Supplemental Material Inventory

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Table S1, Related to Figure 3. PDFR Expression in the DN1ps Does Not Rescue Evening Anticipation Phase Phenotype of *Pdfr* Mutants.

Table S2, Related to Figure 5. Standard Deviation of Within-Cluster DN1 PER intensities in *Pdf*-GAL4,UAS-*CK2βRNAi*/+ Compared to UAS-*CK2βRNAi*/+ Flies.

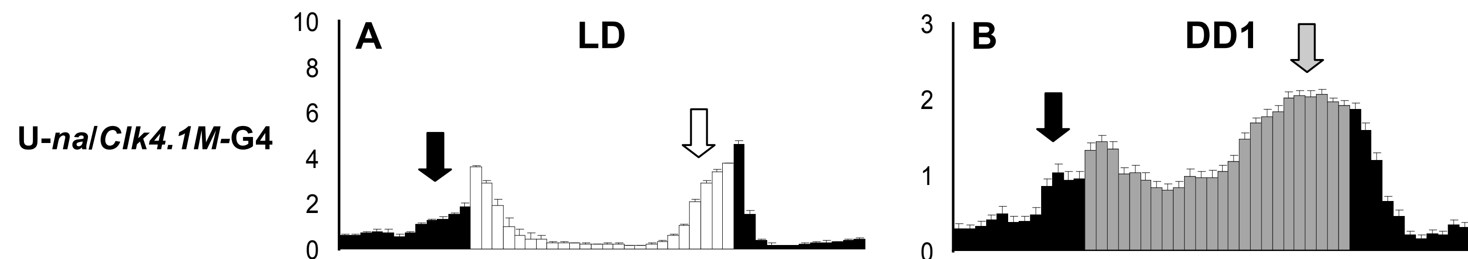


Figure S1, Related to Figure 2. Overexpression of *na* in DN1ps Does Not Alter the Circadian or Masking Response. Normalized activity plots for adult male populations averaged across either (A) 4 days of LD or (B) the first day of DD. For (A), light and dark phases are indicated by horizontal white and black bars, respectively. For (B), subjective light and dark phases are indicated by horizontal gray and black bars, respectively. n=87. Error bars indicate standard error of mean (SEM).

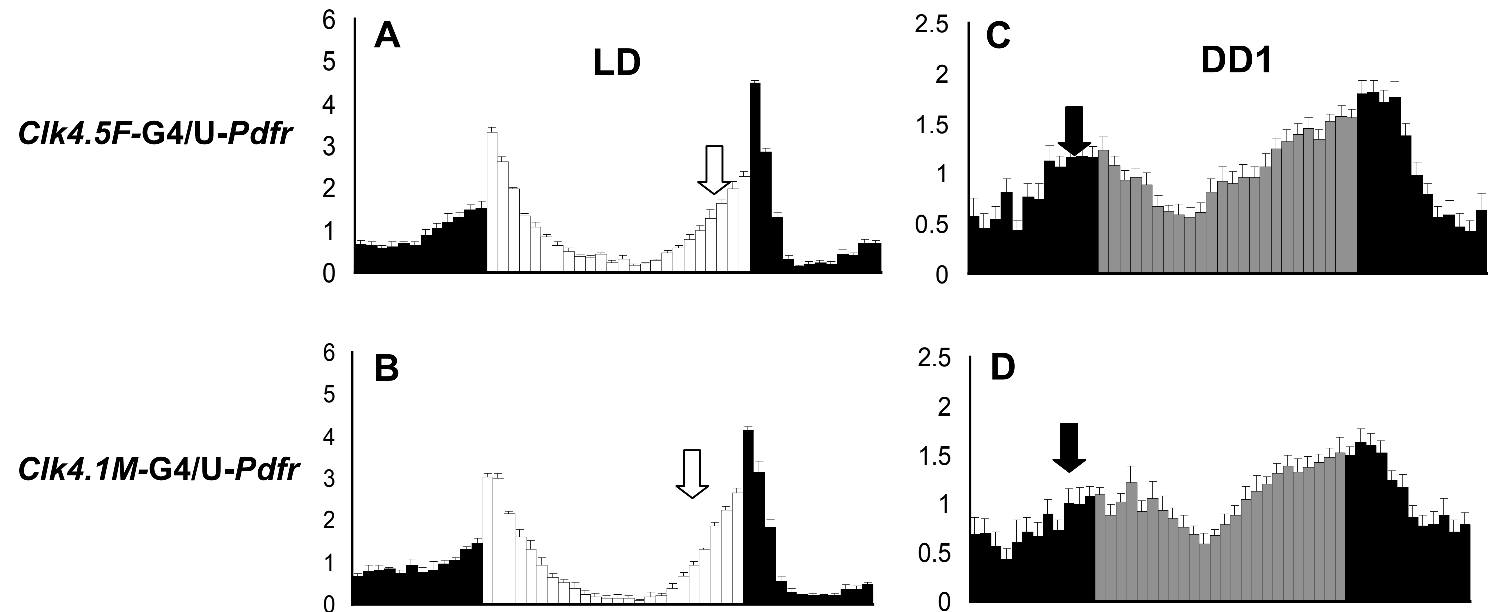
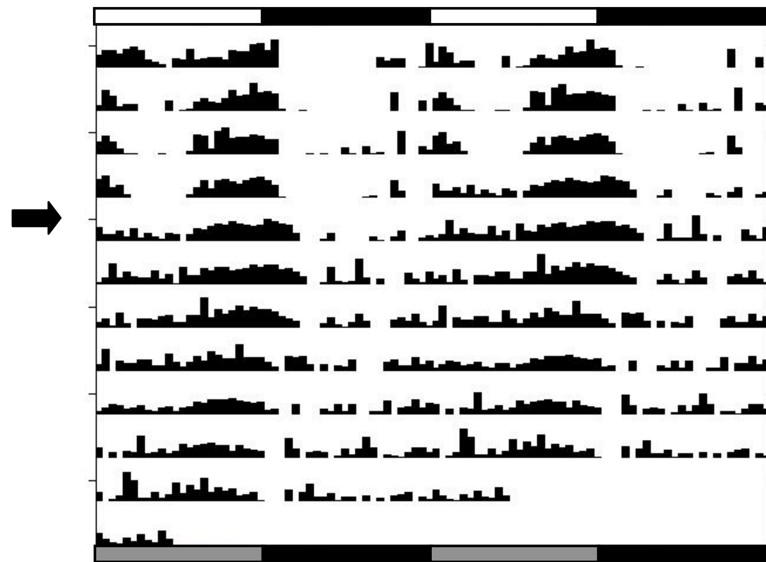


Figure S2, Related to Figure 3. Circadian Behavior of DN1p PDFR Overexpression Controls. (A and B) Normalized activity plots for adult male populations, averaged over four days of 12 hour light: 12 hour dark entrainment. Light phase is indicated by white bars, while dark phase is indicated by black bars. (C and D) Normalized activity plots of adult male populations over the last 6 hours of LD (ZT18-CT0) followed by the first 18 hours of DD (CT0-18). Subjective light phase (CT0-12) is indicated by dark gray bars while subjective dark phase is indicated by black bars. (A and B) *Clk4.5F*-GAL4/UAS-*Pdfr*; (C and D) *Clk4.1M*-GAL4/UAS-*Pdfr.* Error bars represent standard error of the mean (n=17-23). White arrows indicate evening behavior and black arrows indicate morning behavior.

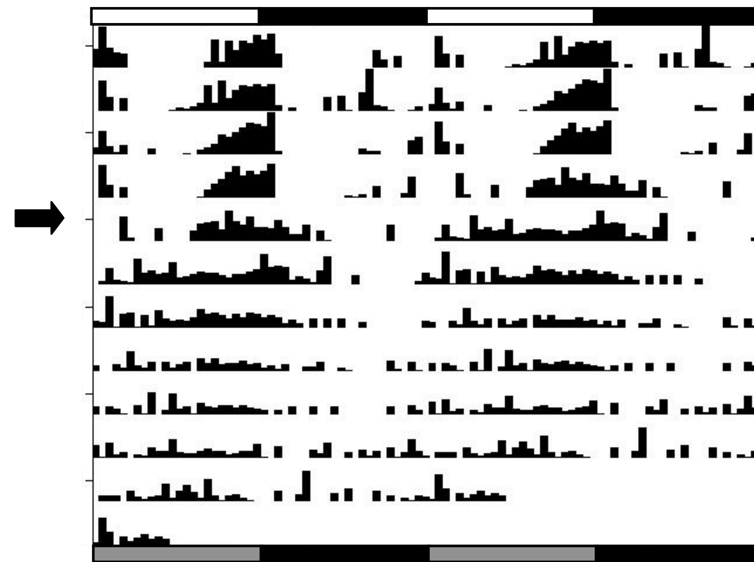
A

Pdfr*-;;*Clk4.1M*-G4/U-*Pdfr



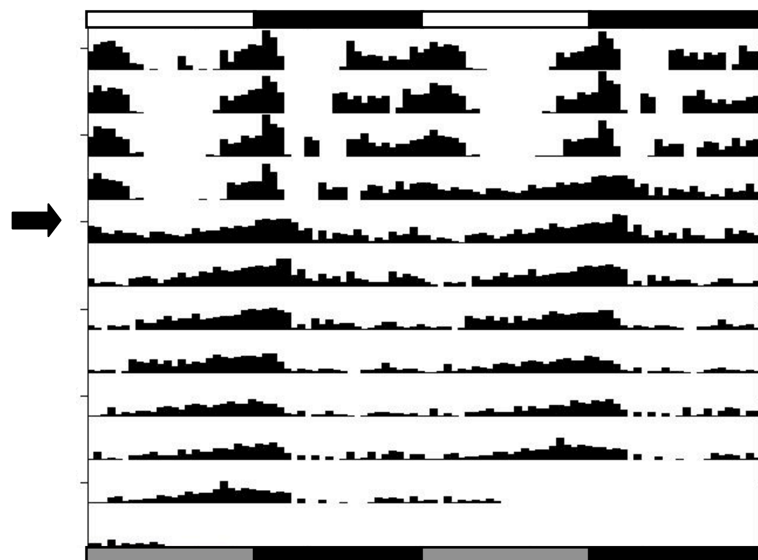
B

Pdfr*-;;*Clk4.5F*-G4/U-*Pdfr



C

U-*Pdfr*/+



D

***Pdfr*-;;U-*Pdfr*/+**

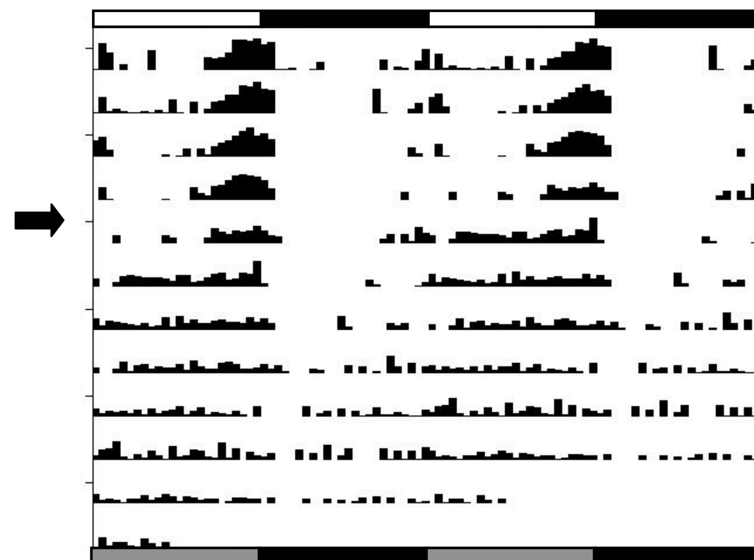


Figure S3, Related to Table 2. Rescue of DD Period Length by PDFR Expression in the DN1ps Using *Clk4.1M*-GAL4. Representative actograms of a single fly tested for 4 days in 12L:12D followed by 7 days in DD are shown for (A) *Clk4.1M*-GAL4 rescue, (B) *Clk4.5F*-GAL4 rescue, (C) *Pdfr*^{han5304} mutant control, and (D) UAS-*Pdfr* wild-type control. Horizontal white and black bars above each actogram denote 12 hr light and dark periods, respectively. Horizontal gray and black bars beneath each actogram denote 12 hr subjective light and subjective dark periods, respectively. The black arrow to the left of each actogram indicates the transition from LD to DD conditions. Vertical black bars represent total activity counts during a 30-min interval. Activity data has been double-plotted to improve visualization of circadian period.

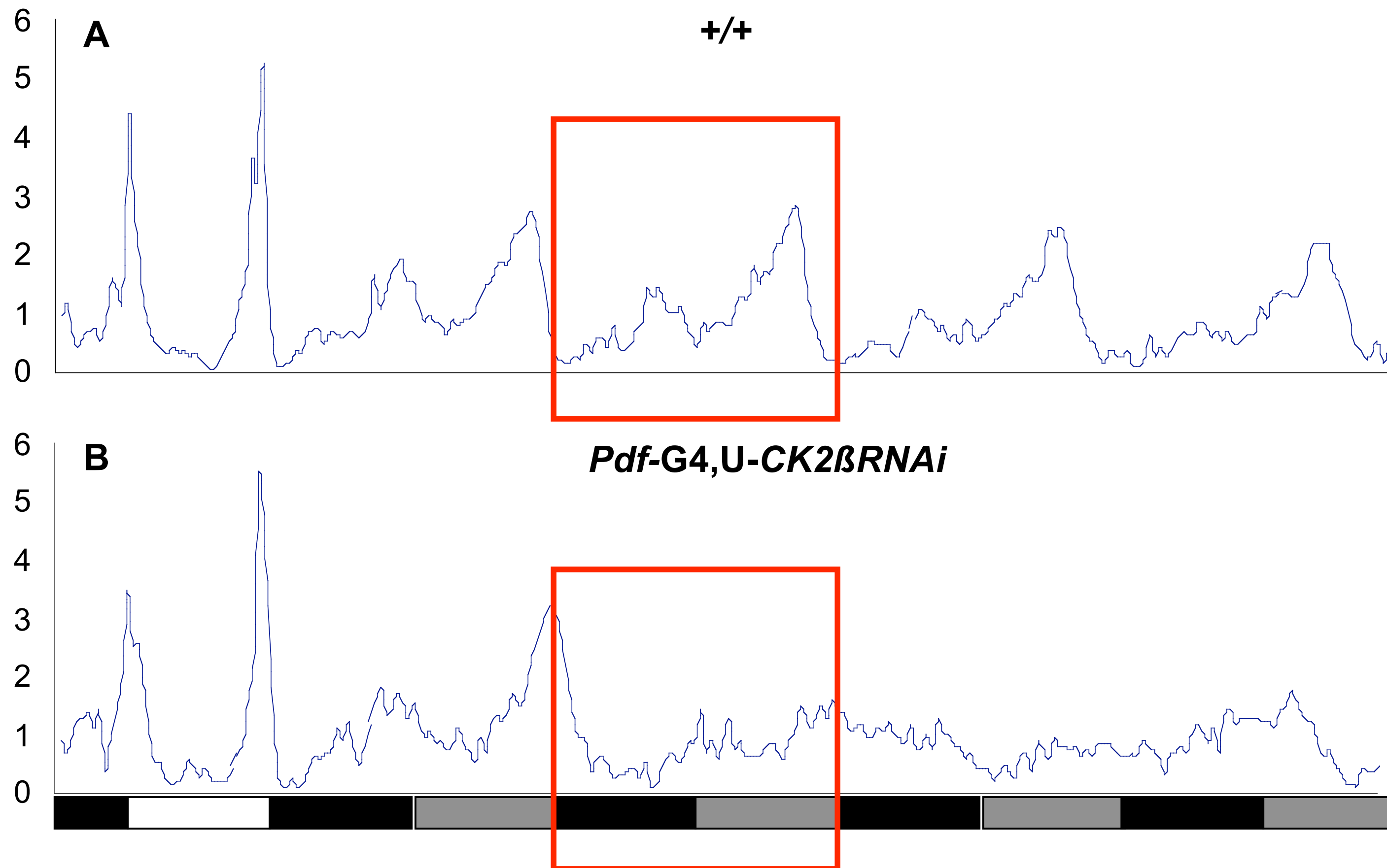
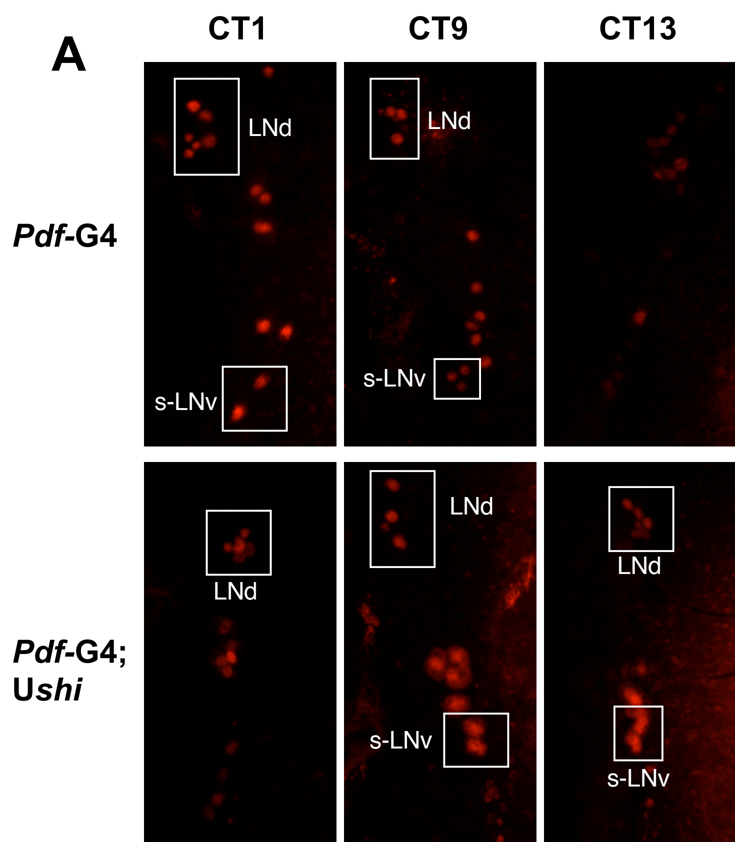
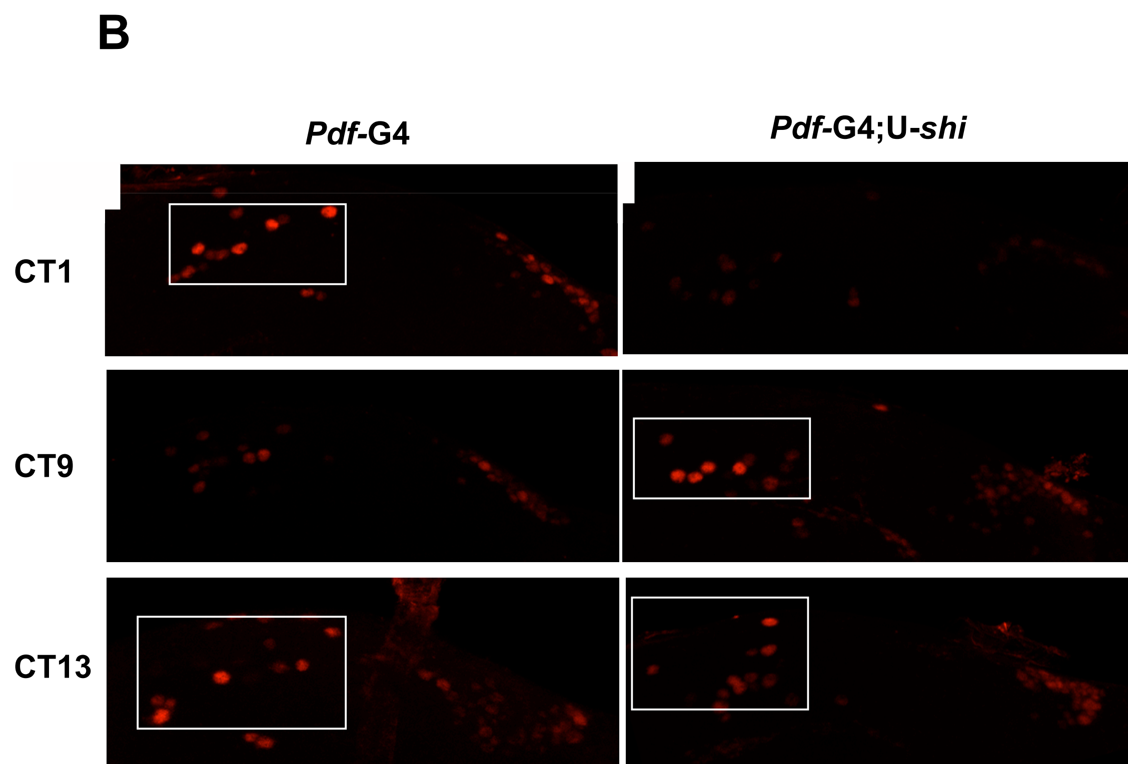


Figure S4, Related to Figure 5. Locomotor Behavioral Trace of Flies Expressing *CK2βRNAi* in PDF Neurons. (A and B) Normalized locomotor activity profiles during 12 hour light: 12 hour dark entrainment for 1 day followed by 4 days of constant darkness (n=18-29). White box indicates light period, black box indicates dark period or subjective dark period, and gray box indicates subjective light period. (A) +/+; (B) *Pdf*-GAL4,UAS-*CK2βRNAi*/+. Red box indicates the day when PER immunolabeling was performed.


A
CT1
CT9
CT13
Pdf-G4
Pdf-G4;
Ushi
LNd
s-LNv


B
Pdf-G4
Pdf-G4;U-shi
CT1
CT9
CT13

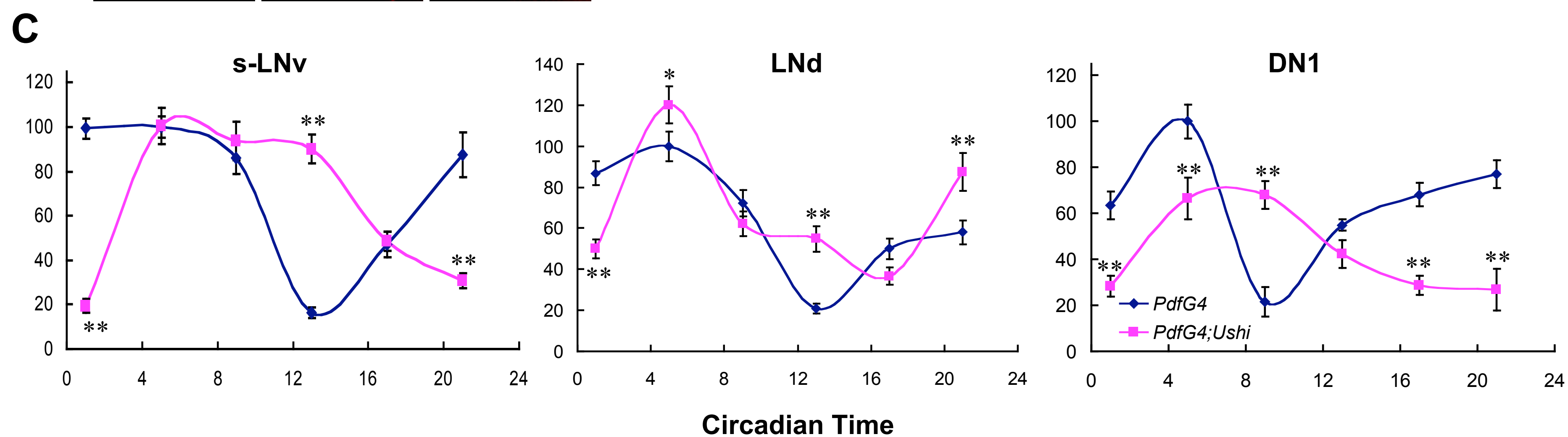

C
s-LNv
LNd
DN1
PdfG4
PdfG4;Ushi
Circadian Time

Figure S5, Related to Figure 5. Changing the Pace of the Clock in the PDF(+) s-LNvs Changes the Pace of the Clock in the DN1s but Not the LNds. (A and B) Maximum projections of confocal sections taken in representative adult *Pdf*GAL4/+ and *Pdf*GAL4/+;UAS*shibire*^{ts1}/+ brains labeled with with PER antibodies. Sections contain the LNs at CT1, 9, and 13. The PDF(+) s-LNvs and LNds are in boxes, while the 5th s-LNv is indicated by line. (B) Maximum projections of confocal sections taken in representative adult *Pdf*GAL4/+ and *Pdf*GAL4/+;UAS*shibire*^{ts1}/+ brains labeled with PER antibody. Sections contain the DNs at CT1, 9, and 13. The DN1s are in a box. (C) Plots of average normalized pixel intensity versus circadian time for each pacemaker cell group. See experimental procedures for details of quantification method. Error bars represent standard error of mean. The results are a combination of two independent experiments: s-LNv, n=30-40; LNd, n=32-79; DN1, n=65-99. Asterisks mark significant differences between genotypes (Student's t-test, *$p<0.05$, **$p<0.01$).

Table S1, Related to Figure 3. PDFR Expression in the DN1ps Does Not Rescue Evening Anticipation Phase Phenotype of *Pdfr* Mutants.

Genotype	Time of Evening Anticipation Zeitgeiber Time	N
UAS-*Pdfr*/+	10.3 ± 0.2	37
Pdfr^{han5304} ; ; UAS-*Pdfr*/+	9.5 ± 0.1	57
Pdfr^{han5304} ; ; *Clk4.5F*-GAL4/UAS-*Pdfr*	9.4 ± 0.1	53
Pdfr^{han5304} ; ; *Clk4.1M*-GAL4/UAS-*Pdfr*	9.4 ± 0.2	26
Clk4.5F-GAL4/UAS*Pdfr*	10.8 ± 0.2	23
Clk4.1M-GAL4/UAS*Pdfr*	11.2 ± 0.2	17

Table S2, Related to Figure 5. Standard Deviation of Within-Cluster DN1 PER Intensities in *Pdf*-GAL4,UAS-*CK2βRNAi*/+ Compared to UAS-*CK2βRNAi*/+ Flies.

	UAS*CK2βRNAi*/+	*Pdf*GAL4-UAS*CK2βRNAi* /+
Circadian Time	**Average Standard Deviation per Hemisphere (± SEM)**	**Average Standard Deviation per Hemisphere (± SEM)**
1	74.8 ± 7.7	41.6 ± 8.8*
7	41.1 ± 3.6	61.2 ± 6.0**
14	61.5 ± 8.9	43.0 ± 2.3
20	74.4 ± 7.4	32.5 ± 5.6***

Asterisks denote statistical differences between the two genotypes as determined by Student's t-test; $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Supplemental Experimental Procedures

Clk4.1M-GAL4 and Clk4.5F-GAL4 transgenic flies. A 1.5 kb fragment of *Clk* genomic DNA that extends from -2.0 kb to -0.5 kb upstream of the *Clk* transcription start site was inserted into pChs-GAL4[1]. The resulting plasmid was then used to generate multiple transgenic fly lines, including the *Clk4.1M*-GAL4 and *Clk4.5M*-GAL4 lines used in this study. The *Clk4.1M*-GAL4 line is the same used in the accompanying paper[2].

Behavioral experiments and analyses. Locomotor activity levels of male flies were monitored using Drosophila Activity Monitoring System (TriKinetics; Waltham, MA) for 5 days under 12 hour light: 12 hour dark conditions (LD) followed by 7 days under constant darkness (DD) at 25°C. For LD circadian analyses (Figures 2, 3, S1 and S2), activity levels from each fly were normalized and averaged within genotypes across the last 4 days of LD, as described previously [3]. For DD Day 1 (DD1) analyses (Figures 2, 3, S1 and S2), activity levels were normalized and averaged across the first day of DD. For LD5-DD4 circadian analyses (Figure S4), activity levels from each fly were normalized and averaged within genotypes over the last day of LD and the first 4 days of DD.

To calculate time of onset of evening anticipation in LD (Table S1), we determined the largest 1.5 hour increase in normalized average activity for each fly over the last six hours of the light phase. The time designation refers to the end point of the maximal activity increase, as averaged among individual flies in each genotype.

To calculate DD1 Morning Anticipation Index (Tables 1 and 2), normalized activity levels were averaged using a moving average over three consecutive 30-minute bins starting at ZT18 of LD5 and ending at CT2 of DD1. Using population averages for each genotype, we determined the beginning (t_{start}) and end-points (t_{end}) of the maximum activity increase, allowing for up to a 5 hour difference between t_{start} and t_{end}. The DD1 Morning Anticipation Index (for each individual fly) was then determined by subtracting its normalized activity at t_{start} from its normalized

activity at t_{end}.

Both the acute masking index (AMI) and sustained masking index (SMI) were developed to quantify the flies' immediate and prolonged response to lights-on conditions, respectively (Table 1). AMI was calculated by first subtracting the amount of activity 1 hr prior to lights-on from the amount of activity 1 hr after lights-on and dividing that value by the total amount of activity for both periods, i.e. AMI = $(Activity_{ZT0\text{-}1} - Activity_{ZT23\text{-}0})/(Activity_{ZT0\text{-}1} + Activity_{ZT23\text{-}0})$. SMI was calculated in a similar manner; the total amount of activity from ZT4-ZT8 was used in place of ZT0-ZT1 and the total amount of activity from ZT16-ZT20 was used in place of ZT23-0, i.e. SMI = $(Activity_{ZT4\text{-}ZT8} - Activity_{ZT16\text{-}ZT20})/(Activity_{ZT4\text{-}ZT8} + Activity_{ZT16\text{-}ZT20})$. A zero value was assigned for instances where the total amount of activity for both periods was equal to zero. Both AMI and SMI were calculated for each of the last 4 days of LD, averaged across all 4 days for each fly, and finally averaged across the population for each genotype.

For DD rhythmicity (Tables 1 and 2), chi-squared periodogram analyses were performed using ClockLab (Actimetrics; Wilmette, IL). Rhythmic flies were defined as those in which the chi-squared power was ≥ 10 above the significance line. Period calculations also considered all flies with rhythmic power ≥ 10.

GFP, *PDF and PER Immunohistochemistry, Microscopy and Quantification*. Male flies were entrained for at least 3 days at 25ºC. Flies were anesthetized with CO_2 and dissected in 3.7% formaldehyde diluted in PBS. After fixing for 30 minutes at room temperature, the brains were rinsed 2 times in PBS and incubated in PBS with 1% Triton for 10 minutes at room temperature. The brains were then incubated with 5% goat serum diluted in PBT (PBS with 0.3% Triton) for 30 minutes at room temperature, followed by overnight incubation of 1:2000 mouse anti-GFP (Invitrogen) 1:500 mouse anti-PDF (Hybridoma Bank, Iowa), or 1:500 rat anti-PDF and 1:2000-1:4000 rabbit anti-PER (rat PDF and PER antibodies are generous gifts from Michael Rosbash) in PBT containing 5% goat serum at 4ºC. After several PBT rinses, the brains were incubated

with 1:500 goat-anti-mouse AlexaFluor488 (Amersham) for GFP/PDF immunostaining and 1:250-1:500 goat-anti-rabbit AlexaFluor 594 (Amersham) for PER immunostaining in PBT overnight at 4ºC. Final rinses in PBT and PBS were followed by mounting in 80% glycerol diluted in PBS. GFP/PER-labeled specimens were photographed with 20x and 60x oil lens on a Nikon Eclipse 800 laser scanning confocal microscope.

For PER intensity quantification, all slides were coded as to sample identity and remained so until the numerical analysis stage. PDF and PER-stained specimens were photographed with 60x oil lens on a Nikon Eclipse 800 laser scanning confocal microscope. For a given experiment the microscope, laser, and filter settings were held constant. PER immunostaining was quantified from single slices of the maximum diameter for each cell using ImageJ (NIH). PER-stained soma were outlined to obtain average pixel intensity. On each projection image an unstained area was used for background subtraction. All background-subtracted intensity measurements within a condition (time and genotype) were averaged. To combine experiments, background subtracted measurements were scaled to a certain time point of a certain genotype in that experiment. Statistical analysis were performed with Excel.

To determine the level of synchrony among DN1s, we determined the variation, or standard deviation, of PER immunofluorescence pixel intensity between single DN1s within a hemisphere[4]. Thus, each hemisphere yielded a single standard deviation value. For a given genotype and time point, we then determined the average of these standard deviation values as well as the variance (expressed as standard error of the mean). Desynchrony among DN1s would be reflected as greater variation among single DN1s, and thus a higher standard deviation.

Statistical Analysis. Quantitative data analysis and statistical tests described in the text were performed using Excel (Microsoft) and the STATISTICA 6.0 software suite (StatSoft).

Supplemental References

1. Apitz, H. (2002). pChs-Gal4, a vector for the generation of Drosophila Gal4 lines driven by identified enhancer elements. Drosophila Information Service *85*, 118-120.
2. Zhang, Y.e.a. (2010). Light and temperature control the contribution of a subset of Dorsal Neurons 1 to Drosophila circadian behavior. Curr Biol.
3. Zhao, J., Kilman, V.L., Keegan, K.P., Peng, Y., Emery, P., Rosbash, M., and Allada, R. (2003). Drosophila clock can generate ectopic circadian clocks. Cell *113*, 755-766.
4. Zhang, L., Lear, B.C., Seluzicki, A., and Allada, R. (2009). The CRYPTOCHROME photoreceptor gates PDF neuropeptide signaling to set circadian network hierarchy in Drosophila. Curr Biol *19*, 2050-2055.