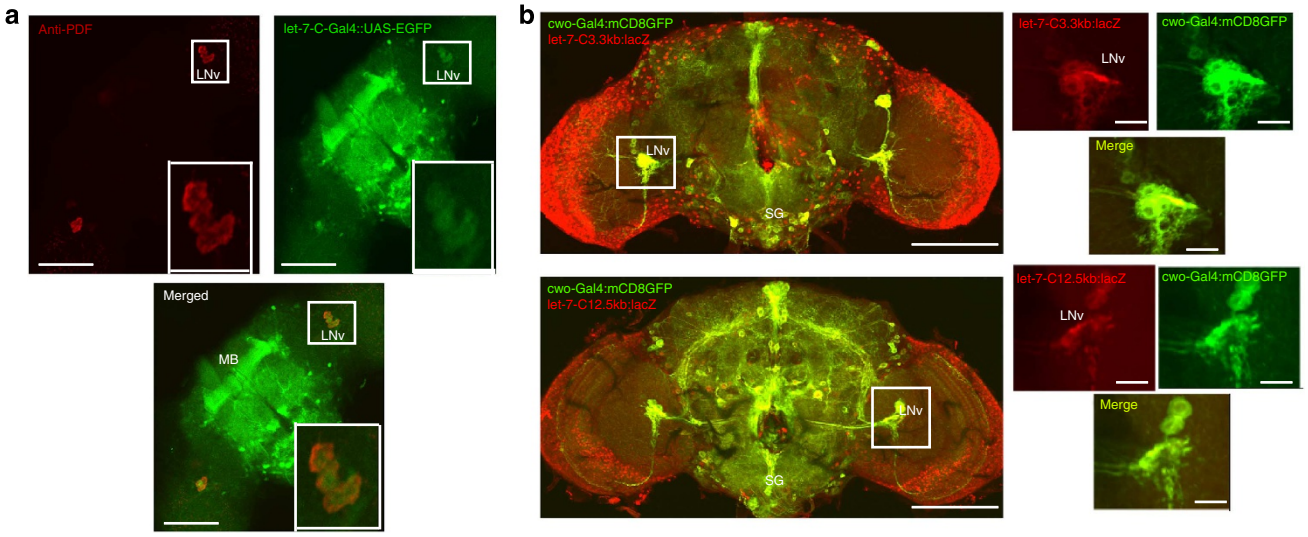

Figure 4 | *let-7* is expressed in central clock cells. (**a**) *let-7-C-Gal4* is expressed in LNvs. Three- to five-day-old files expressing enhanced green fluorescent protein were entrained to LD for 3 days. Brains were dissected and stained with anti-PDF (red) antibody at ZT1 on the 3rd day in LD, while GFP was detected directly by its fluorescence. The regions in the boxes are magnified in the panels at the bottom right. MB, mushroom bodies. (**b**) *let-7-C* intron 1 transcriptional reporters were used to detect *let-7* expression in LNvs. A membrane-targeted mCD8-GFP was expressed in *Cwo* neurons and detected *Cwo* expression sites. Brains from the transgenic mutants (*cwo-Gal4/UAS-mCD8-GFP;let-7-C3.3kb:lacZ/+* and *cwo-Gal4/UAS-mCD8-GFP;let-7-C12.5kb:lacZ/+*) were dissected and stained with anti-β-galactosidase at ZT1 on the 3rd day in LD. The region in the box is magnified in the right panels. Each experiment was conducted at least three times, and 10–15 flies of each genotype were used for immunohistochemical assays. Scale bars, 100 μm for the whole-mount figure and 20 μm for the magnified images.

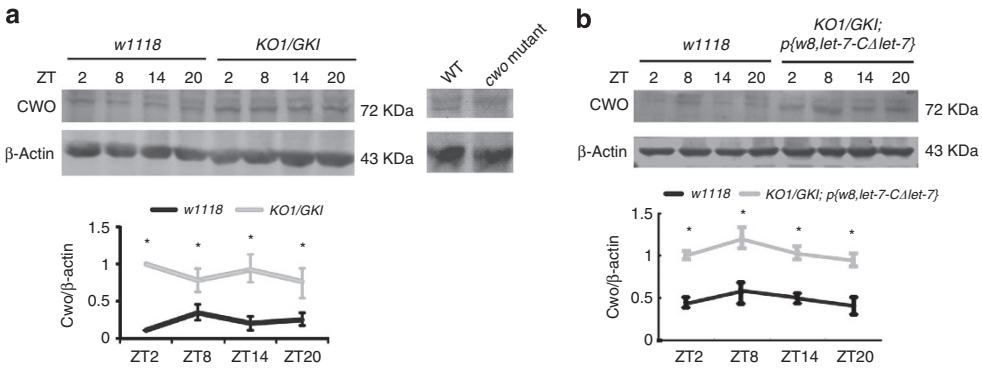

Figure 5 | *let-7* affects cwo expression in head. (**a**) The heads of wild-type (w^{1118}), *let-7-C* mutant (*KO1/GKI*); and (**b**) △*let-7* mutant (*KO1/GKI;p{w8, let-7-C*$^{\triangle let\text{-}7}$*}*; flies were collected at indicated times on the 3rd day of LD. Protein extracts of heads were subjected to western blot analysis using antibodies specific for CWO. β-actin is a loading control. Each experiment was conducted three times, and quantified protein normalized to loading control levels was further normalized to those of the maximum level time point of *KO1/GKI* and *KO1/GKI;p{w8,let-7-C*$^{\triangle let\text{-}7}$*}* in each gel. In flies, the homozygous *Cwo* mutants cwo^{e04207} were used as control. The quantification curves were plotted as the mean ± s.d. of three independent western blots. A significant difference by Student's *t*-test between wild-type and mutant at the same time point is indicated (*$P<0.05$).

hs-Gal4-usp.LBD, in which heat shock-induced expression of this USP dominant-negative form disrupts ecdysteroid signalling during adulthood. This resulted in the absence of *let-7* oscillations in the fly head and depressed the daytime levels of let-7 (Fig. 8b). We further tested the morning and evening peaks of activity in *hs-Gal4-usp.LBD* flies under LD conditions. Interestingly, we found that adult flies with ecdysteroid-signalling disruption displayed suppressed morning peak anticipation before lights-on (Fig. 8c) and became largely arrhythmic in DD (Supplementary Table 1), which mimicked the phenotype of *let-7* mutants. Furthermore, lack of 20E signal by inhibiting EcR expression results in decrease of *let-7* levels (Supplementary Fig. 7a) and increase of *Cwo* levels (Supplementary Fig. 7b), while activation of 20E signal by promoting *ptth* expression (Supplementary Fig. 7c) results in decrease of *Cwo* levels (Supplementary Fig. 7d), and lengthening of circadian period (Supplementary Fig. 7e). All of these findings indicate that *EcR* signalling is required for oscillations of *let-7* expression in the fly head and contributes to adult circadian behaviour.

From all of these data in this study, we conclude that *let-7* functionally regulates circadian rhythm via the core clock component CWO, and we find a novel regulatory cycle for the circadian rhythm mechanism. This cycle is regulated by CLOCK/CYCLE via a pathway involving PTTH, ecdysteroid, its dimeric receptor (EcR/USP), *let-7* and CWO (Fig. 9).

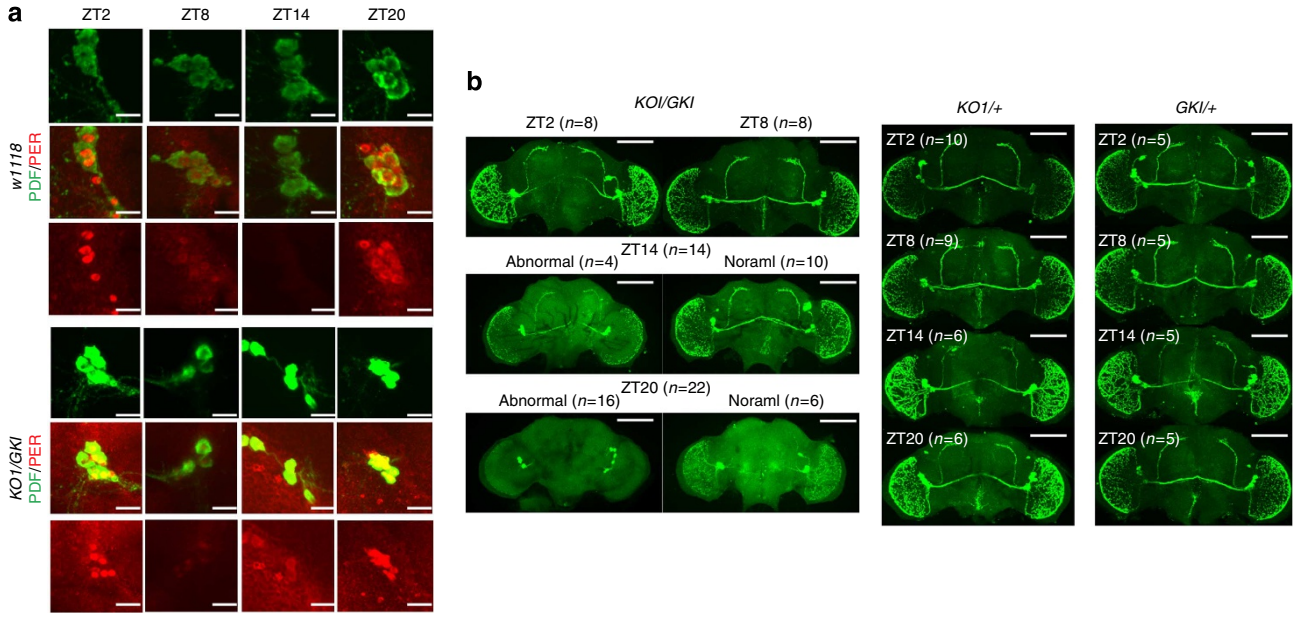

Figure 6 | *let-7-C* mutant flies display abnormal PER and PDF expression. (**a**) *let-7-C* mutant flies show abnormal PER expression in LNvs. The brains of wild-type (w^{1118}) and *let-7-C* mutant (*KO1/GKI*) flies were collected at the indicated times on the third day of LD and stained with PER (red) and PDF (green) antibodies. (**b**) *let-7-C* mutant flies show abnormal PDF expression in the projections from LNvs. The brains of control heterozygous files (*KO1/+* and *GKI/+*) and *let-7-C* mutant (*KO1/GKI*) flies were collected at the indicated times on the 3rd day of LD and stained with PDF antibodies. Each experiment was conducted at least three times, and 8–22 flies of each genotype were used for immunohistochemical assays. Scale bars, 100 μm for the whole-mount figure and 20 μm for the magnified images.

Discussion

A prior study screened for daily rhythms of miRNAs from wild flies and the mutant cyc^{01} by using the microarray method. However, only two *Drosophila* miRNAs (*miR-263a* and *miR-263b*) were found to exhibit circadian oscillations[29]. To further investigate miRNAs that exhibit circadian oscillations, Vodala and colleagues used deep sequencing technology to study 18–29 nt RNAs in a 24 h cycle and found a few miRNAs showed significant oscillations. In this study, we found that *let-7* showed significant rhythmic oscillation, consistent with the sequencing data from Vodala[31]. However, some miRNAs have been found to play an important role in circadian regulation even though they do not show significant daily oscillations. For example, two important miRNAs (*bantam* and *miR-279*) participate in circadian regulation but are not cycling[28,30,31].

The *let-7-C* pri-miRNA consisted of *miR-100*, *let-7* and *miR-125*, and these three miRNAs are produced by differential processing in fly heads. Previous studies showed that *miR-100* expression was delayed and its expression level was reduced during development compared to *let-7* and *miR-125* (ref. 37). In this study, we found that the expression of *pri-let-7-C* did not exhibit cycling. However, the expression levels of both the mature *let-7* and *miR-125* exhibited circadian oscillations, with levels increasing from the late night and reaching a peak in the day, while the *miR-100* had no such oscillation (Fig. 7 and Supplementary Fig. 8). Moreover, the expression rhythm of *let-7* in the brain is consistent with the expression rhythm of E23—a novel clock gene also induced by ecdyseroid (20E)[42]—and was almost antiphase to that of the 20E. This phase delay is probably due to the time required for post-transcriptional processing of *let-7*.

It is thought that miRNAs mainly function to fine-tune the levels of key proteins. The miRNAs may have a profound effect on the dynamics of regulatory modules by effects on the amplitude of gene expression and alteration of the frequency in the temporal sequence of gene production or delivery[43]. In this study, we found that the CWO levels were higher in *let-7-C* null mutant flies (Fig. 5). Additionally, our AGO1 immunoprecipitation in △*let-7* showed that *cwo* is a direct target of *let-7* in fly heads (Fig. 3). So *let-7* probably contributes to the clock rhythms via regulation of *cwo*. From our CWO/let-7 co-staining it is clear that both of them are co-present in only some brain cells (Fig. 4). This probably means that the effects on CWO levels observed in *let-7* mutants are partially due to expression of both genes in other tissues, such as eyes. Overexpression of CWO in the *cwo*-expressing cells didn't generate a *let-7* mutant phenotype, but it rescued the rhythm of *let-7* overexpression flies (Fig. 3). Moreover, previous studies showed that two copies of *Cwo* producing overexpression by *tim-Gal4* (*tim-Gal4/+*; *UAS-cwo*) generate a modest reduction of rhythmic power[12–15], which can mimic the *let-7* loss–of –function phenotype in this study, indicating that the *let-7* mutant phenotype may be due to increased CWO expression. On the contrary, our results show that *let-7* overexpression in *Cwo* cells lengthened circadian period, which is consistent with the effect of *cwo-RNAi* in both *Cwo* cells and *Tim* cells, and it was able to be partially rescued by *Cwo* overexpression, indicating regulation of *Cwo* by *let-7*. CWO function in regulating expression of *per/tim* has been clear from previous reports[12–15]. In this study, results showed that *let-7* is expressed in circadian sLNv neurons, behavioural data showed that the fly's circadian period when let-7-C is overexpressed in endogenous neurons (*let-7-C-Gal4/+*;*UAS-let-7-C/+*) is longer than wild-type (25.04 ± 0.26), and when it is overexpressed in PDF neurons the period is even longer (26.12 ± 0.24, Table 1). The *let-7* mutant phenotype is successfully rescued by *let-7* re-expression in PDF neuron (Fig. 2). These results suggest that sLNvs are important circadian neurons for regulation of *let-7*. Because the *UAS-let-7-C* seems to have a stronger phenotype that *UAS-let-7*, we speculate that three miRNAs (*miR-125*, *miR-100* and *let-7*) together may enhance function of *let-7*, although *miR-125* and *miR-100* do not act by themselves.

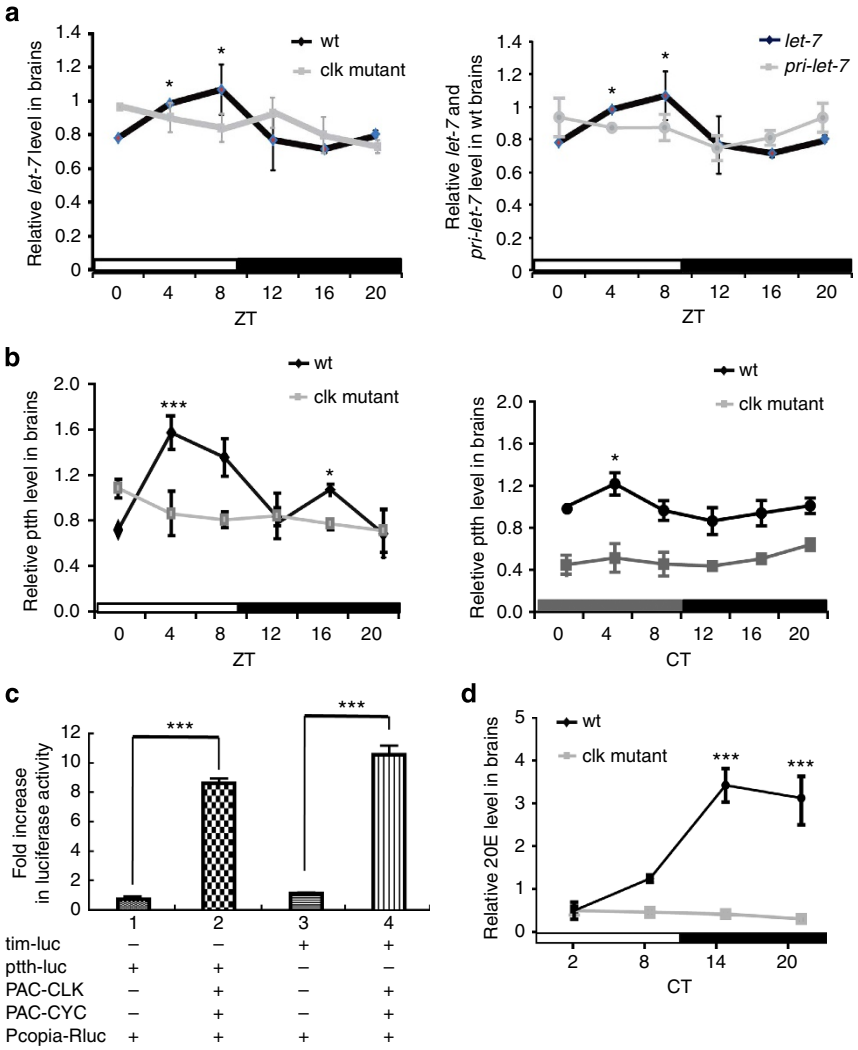

Figure 7 | *Let-7* is regulated via PTTH and ecdysteroid signalling. (**a**) Levels of *let-7* are altered in *Clk*^{Jrk} mutant flies. Real time quantitative reverse transcriptase PCR analysis of total RNA prepared from adult brains of the indicated genotypes at the indicated time points. The relative expression levels were normalized to 2S RNA levels and were further normalized to *Clk*^{Jrk} at ZT0 (left panel). Each time point was compared to ZT0 with Student's *t*-test. $^{*}P<0.05$. The level of *let-7* in wild-type fly brains reached a peak in the day but did not in *Clk*^{Jrk} flies (left panel). The *pri-let-7-C* didn't show oscillation (right panel). (**b**) Levels of *ptth* mRNA are altered in *Clk*^{Jrk} mutant flies in LD (left panel) and DD (right panel) conditions. The relative expression levels were normalized to *rp49* levels and were further normalized to *Clk*^{Jrk} at ZT0. Each time point was compared to ZT0 with Student's *t*-test. $^{*}P<0.05$ and $^{***}P<0.001$. The levels of ptth in wild-type fly brains had rhythmic peaks at ZT4 and ZT16, while they were eliminated in *Clk*^{Jrk} flies. (**c**) The *ptth* promoter responds to CLK/CYC. Fold increase in luciferase activity of S2 cells transfected with luciferase reporter constructs containing the promoter of *ptth* and *Clk/Cyc*-expressing plasmids. A *tim* promoter fused to luciferase reporter (tim-luc) was used as positive control. The histogram plot representing fold increase in luciferase activity ± s.e.m. ($n=3$) is shown. A significant difference with Student's *t*-test analysis is indicated ($^{***}P<0.001$). (**d**) Levels of 20E are under the control of the central clock. Wild-type and *Clk*^{Jrk} mutant fly heads were collected from males at the indicated times for 3 days and 20E levels were measured using radioimmunoassay (RIA). Three experiments with duplicates for each time point were combined and are presented as the mean ± s.e.m. The 20E levels at ZT14 and ZT20 were higher than those of other time points by one-way analysis of variance analysis ($^{***}P<0.001$). Each experiment was conducted at least three times, and 25–30 fly heads of each genotype were used for the mRNA and RIA detections.

EcR-mediated signalling in the adult fly nervous system is critical for adult behaviour. Immunohistochemical detection showed that EcR was widely expressed in the adult brain[44]. EcR-mediated signalling in adult flies has an important role in the formation of long-term memory) and sleep regulation[44,45], and defective long-term memory and sleep in EcR mutants is thought to be caused by insufficient EcR-mediated signalling. Previous studies showed that deletion of EcREs in the *pri-let-7-C* locus caused a reduction and delay of *let-7-C* miRNA expression in adult flies and is associated with a *let-7-C* mutant phenotype[37]. EcREs in intron 1 of the *let-7-C* locus play an important role in onset and maintenance of *let-7-C* expression in a tissue-specific manner[37]. In addition, the *let-7-C* locus was found to be a direct transcriptional target of 20E because *pri-let-7-C* RNA could be detected in KC167 cells 30 min after exposure to 20E[37]. Taken together, these data strongly suggest that EcR binds to the endogenous *let-7-C* locus and activates its transcription in response to 20E, and that this transcriptional regulation is required for miRNA let-7 function[37]. In this study, we showed that CLOCK-PTTH-20E-EcR/USP-mediated signalling contributes to circadian regulation, and 20E mediates circadian expression of *let-7* in the adult brain. Moreover, the 20E response is necessary for circadian oscillations in adult pacemaker neurons because the double knockdown of *EcR* and *usp* produced an

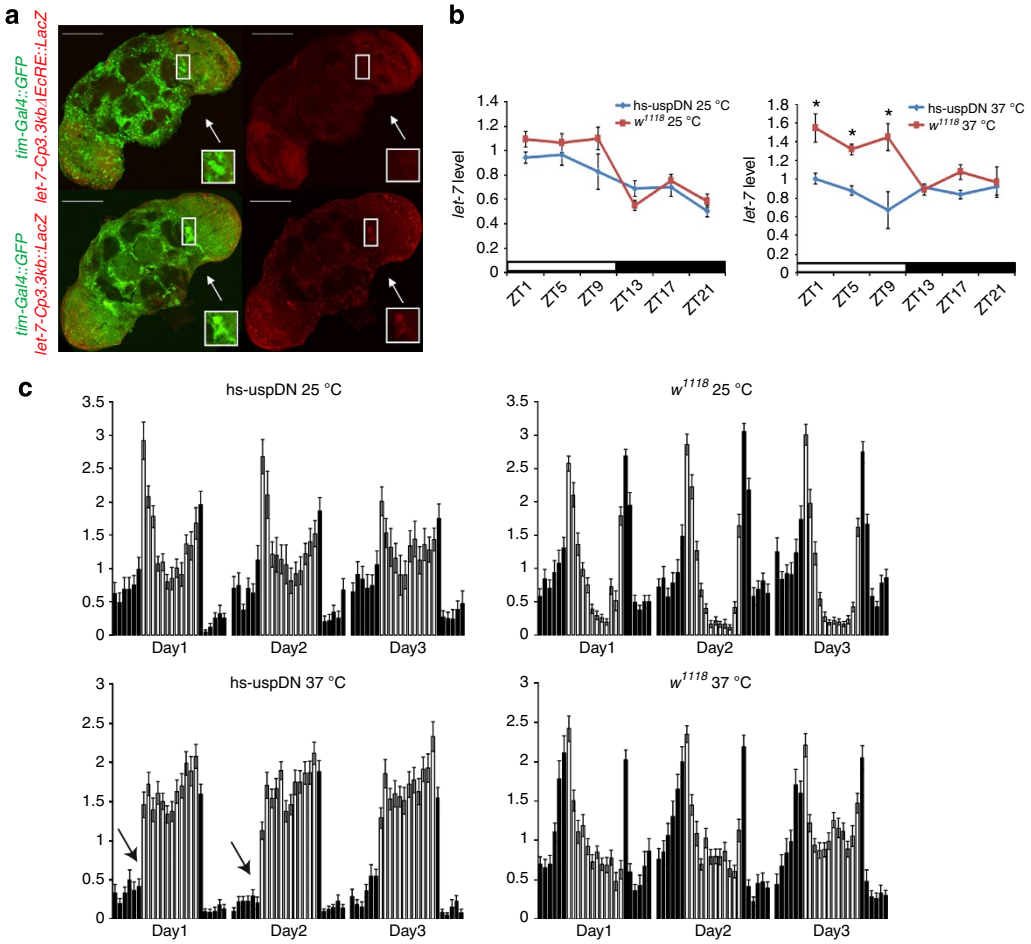

Figure 8 | Ecdysteroid signalling regulates *let-7* and rhythmic behaviour. (**a**) EcR signalling is required for *let-7-C* expression in fly heads. The transgenic line *let-7-Cp12.5kb△EcRE1-3::lacZ*[37], in which each of the EcREs sequences was deleted, was coupled with a *tim-Gal4::YFP* line to monitor the *let-7* expression in clock neurons. In contrast to the wild-type lacZ reporter, expression of the mutated reporter was strongly blocked in the pacemaker neurons. Scale bars, 100 μm for the whole-mount figure. (**b**) Blocking ecdysteroid signalling affects *let-7* circadian expression in heads. Three-day-old w^{1118} and w^{1118};*hs-Gal4-usp.LBD* adult flies were heat shocked (starting from ZT12) at 37 °C for 4 h and allowed to recover to 25 °C for 4 h, and this regime was repeated three times per day for 2 days. After heat shock pulses ended for 12 h, samples (30 heads per each sample) were collected at the indicated time points for RNA extraction and real time quantitative reverse transcriptase PCR analysis. The heat shock method was taken from a reference[62] with some revision. The relative expression level was plotted as mean ± s.e.m. Levels were normalized relative to those of *hs-Gal4-usp.LBD* flies seen at ZT1. The *let-7* level at each time point was compared to ZT21 by one-way analysis of variance with Tukey *post-hoc* tests (*$P<0.05$). (**c**) Blocking ecdysteroid signalling in adult flies results in the loss of the anticipatory morning locomotor activity, thereby mimicking the phenotype of *let-7* mutants. The heat shock regime used here was same as that used for panel **b**. The arrows indicate the loss of the anticipatory morning peaks.

abnormal circadian phenotype[42]. So EcR/USP in circadian neurons receives 20E and mediates the signalling via *let-7*, thereby contributing to circadian regulation. However, one complexity to this model is our finding that *pri-let-7* mRNA did not exhibit circadian cycling, while *let-7* mRNA did cycle. This finding suggests that additional post-transcriptional regulation is necessary to produce cycling of *let-7* miRNA, and that *pri-let-7* is expressed in additional non-EcR-expressing cells in which it may not be transcribed in a circadian manner and not processed to *let-7* (expression in these cells could mask the *pri-let-7* oscillation in circadian cells).

VRI, a bZIP transcription factor whose expression is controlled by the clock in adult flies[46], also responds to 20E both in cell culture and *in vivo*[47]. Interestingly, the ecdysone-signalling pathway was found to function in the adult circadian system; E23—a novel clock gene that encodes the membrane-bound ABC transporter—was induced by ecdysone, and its knockdown in flies increased expression of *vri*. *E23 and vri* responded to both ecdysone and clock signals, whereas E23 protein suppressed the ecdysone response and controlled the circadian oscillation through ecdysone-mediated *vri* expression[42]. The prothoracic gland in *Drosophila* larvae and pupae, where ecdysone is secreted, is reported to strongly express clock genes[48,49]. The rhythmic changes in 20E levels were also regulated by an endogenous circadian oscillator in the haemolymph and testes of adult males in cotton leafworm and in *Rhodnius prolixus*[50,51], and 20E is also present in *Drosophila* adults[52]. In this study, we found that the PTTH is a direct target of CLK/CYC, most likely because the 2-kb promoter region of *ptth* contains 10 noncanonical E-box sequences (CANNTG other than CACGTG). Furthermore, circadian oscillations of PTTH, 20E and *let-7* in the brain are caused by CLK. Thus, a novel cycle mediated by ecdysteroid was shown to regulate circadian rhythms in this study. The ultimate target of *let-7* miRNA is CWO, and this does not appear to cycle by immunoblot analysis of heads (Fig. 5). However, it is likely that CWO does oscillate in a circadian subset of the cells in which

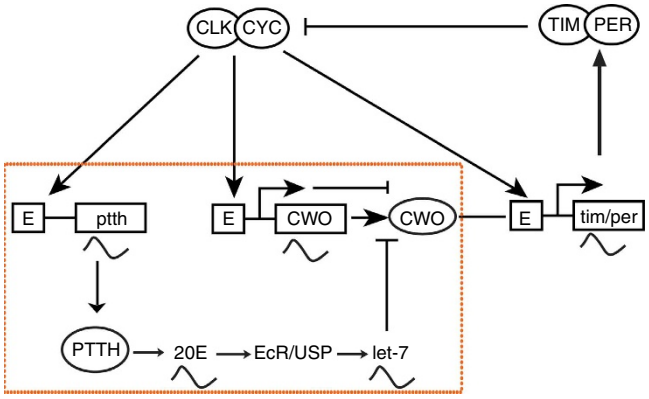

Figure 9 | A regulatory cycle in the circadian clock. A regulatory cycle for the *let-7* pathway (orange dotted line), in which *let-7* functionally regulates circadian rhythm via core clock component CWO, and *let-7* is regulated by CLOCK/CYCLE via a pathway involving PTTH, ecdysteroid and its dimer receptor (EcR/USP). The model for CWO downstream effects in the circadian system is adapted from previous studies[12,14].

it is expressed, and that constitutive expression in non-circadian cells masks this oscillation in head extracts (note that Fig. 4 shows both *let-7* and *cwo* promoter activity that is more widespread than just circadian cell expression, and Fig. 8 shows that not all *let-7* promoter activity is eliminated by deletion of the *EcR* elements).

miRNAs appear to be major players in the regulation of circadian timekeeping. High throughput sequencing experiments have raised the number of miRNAs to 240 in the *Drosophila* genome[53]. However, only three miRNAs in *Drosophila* have been clearly reported to function in regulation of circadian rhythm[28,30,31]. Thus, it may be necessary to perform a genetic screen of individual miRNAs in fly head circadian cells to investigate their potential role in circadian regulation. Recently, a transgenic *UAS-miRNA* library in *Drosophila* has been constructed[54–56]. This library circumvents the redundancy issues inherent in loss-of-function screens by facilitating the controlled misexpression of individual miRNAs and is a useful tool to complement loss-of-function approaches. In *C.elegans* only about 10% of all miRNA deletions had detectable phenotypic consequences, and Schertel *et al.*[55] validated misexpression as an approach to probe miRNA function. Our analysis of *let-7* demonstrates the value of this approach for understanding the role of miRNAs in the fly circadian rhythm.

Methods

Fly strains. Fly strains were maintained on standard molasses-cornmeal-yeast food in a 12 h:12 h LD cycle at 25 °C. The fly lines contain: *tim-Gal4*, *pdf-Gal4*, *cwo-Gal4*, *tubulin-Gal80ts*, *tim-Gal4,UAS-2xYFP/Cyo*, *yw*, w^{1118}, Clk^{Jrk}, *hs-Gal4-usp.LBD*, *UAS-let-7C*, *UAS-let-7*, *UAS-miR-125*, *UAS-mut-let-7*, *UAS-EGFP*, *UAS-cwo-RNAi*, *let-7-C*^{GKI}/*CyO-TM6b*, *let-7-C*^{KO1}/*CyO-GFP*, *let-7-C*^{KO1}/*CyO;p{w8,let-7-C*$^{\triangle let\text{-}7}$*}*, *let-7-C*^{KO1}*;p{w8,let-7-C*$^{\triangle miR\text{-}125}$*}/ CyO-TM6b*, *let-7-C*^{KO1}/*CyO;p{w8,let-7-C*$^{\triangle miR\text{-}100}$*}/TM6b*, *p{w8,let-7-C*$^{12.5kb::lacZ}$*}*, *p{w8,let-7- C*$^{3.3kb::lacZ}$*}*, *p{w8,let-7-C*$^{12.5kb\triangle EcRE::lacZ}$*}*.

Locomotor behavioural assays. Male fly lines were monitored in a 12 h:12 h LD cycle at 25 °C for 3–4 days, followed by 6–7 days in DD using Trikinetics *Drosophila* Activity Monitors. Activity records were collected in 1 min bins and analysed using Faas (Fly activity analysis suite) software developed by M. Boudinot and derived from the Brandeis Rhythm Package (http://hawk.bcm.tmc.edu) described by D. Wheeler[57]. Circadian periods were determined by periodogram analysis[57]. A signal-processing toolbox was used to plot actograms[58]. The morning anticipation was determined by assaying of the locomotor activity[13].

S2 cell culture and luciferase reporter assay. The full length 3′UTR of *Cwo* and an ~500 bp coding region of *let-7* (including *pre-miR-125*) were amplified by the PCRusing PrimeSTAR HS DNA Polymerase (TaKaRa). The *Cwo* 3′UTR was cloned into a pAc5.1-firefly luciferase-V5-His vector and the *let-7* coding region was cloned into a pAc5.1-V5-His vector (Invitrogen). The *let-7* 'seed'-targeted sequence in the *Cwo* 3′UTR was mutated from CTACCTCA to GTTCGTGG using Site-directed, Ligase-Independent Mutagenesis[59]. S2 cells were maintained in 10% fetal bovine serum (HyClone) Schneider's Insect Medium (Sigma). Cells were seeded in a 12-well plate. Transfection was performed at 70–90% confluence with Cellfectin (Invitrogen) according to the manufacturer's instructions. In all experiments, 1μg of miRNA expression plasmid, 100 ng of pCopia-Renilla Luciferase and 100 ng of the luciferase firefly reporter were used. Luciferase assays (Dual Luciferase System, Promega) were performed 2 days after transfection. Renilla luciferase activity provided normalization for firefly luciferase activity.

AGO1 immunoprecipitation. 60 fly heads were homogenized in 700 μl of lysis buffer (30 mM HEPES KOH at pH 7.4, 100 mM KAcetate, 2 mM MgAcetate, 5% glycerol, 0.1% Triton X-100, 1 mM ethylene glycol tetraacetic acid, 5 mM dithiothreitol, 0.4 U μl^{-1} RNase OUT (Invitrogen), 1 × ethylenediaminetetraacetic acid proteinase inhibitor (Roche)). The lysates were incubated for 10 min on ice and centrifuged at 14,000 r.p.m. for 15 min at 4 °C. One hundred microliters of the supernatant were used as input. To the rest of the supernatant (~500 μl), 1/10 volume of anti-AGO1 antibody (2.0 ug ul^{-1}, from Miyoshi's lab) was added and incubated at 4 °C with rotation. After 2 h, 100 μl of protein G plus bead slurry (Invitrogen; previously washed with lysis buffer) was added and incubated for 1 h at 4 °C with rotation. The beads were then recovered by gentle centrifugation. The beads were washed five times (beads plus 500 μl of lysis buffer were rotated for 5 min at 4 °C), before proceeding to RNA extraction. Total RNA from beads of the w^{1118} wild-type control and △*let-7* mutant (*KO1/GKI;p{w8,let-7-C*△*let-7}*) at the same circadian time (ZT8) was extracted using Trizol.

Real time quantitative reverse transcriptase PCR. Flies were collected at the indicated time points and isolated heads were stored at −80 °C. Total RNA (including miRNA) was isolated from 30–50 heads with miRcute miRNA isolation kits (TIANGEN). For reverse transcription and real-time PCR (qRT-PCR) of *let-7*, *miR-125*, *miR-100* and 2s rRNA, we used a miRcute miRNA first-stand cDNA synthesis kit and a miRcute miRNA qPCR detection kit (SYBR Green) (TIANGEN). The miRNA-specific forward primers used for qPCR are shown in Supplementary Table 2. The reverse primer was a primer complementary to the poly(T) adapter that was provided in the miRcute miRNA qPCR detection kit. For reverse transcription and real-time PCR of *Cwo*, we used the Quantscript RT kit and RealMasterMix (SYBR Green) (TIANGEN). The sequences of primers are shown in Supplementary Table 2. All the experiments were performed in the ABI prism 7500 (Applied Biosystems).

Western blot analysis. Fly heads were homogenized with a pestle, and protein extracts were prepared with radioimmunoprecipitation assay buffer buffer (50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1%NP-40, 0.1% SDS). For immunoblot analysis, proteins were transferred to NC membranes (Pall) and incubated with anti-CWO (1:1,000, from Hardin's lab) in blocking solution. Band intensity was calculated and analysed with the Gel-Pro Analyzer 4.0.

Whole-mount brain immunostaining and microsocopy. Adult flies were collected for brain dissection at the indicated times. Brains were fixed for 30–45 min in 4% buffered formaldehyde, washed in phosphate-buffered saline (pH 7.4) with 0.3% Triton X-100 (polybutylene terephthalate), blocked in 5% goat serum in PBT (phosphate-buffered saline tween-20) for 2 h and incubated in primary antibody overnight at 4 °C. Indicated dilutions of the following primary antibodies were made in phosphate-buffered saline tween-20: rabbit anti-PER(1:2,000, from Dr Rosbash), rat anti-PDF (1:2,000 from Dr Ceriani) and rabbit anti-β-galactosidase (1:2,000, Promega). After three 15 min washes, brains were incubated with secondary goat antibodies for 2 h at room temperature, followed by extensive washes. Secondary antibodies included goat anti-rabbit tetramethylrhodamine and goat anti-rabbit fluorescein isothiocyanate. The GFP and yellow fluorescent protein (YFP) were visualized directly by their excitation. All experiments were imaged on a Nikon ECLIPSE TE2000-E and a Nikon D-ECLIPSE confocal microscope (Nikon, Japan). Confocal images were obtained at an optical section thickness of 1–2 μm and finally analysed with Image J. Staining intensity of PER was calculated and normalized as described[60].

20E quantification. To determine the titre of 20E in the heads, we performed a radioimmunoassay developed by David Borst[61]. Briefly, samples were extracted from 50 adult heads by homogenization in 90% methanol. The homogenates were dried at 70 °C using a block heater and then dissolved in 200 μl borate buffer (0.05 M boric acid; 0.9% NaCl; 0.1% gelatin; 0.05% Triton X-100; 7.7 mM NaAzide; pH 8.4) with ~3,000 disintegrations per minute (DPM) [^{3}H]ecdysone and a monoclonal anti-ecdysteroid antibody (diluted 1:12,000). After incubating for 1 h at room temperature and then overnight at 4 °C in the refrigerator, the chilled assay tubes was placed on ice for 5 min, and 500 μl of stirred, cold dextran-coated charcoal was added (2.5 mM boric acid; 0.045% NaCl; 12.5 mg dextran; 38 mM

ethylenediaminetetraacetic acid; 7.7 mM NaAzide; 0.5 g charcoal; pH 8.4) to each tube. After 5 min, the samples were centrifuged at 2,000 *g* for 5 min and the 400 µl supernatant was decanted into a new tube. Two millilitres of scintillation cocktail was added, and the each sample was counted in an LS 6500 scintillation counter (Beckman) for 5 min. Calibration curves were generated using the commercially obtained 20E (Sigma, USA).

Statistics analysis. Statistical analysis of two data points was performed with Student's *t*-test. Statistical analysis of multiple data points was performed with one-way analysis of variance with Tukey *post-hoc* tests.

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Author contributions

W.C. and Z.Z. conceived and designed the experiments. W.C., Z.L., T.L., R.Z., Y.X., Y.Z., W.B. and D.Z. performed the experiments. W.C. and Z.L analysed the data. Z.Z. contributed reagents/materials/analysis tools. W.C. and Z.Z. wrote the manuscript.

Additional information

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