Supporting Online Material for

Control of Sleep by Cyclin A and its Regulator

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Materials and Methods

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Materials and Methods

Fly Genetics

Flies were raised on standard cornmeal/molasses food at 25^{o}C in a 12-hour light/12-hour dark (LD) cycle.

Transgenes were introgressed into the wild type *iso31* genetic background via backcrossing for at least 5 generations. ln all experiments, *Gal4* and *UAS* parental controls were tested as hemizygotes.

In all RNAi experiments except those performed in the *CycA* heterozygous background (Fig. 2E), *Dicer2* was overexpressed along with the indicated *UAS-RNAi*. In all experiments except *PDFrGal4*-driven *CycA-RNAi* (Fig. S6B), male flies were tested in the sleep assay. In the case of *PDFrGal4*, female flies were tested as the *PDFrGal4* insertion is on the X chromosome and creates an allele of *PDFr*.

UAS-RNAi lines were obtained from NIG-Fly Stock Center (10800R-1, 5940R-1), the *CycA* null allele (C8LR1), *elavGal4*, *ActinGal4* and *UAS-CycA* transgenic flies were obtained from the Bloomington Drosophila Stock Center. The *CycAGal4* enhancer trap line was obtained from the Drosophila Genetic Resource Center, *PDFrGal4* and *UAS-GFP:nls* flies were a gift from Ravi Allada, *perGal4* flies were a gift from Jeffrey Hall, *UAS-Dicer2* transgenic flies were a gift from Barry Dickson and *UAS-Rca1* flies were a gift from Frank Sprenger.

Sleep and circadian assays

In the initial screen, *elavGal4* flies were crossed to individual *UAS-RNAi* lines and for each genotype four male progeny flies, 1-5 days old, were screened for sleep phenotypes. Because RNAi is more efficient, and the background levels of activity are lower at higher temperature, our screen and subsequent analysis were performed at 26-27^{o}C. All the flies that exhibited sleep phenotypes at this temperature did so at 25^{o}C as well. Locomotor activity levels were monitored using the *Drosophila* Activity Monitoring System (Trikinetics). For sleep measurements, activity counts were collected in 1 minute bins for at least 4 days in LD and sleep was identified as at least 5 minutes of inactivity using a sliding window. All the sleep parameters were determined using custom-written MATLAB software (gift from Nicholas Stavropoulos). All the preliminary hits underwent at least 3 subsequent rounds of retesting, with 50 or more flies monitored. For analysis of sleep and circadian rhythmicity in constant darkness (DD), activity counts were collected for five days at 1 minute intervals. For circadian analysis, data were binned at 30 minutes and analyzed using Clocklab (Actimetrics).

Sleep deprivation

Flies were mechanically stimulated overnight during their normal sleep hours (ZT 12-24) using a custom-built machine, and rebound sleep was measured the following morning

(ZT 0-6). Mechanical stimuli were applied for 2 seconds at random intervals averaging 20 seconds. For each fly, the amount of sleep lost was calculated by subtracting sleep on the night (ZT 12-24) of the deprivation from undisturbed sleep the night (ZT 12-24) before. Only flies that lost at least 95% of sleep compared to baseline were analyzed. Excluded from the analysis were flies that did not sleep on the undisturbed night (<100 minutes of sleep), as there was not much sleep to deprive in these animals. For each fly, rebound sleep was calculated by subtracting sleep on the morning (ZT 0-6) before deprivation from sleep on the morning (ZT 0-6) following deprivation. To account for differences in baseline sleep between the genotypes, the recovery data were normalized relative to sleep lost for each genotype.

Longevity Assay
Flies were raised at 25^{o}C in an LD cycle and within 1 day after eclosion transferred into fresh vials at 27^{o}C (10 males/vial). Flies were transferred to new food and the number of dead flies counted every 1-2 days.

Eclosion Assay
Flies were raised at 25^{o}C in an LD cycle and transferred to a 27^{o}C DD cycle after 1 day of eclosion. To monitor eclosion rhythms, vials were emptied every 2-5 hours and the number of emerged flies counted over the course of 4 days.

Statistics
Statistical analysis was performed using GraphPad Prism. For comparisons between two genotypes, Student's T-tests were used. For comparisons between three or more genotypes, One-way ANOVAs with Tukey's post-tests were used. Log-rank tests were used to compare longevity of experimental and control animals. The chi square test was used to examine rhythmicity in constant darkness.

Immunostaining
For each staining pattern shown, at least 20 individual brains were examined. Adult heads were fixed for 2-3 hours on ice in fixative containing 4% paraformaldehyde (PF) and 0.2% Triton X-100 in PBS. Brains were dissected after an overnight rinse at 4^{o}C in PBS/0.2% Triton X-100. Staining with both primary and secondary antibodies was carried out PBS/0.2% Triton X-100.

Rabbit anti-CycA FL serum was a gift from Christian Lehner (1:100), goat anti-CycA NT antibody was obtained from Santa Cruz (sc-15869, 1:100). Rat anti-PER antibody was a gift from Amita Sehgal (1:1500). Mouse anti-PDF antibody was obtained from Developmental Studies Hybridoma Bank (1:1000).
For embryo staining, standard procedures were used.

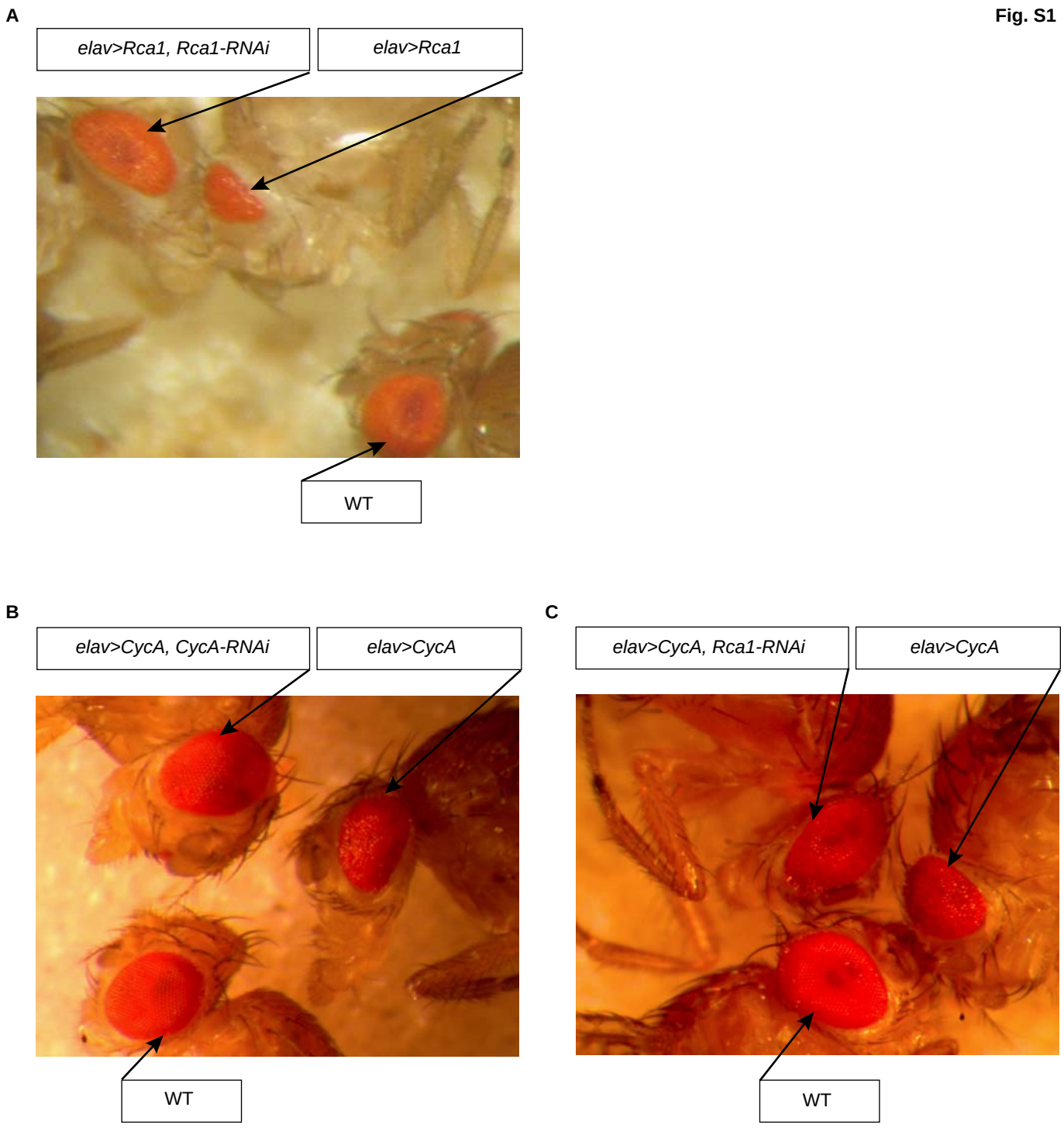


Figure S1
Functional tests of *Rca1*- and *CycA*- *RNAi* constructs
(A) Small and rough eye phenotypes resulting from ectopic pan-neuronal *Rca1* expression (*elav>Rca1*) were suppressed by simultaneously driving *Rca1-RNAi* (*elav>Rca1*, *Rca1-RNAi*). **(B, C)** Small and rough eye phenotypes resulting from ectopic pan-neuronal CycA expression (*elav>CycA*) were suppressed by simultaneously driving either *CycA-RNAi* [*elav>CycA*, *CycA-RNAi* (B)], or *Rca1-RNAi* [*elav>CycA*, *Rca1-RNAi* (C)]. In all experiments described in this figure, *elavGal4* drove the expression of *UAS-Dicer2*. Wild type is denoted as WT.

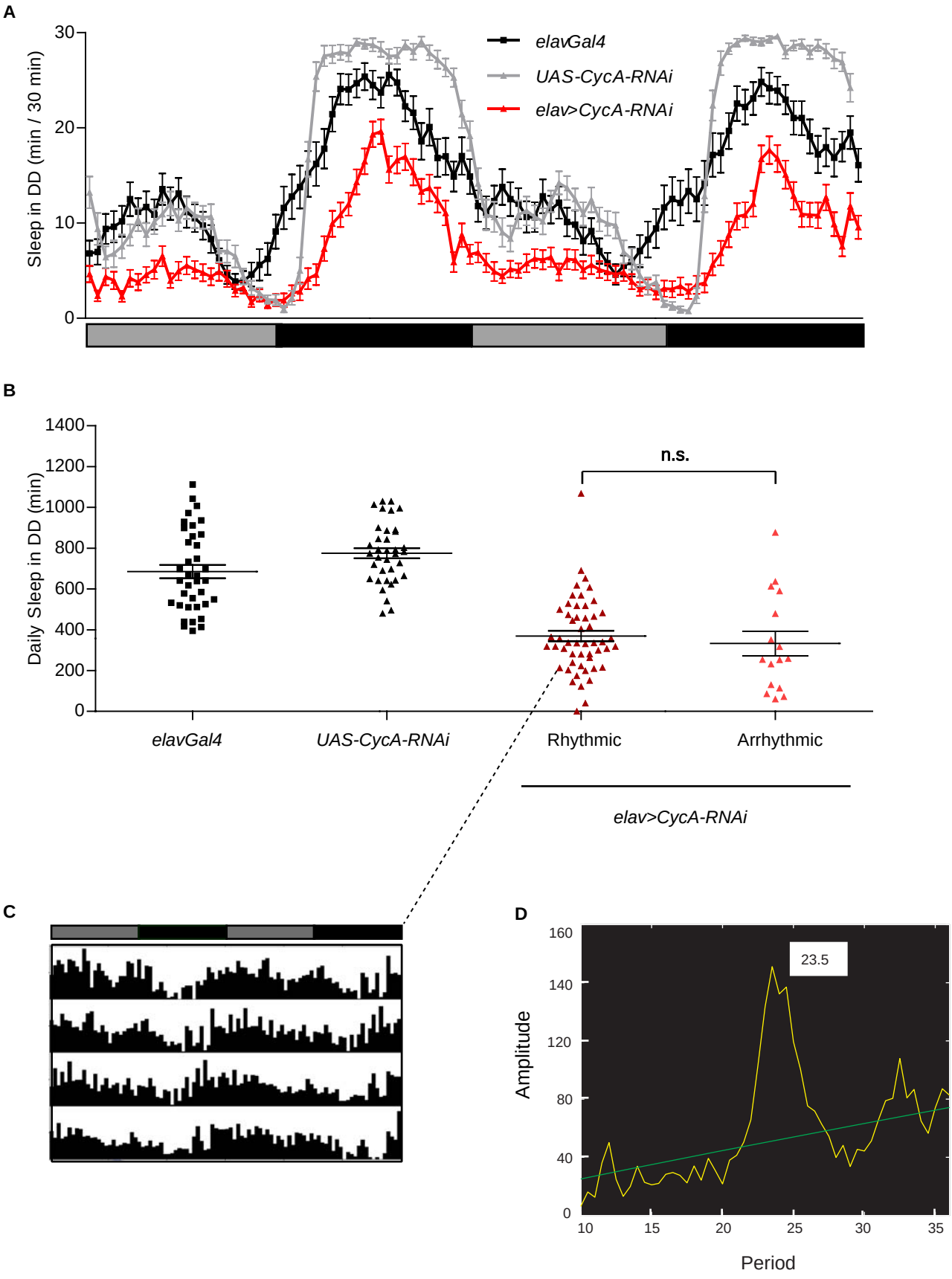

A
Sleep in DD (min / 30 min)
30
20
10
0
elavGal4
UAS-CycA-RNAi
elav>CycA-RNAi
B
Daily Sleep in DD (min)
1400
1200
1000
800
600
400
200
0
n.s.
elavGal4
UAS-CycA-RNAi
Rhythmic
Arrhythmic
elav>CycA-RNAi
C
D
Amplitude
160
140
120
80
40
0
23.5
10
15
20
25
30
35
Period

Figure S2

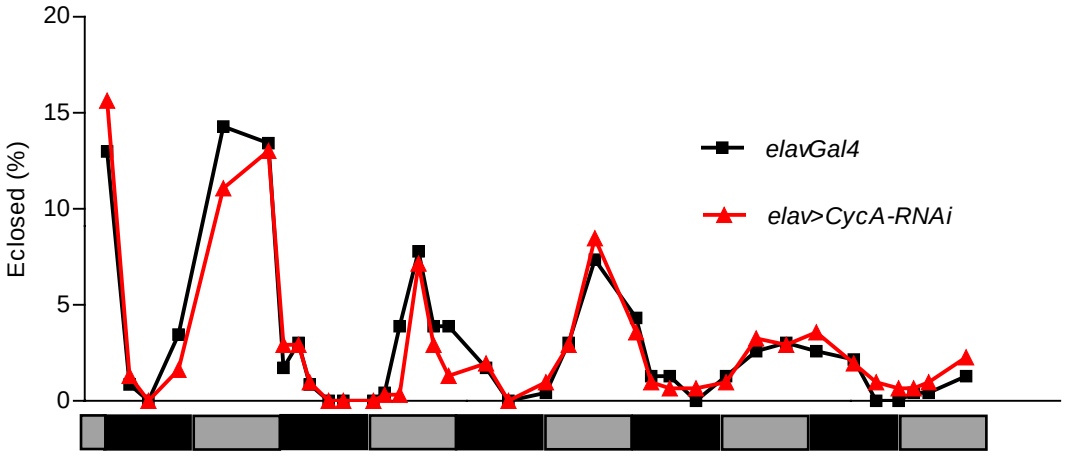
Circadian rhythms in *elav>CycA-RNAi* animals

(A) Two-day sleep profile in constant darkness (DD) of *elav>CycA-RNAi* (n=68), *elavGal4* (n=37) and *UAS-CycA-RNAi* (n=36) animals. Gray and black portions of the bar shown below the X-axis indicate subjective day and night, respectively. **(B)** Sleep amount in rhythmic vs. arrhythmic *elav>CycA-RNAi* animals. **(C, D)** An actogram (C) and a periodogram (D) of a short-sleeping, rhythmic *elav>CycA-RNAi* fly with a normal daily rhythm of 23.5 hours. Gray and black portions of the bar above the actogram indicate subjective day and night, respectively. Daily activity profiles are double-plotted so that each horizontal line in the actogram represents two days of data collection. In all experiments described in this figure, *elavGal4* drove the expression of *UAS-Dicer2*.

A

Genotype	Number of flies	% Rhythmic	Tau +/- SEM (hr)	Power +/- SEM
elavGal4	37	100	23.75 +/- 0.04	182.3 +/- 5.4
UAS-CycA-RNAi	36	100	24 +/- 0.07	169.2 +/- 4.5
elav>CycA-RNAi	68	75	23.9 +/- 0.06	141.1 +/- 5.5

B



C

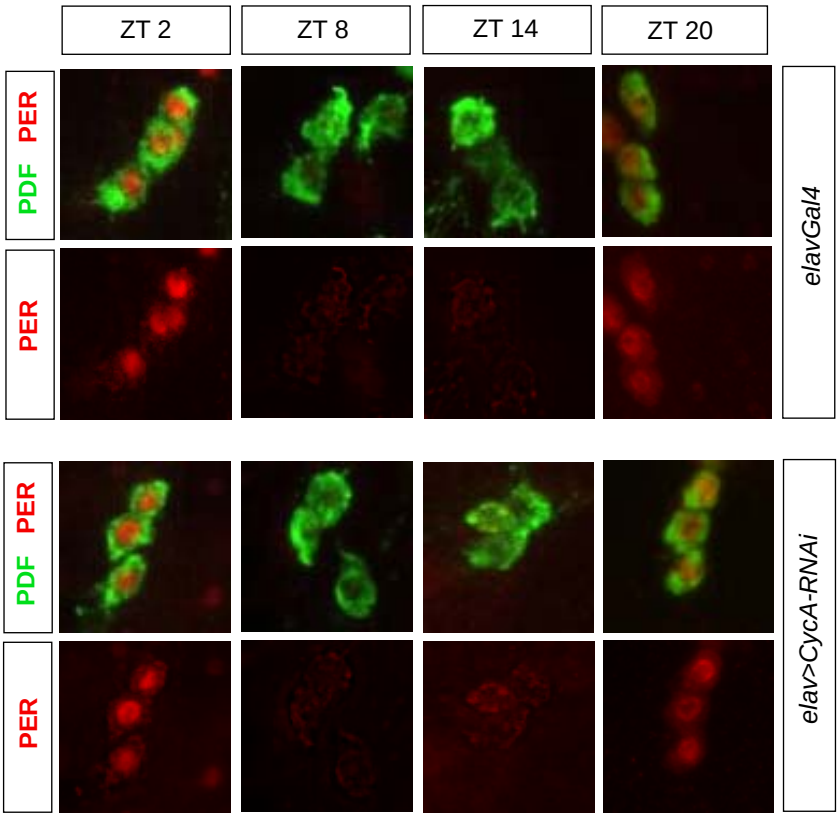
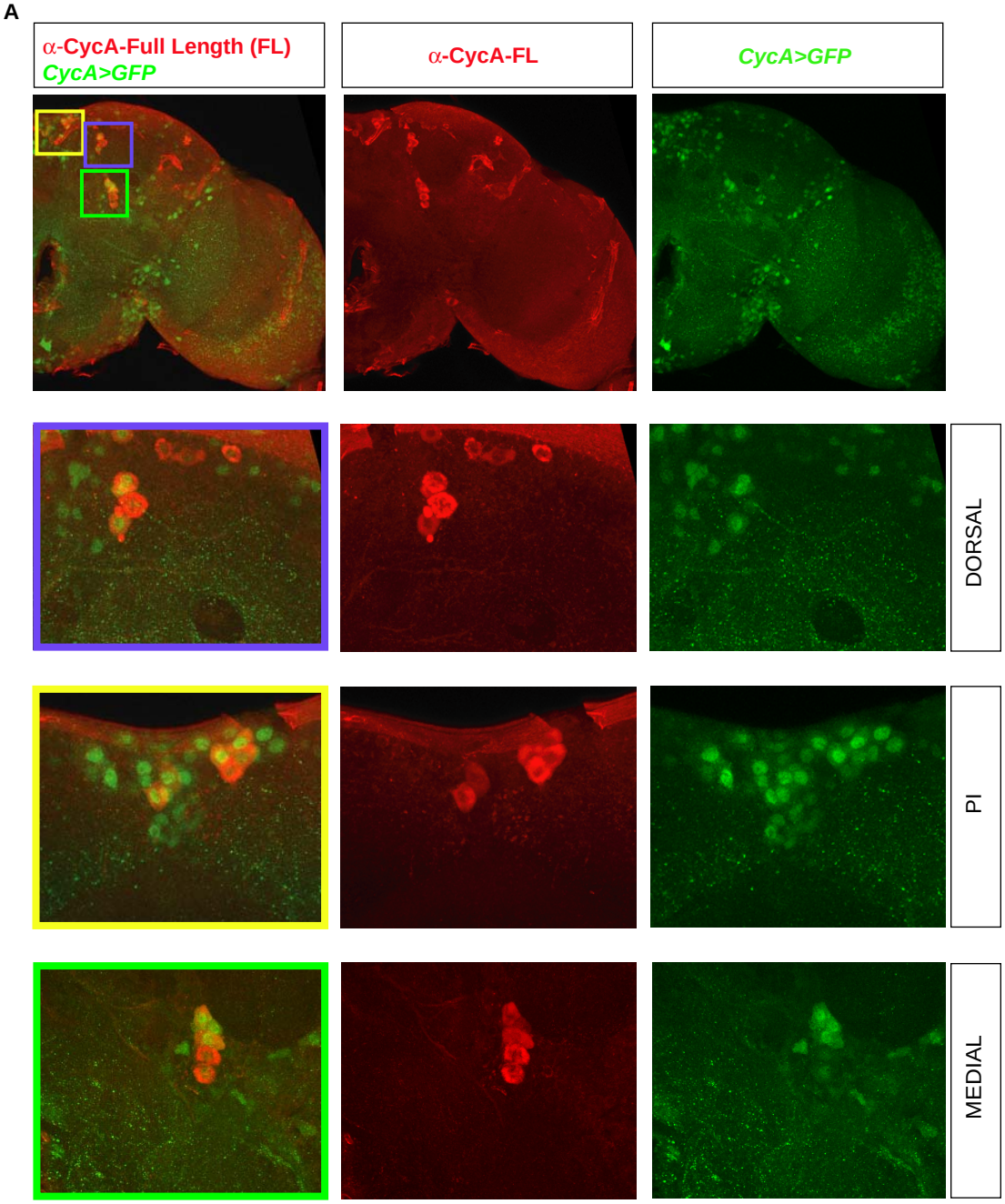
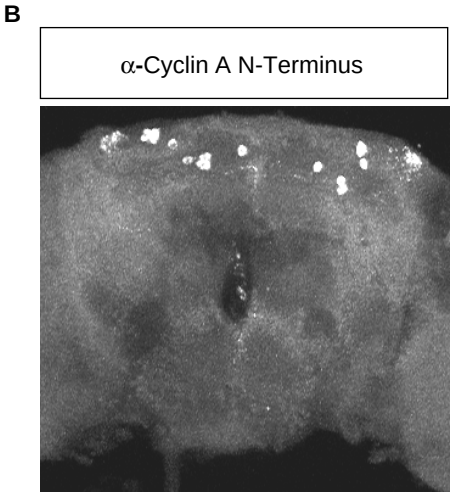


Figure S3

Functional circadian clocks in *elav>CycA-RNAi* flies

(A) The majority of *elav>CycA-RNAi* flies had rhythmic locomotor activity in free-running conditions (constant darkness) with a normal period (tau). Power reflects the rhythm strength, with higher numbers denoting stronger rhythms. **(B)** Eclosion rhythms in constant darkness were normal in *elav>CycA-RNAi* flies (n=307) flies compared to the control [*elavGal4*, (n=240)]. Gray and black portions of the bar shown below the X-axis indicate subjective day and night, respectively. **(C)** PER (red) protein oscillations were intact in pacemaker neurons of *elav>CycA-RNAi* animals, as detected by an anti-PER antibody. PDF staining (green) is shown as a marker of lateral (pacemaker) neurons, as detected by an anti-PDF antibody. All pictures were taken with the same confocal settings. Brains were fixed at indicated Zeitgeber times (ZT). For each genotype and each time point, at least 20 brains were examined. In all experiments described in this figure, *elavGal4* drove the expression of *UAS-Dicer2*.


A
α-CycA-Full Length (FL)
CycA>GFP
α-CycA-FL
CycA>GFP
DORSAL
PI
MEDIAL


B
α-Cyclin A N-Terminus

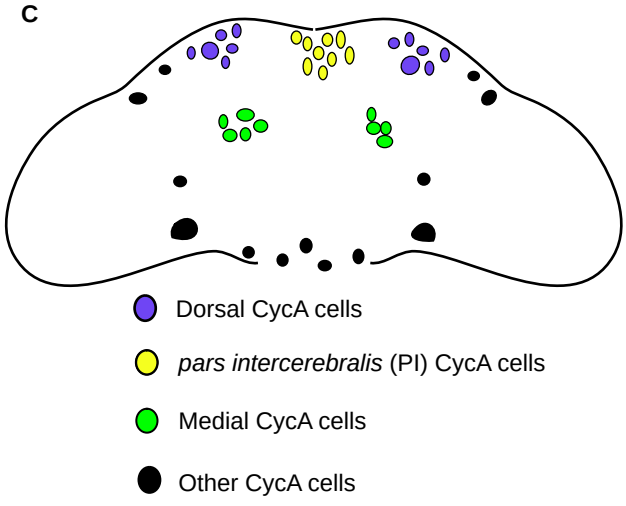

C
Dorsal CycA cells
pars intercerebralis (PI) CycA cells
Medial CycA cells
Other CycA cells

Figure S4
Expression of CycA in adult brains
(A) Anti-CycA antibody raised against the full-length protein marked a few small groups of cells in the adult fly brain (red). This pattern was confirmed by *CycAGal4*-driven *GFP* (*CycA>GFP*, green), which also labeled additional cells. Higher magnification images of the dorsal region of the brain ("Dorsal"), *pars intercerebralis* ("PI"), and the medial region of the brain ("Medial") are shown. Different colors denote different brain areas in this and subsequent figures. Maximal projections of the relevant confocal sections are shown for the higher magnification panels. For simplicity, only one brain hemisphere is shown. **(B)** Anti-CycA antibody raised against the N-terminus of the protein recognized only the dorsal subset of CycA cells. **(C)** A schematic representation of an adult brain with CycA cells indicated. Accented by color are CycA cells shown in panel (A).

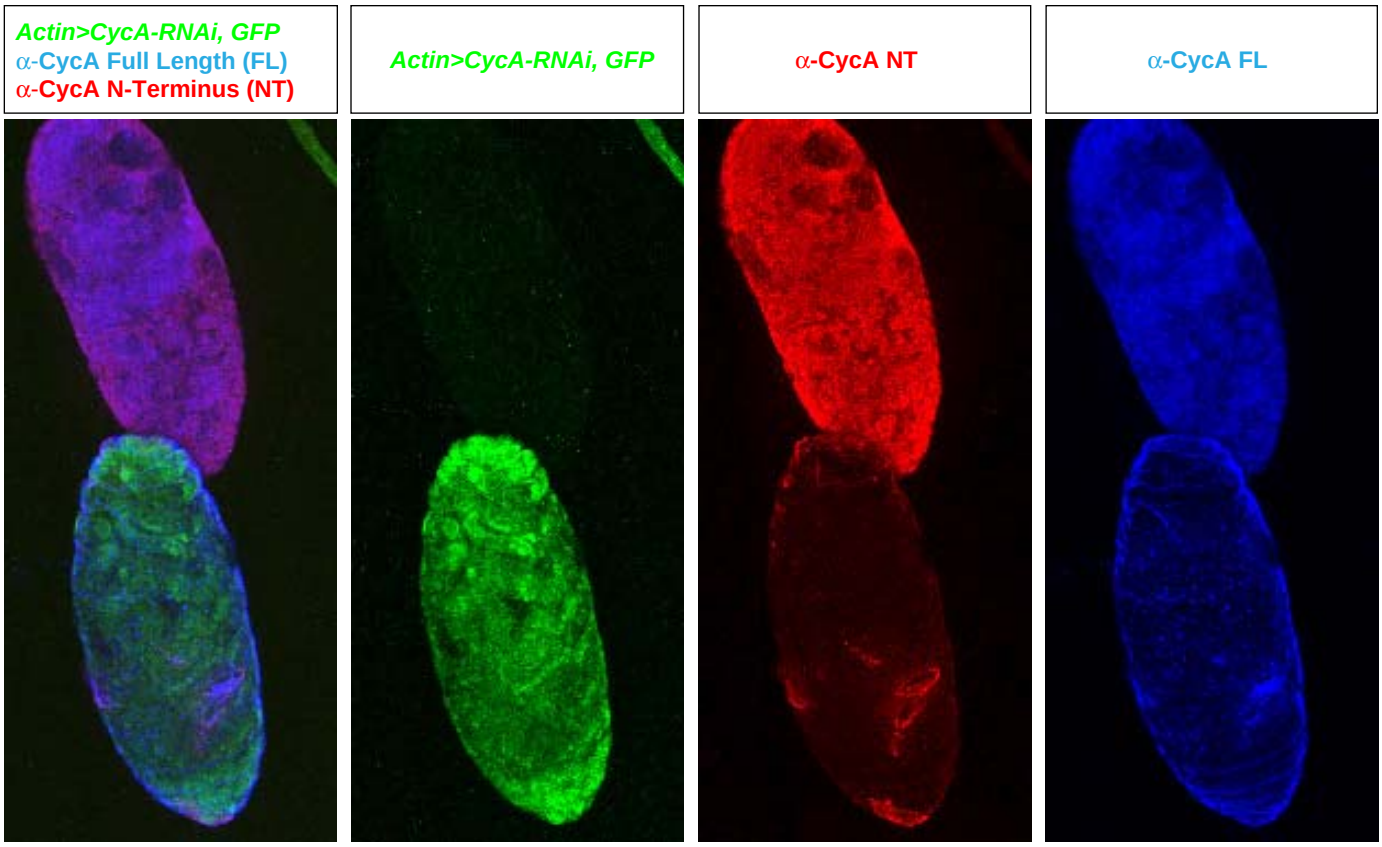
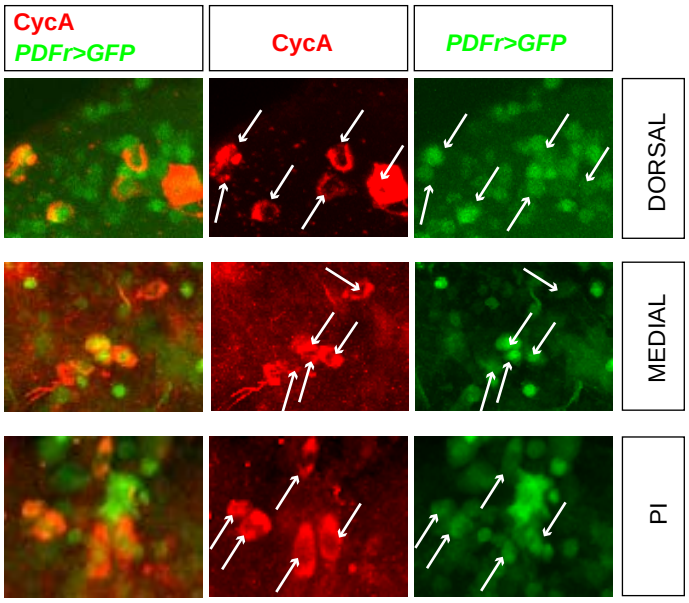
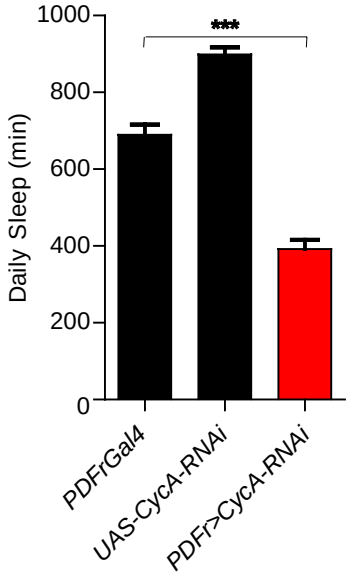


Figure S5
Specificity of Anti-CycA antibodies
GFP+ embryos (green) in which *CycA-RNAi* was driven by *ActinGal4* (*Actin>CycA-RNAi, GFP*) had strongly reduced CycA staining as judged by both an antibody raised against the full-length CycA protein (α-CycA FL, blue) and an antibody raised against the N-terminus of CycA (α-CycA NT, red), when compared to their siblings lacking *ActinGal4* (GFP-).

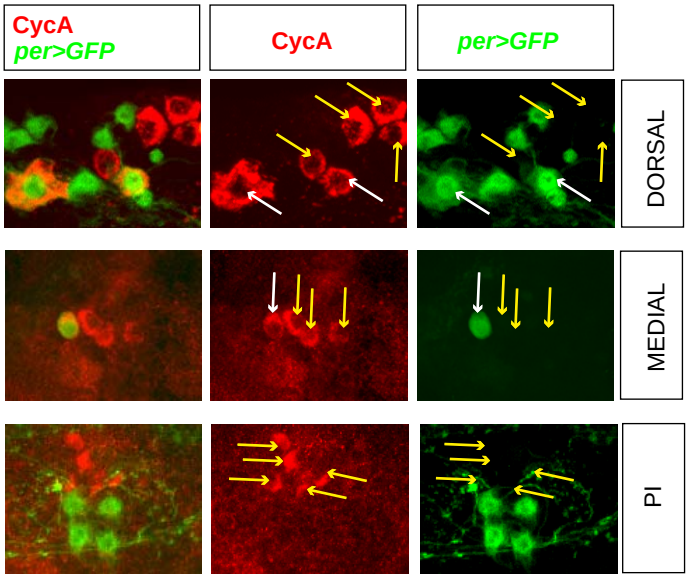
A


CycA
PDFr>GFP
CycA
PDFr>GFP
DORSAL
MEDIAL
PI

B


Daily Sleep (min)
1000
800
600
400
200
0
***
PDFrGal4
UAS-CycA-RNAi
PDFr>CycA-RNAi

C


CycA
per>GFP
CycA
per>GFP
DORSAL
MEDIAL
PI

D

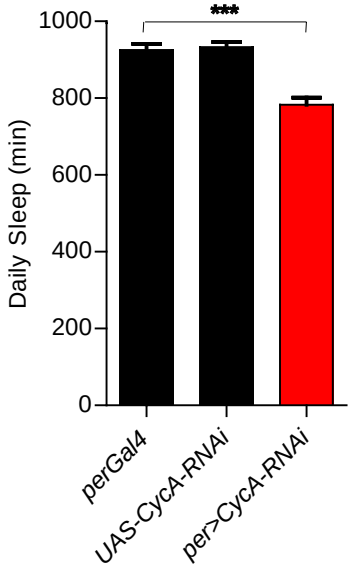

Daily Sleep (min)
1000
800
600
400
200
0
***
perGal4
UAS-CycA-RNAi
per>CycA-RNAi

Figure S6

Co-labeling of CycA with *PDFr-Gal4* and *per-Gal4*

A) CycA+ neurons (red) labeled for the expression of PDF receptor (*PDFr*), as judged by *PDFrGal4*-driven *GFP* (*PDFr>GFP*, green) in different regions of the brain. White arrows point to overlapping cells. **(B)** *PDFrGal4*-driven *CycA-RNAi* [*PDFr>CycA-RNAi* (n=63)] reduced sleep when compared to the controls [*PDFrGal4* (n=84), *UAS-CycA-RNAi* (n=104)]. **(C)** A subset of the CycA+ cells (red) labeled positive for the activity of *perGal4* (*per>GFP*, green). White and yellow arrows point to overlapping and non-overlapping cells, respectively. **(D)** *perGal4*-driven *CycA-RNAi* [*per>CycA-RNAi* (n=131)] reduced sleep when compared to the controls [*perGal4* (n= 126), *UAS-CycA-RNAi* (n=156)]. In all experiments described in this figure, the indicated *Gal4* lines drove the expression of *UAS-Dicer2*.

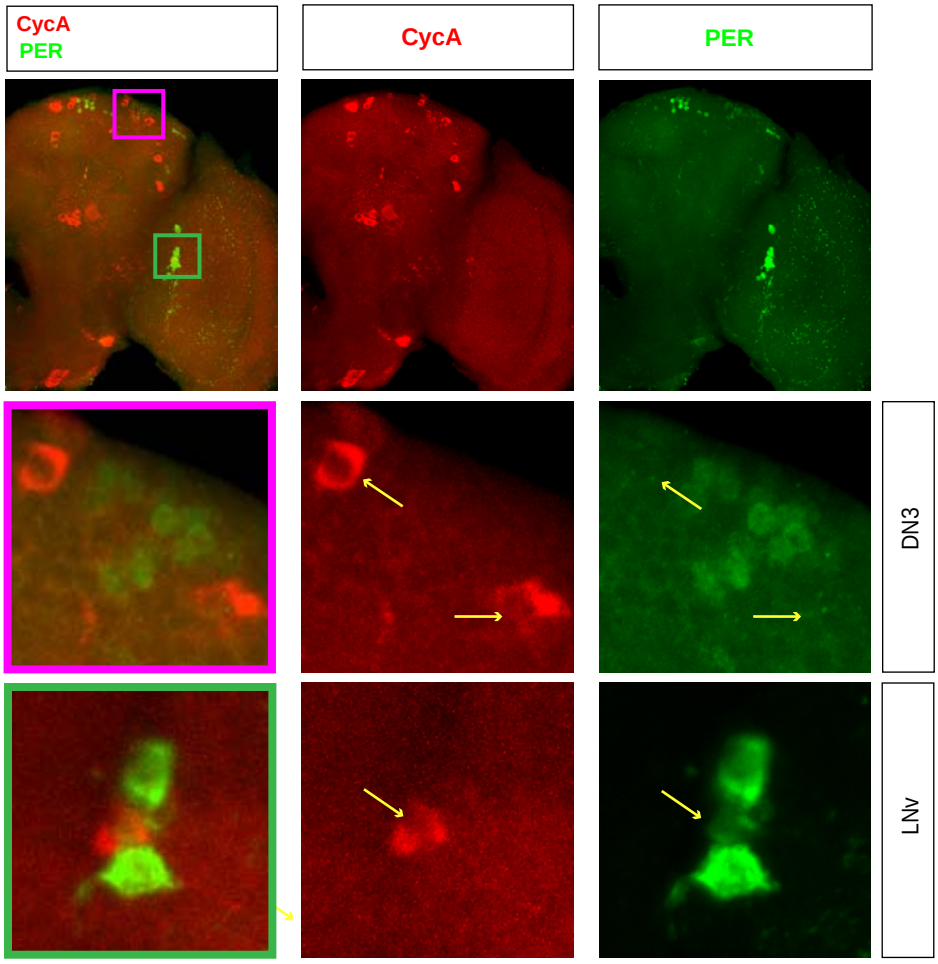


Figure S7
Close association of CycA producing cells and circadian clock neurons
CycA+ cells (red) clustered with both DN3 and LNv circadian neurons (green, marked by an anti-PER antibody) in the adult brain (yellow arrows). Note that the CycA-labeled neuron is in a different confocal plane than the middle LNv.