SUPPLEMENTAL INFORMATION

yw;pdf-GAL4:UAS-Cry;Cry^{b}

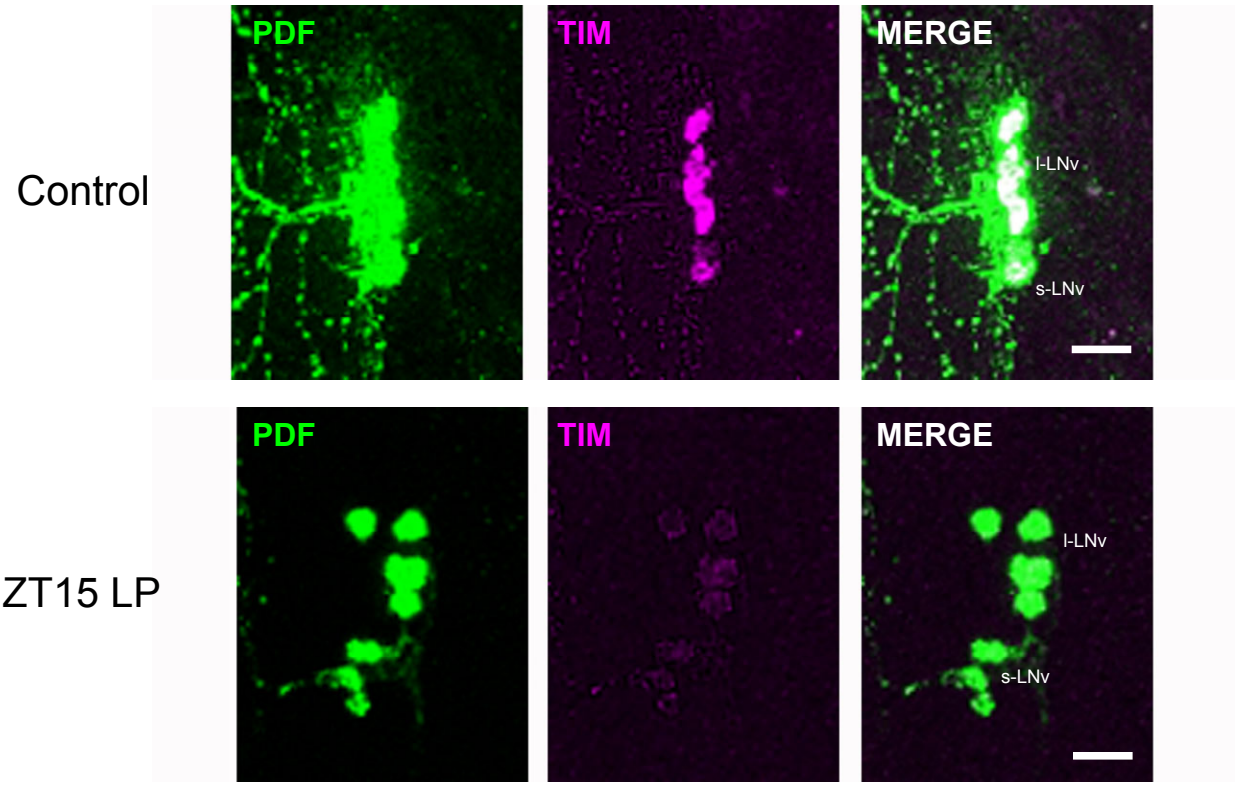


Figure S1.

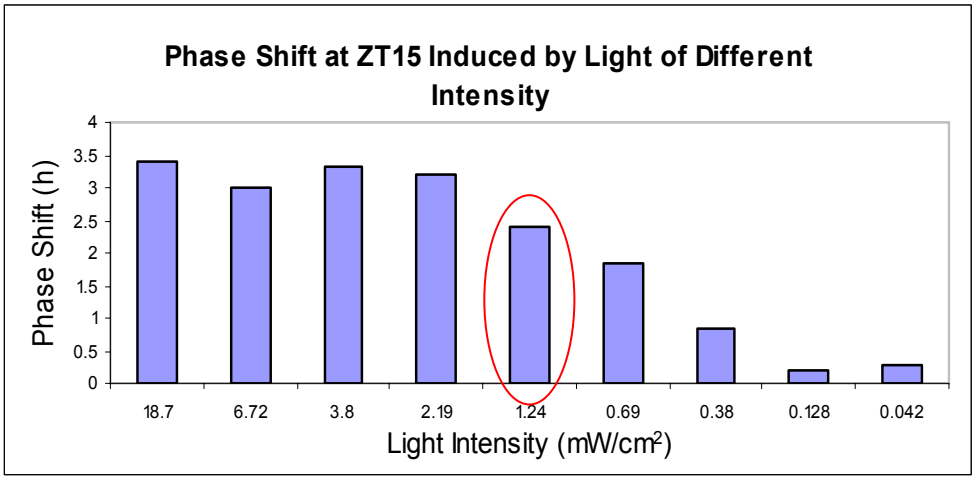

Phase Shift at ZT15 Induced by Light of Different Intensity
Phase Shift (h)
4
3.5
3
2.5
2
1.5
1
0.5
0
18.7
6.72
3.8
2.19
1.24
0.69
0.38
0.128
0.042
Light Intensity (mW/cm2)

Figure S2.

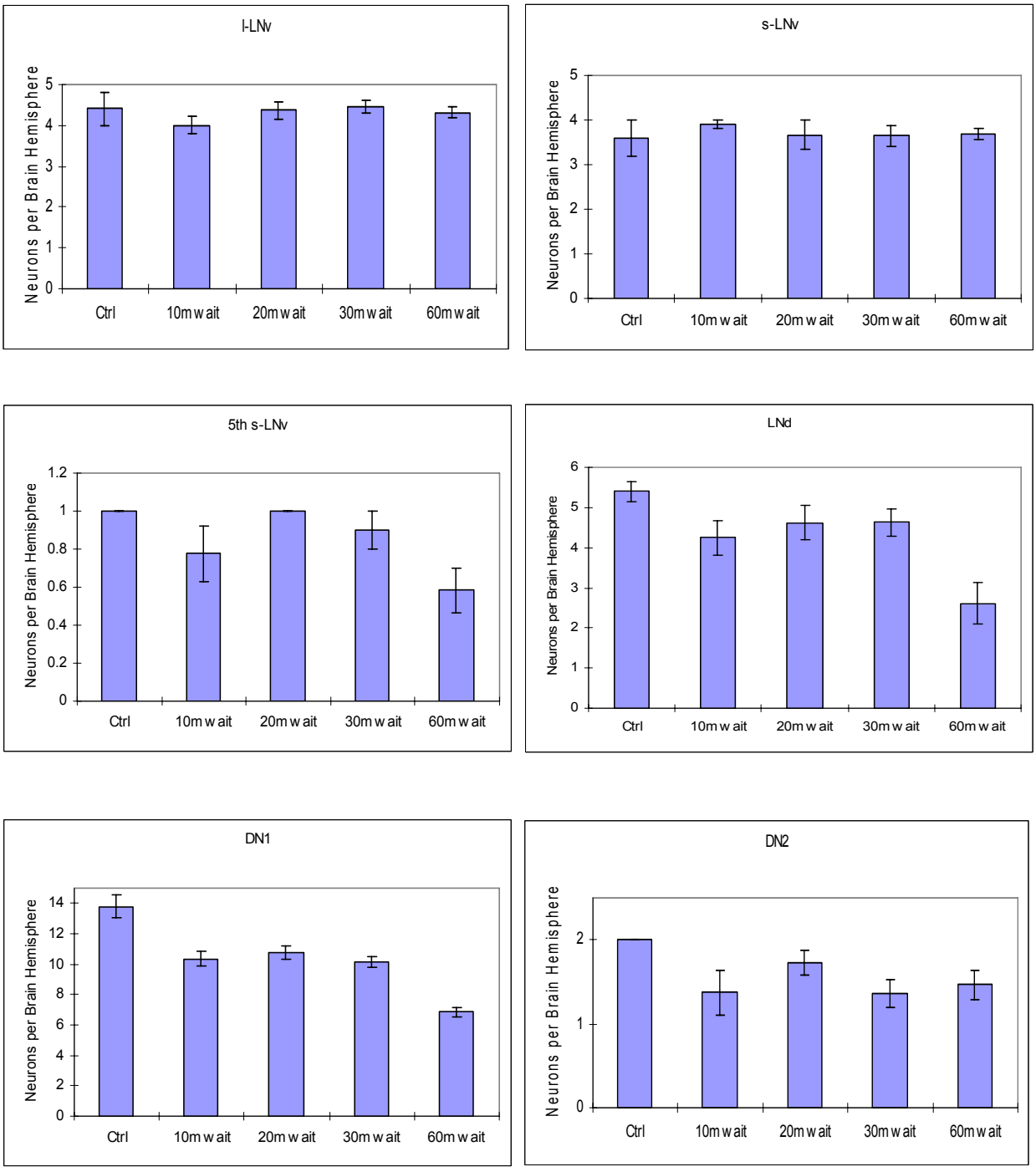

l-LNv
Neurons per Brain Hemisphere
0
1
2
3
4
5
Ctrl
10m wait
20m wait
30m wait
60m wait
s-LNv
Neurons per Brain Hemisphere
0
1
2
3
4
5
Ctrl
10m wait
20m wait
30m wait
60m wait
5th s-LNv
Neurons per Brain Hemisphere
0
0.2
0.4
0.6
0.8
1
1.2
Ctrl
10m wait
20m wait
30m wait
60m wait
LNd
Neurons per Brain Hemisphere
0
1
2
3
4
5
6
Ctrl
10m wait
20m wait
30m wait
60m wait
DN1
Neurons per Brain Hemisphere
0
2
4
6
8
10
12
14
Ctrl
10m wait
20m wait
30m wait
60m wait
DN2
Neurons per Brain Hemisphere
0
1
2
Ctrl
10m wait
20m wait
30m wait
60m wait

Figure S3.

ZT15 Light Pulse After 3 h Wait

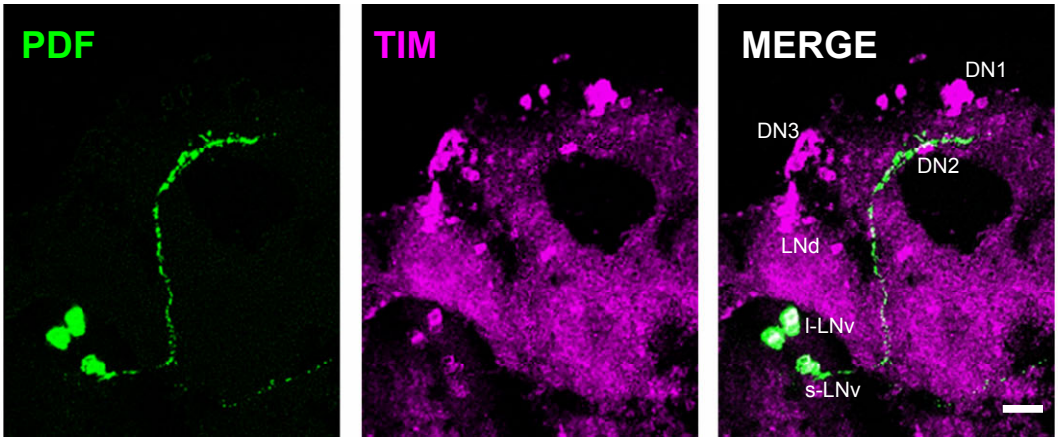


Figure S4.

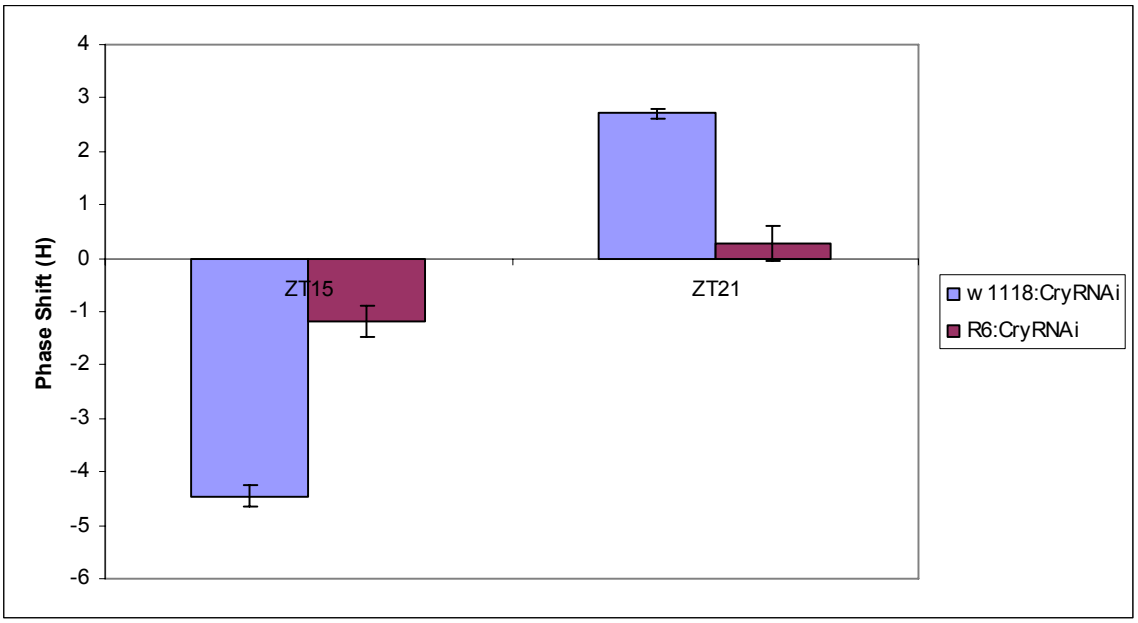
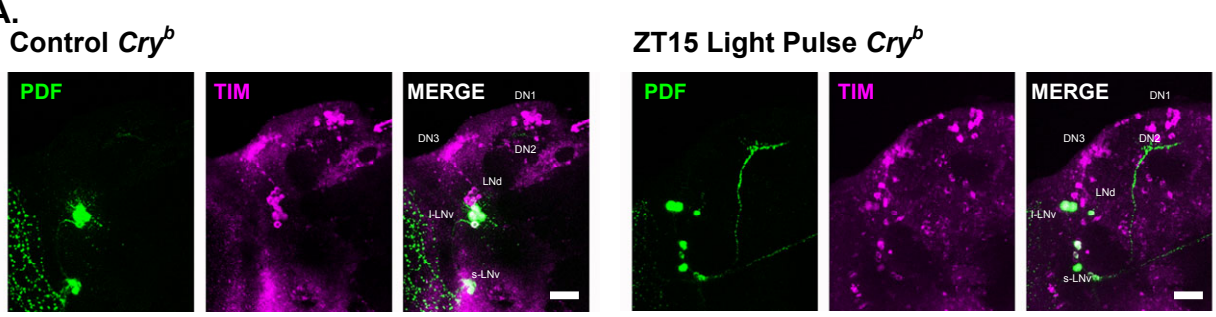

Phase Shift (H)
4
3
2
1
0
-1
-2
-3
-4
-5
-6
ZT15
ZT21
w 1118:CryRNAi
R6:CryRNAi

Figure S5.

A.


Control Cry^b
ZT15 Light Pulse Cry^b
PDF
TIM
MERGE
DN1
DN3
DN2
LNd
l-LNv
s-LNv

B.

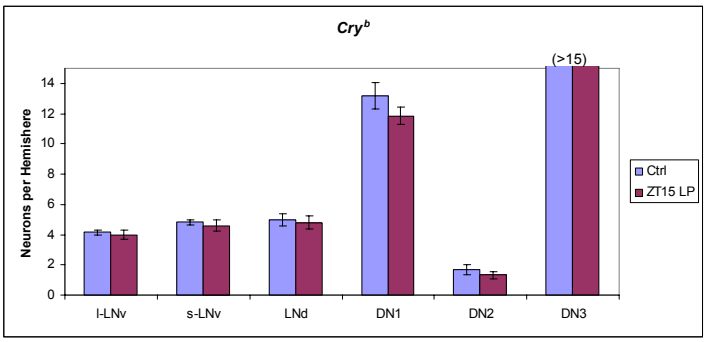

Cry^b
Neurons per Hemishere
(>15)
Ctrl
ZT15 LP
l-LNv
s-LNv
LNd
DN1
DN2
DN3

Figure S6.

ZT21 Heat Pulse

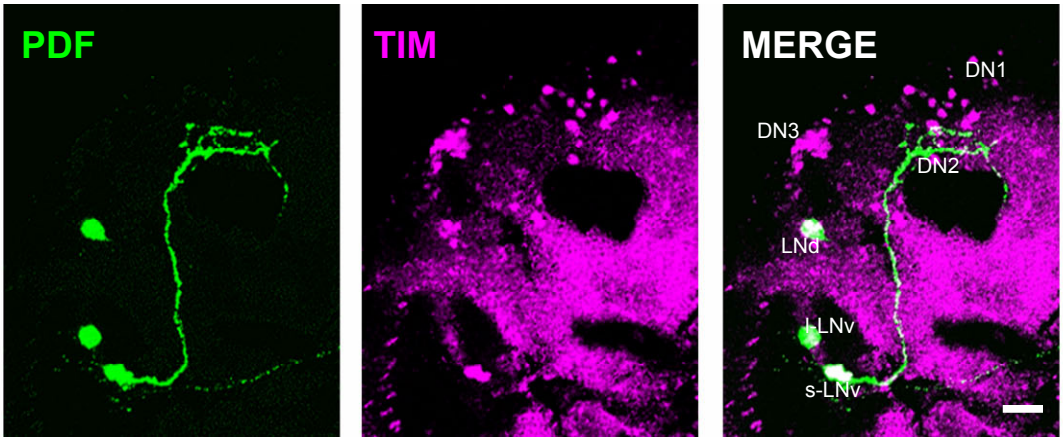


Figure S7.

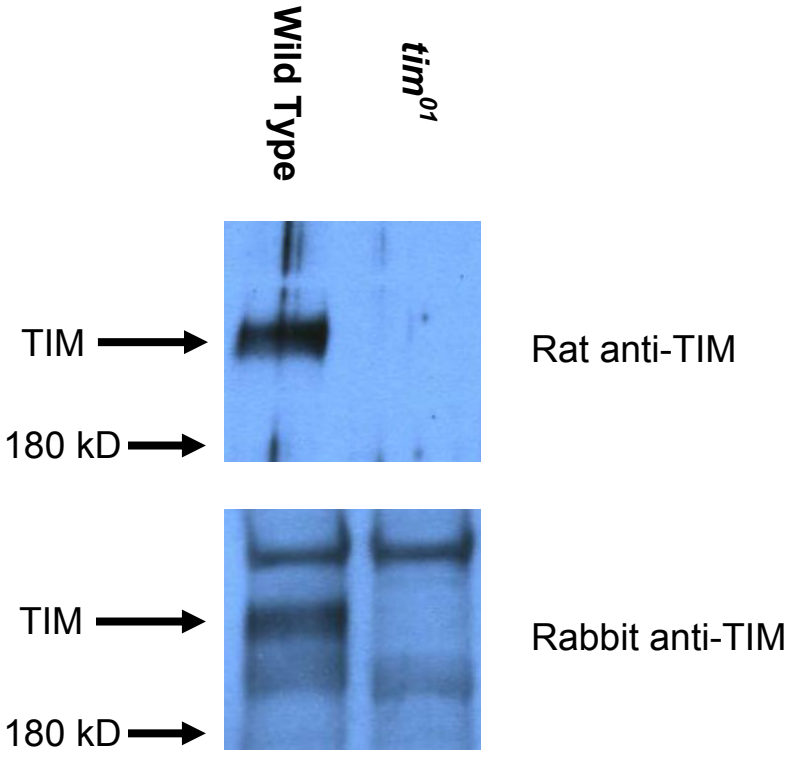

Wild Type
tim01
TIM
180 kD
Rat anti-TIM
TIM
180 kD
Rabbit anti-TIM

Figure S8.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Overexpression of CRY in LNvs under *cry*^{b} background also promotes light-induced TIM degradation in LNv at ZT15. CRY was overexpressed in the LNvs (*yw;pdf-GAL4:UAS-cry;cry*^{b}), causing TIM degradation in the LNvs after a light pulse at ZT15. The results are similar to that over the wild-type background (Figure 2). Scale bar = 20 μm

Figure S2. Flies were pulsed with a single light pulse of different intensities at ZT15. Phase shifts were measured as a function of light intensity. Light intensity at 0.69 mW/cm^2 (red circle) causes ca. 2 hour phase delay and was selected as a "half light pulse".

Figure S3. TIM-positive cell numbers decrease as a function of time in darkness after a half light pulse. To identify the most light-sensitive or insensitive subsets of the clock neurons, flies were subjected to a half light pulse and then dissected after 10, 20, 30, and 60 min in darkness. TIM-positive cells were counted in each neuronal group (±SEM). The most significant TIM degradation occurred between 30 to 60 min.

Figure S4. TIM expression pattern after 3 h after a light pulse at ZT15. TIM is detected in all clock neurons 3 h after 10 minutes light pulse at ZT15. The result can be compared with TIM staining at ZT15 which was done after 1 h wait (Figure 1A) (see Experimental Procedures). Scale bar = 20 μm.

Figure S5. CRY is necessary within the s-LNvs for phase shifting.

cry mRNA was knocked down specifically in s-LNvs with the R3 *UAS-cryRNAi* line (Picot et al., 2007) crossed to the *R6-GAL4* driver line (Helfrich-Forster et al., 2007). The knockdown line (red bars) was assayed in parallel with the control line (*w1118:UAS-cryRNAi*) (blue bars).

Figure S6. Light-induced TIM degradation in clock neurons is abolished on cry^b flies at ZT15. (A) The TIM staining pattern (magenta) with or without a light pulse at ZT15. LNvs are also stained with PDF (green). TIM can still be easily detected in the clock neurons after a light pulse at ZT15. Scale bar = 20 μm. (B) Quantification of TIM-stained cells in each group of clock neurons with or without a light pulse at ZT15 in cry^b flies (±SEM).

Figure S7. Heat pulse at ZT21. The TIM staining pattern (magenta) after a 30 min heat pulse at ZT21. LNvs are also stained with PDF (green). No detectable TIM degradation is found among all clock neurons. Scale bar = 20 μm.

Figure S8. Characterization of the new rat anti-TIM antibody. Rat anti-TIM antibody was examined by western blotting. Flies of wild type background and tim^{01} were entrained for 3 days in LD and collected at ZT21. Fly heads extract was loaded on a SDS-PAGE gel and transferred to a nitrocellulose membrane. The new rat anti-TIM and rabbit anti-TIM antibodies can detect the presence of TIM in wild type flies but not in tim^{01}.

EXPERIMENTAL PROCEDURES

Drosophila Stocks and Genetics

Drosophila melanogaster were reared on standard cornmeal/agar medium supplemented with yeast and kept in 12:12 light-dark (LD) cycles at 25°C. Wild-type Canton-S flies were used for immunocytochemistry and phase response analyses (see below). The *yw;pdf-GAL4* and *yw;UAS-cry* transgenic flies have been described previously (Emery et al., 2000). *Jetlag (common)* mutant and jet^c*,tim-GAL4*, and jet^c*,UAS-jet* were kindly provided by Amita Sehgal. Jet^c*,pdf-GAL4* was produced by introducing *pdf-GAL4* into jet^c background. *R6-GAL4* and *UAS-cryRNAi* (R3 line) were generous gifts from Paul Taghert and François Rouyer, respectively.

Behavioral Analysis

Locomotor activity of individual male flies (1–5 days old) were measured with Trikinetics Activity Monitors (Waltham, MA) for 3 days under 12:12 LD conditions followed by 6 days of constant darkness at 25°C. For light pulsed flies, a single light pulse was delivered on the third night of LD, at ZT15 or ZT21, and the flies were then maintained in constant darkness to record phase shifts relative to non-light pulse-treated flies. Light intensities were varied using neutral density filters (Oriel Instruments, CT) in combination with an adjustment of the distance between the light source and the target. Light intensity was measured with a radiometer IL1350 (International Light, MA). By this means, a variable light intensity system was calibrated to generate a light-induced phase shift of 2 h. An intensity of 0.69mW/cm^2 for 10 min at ZT15 (half light pulse) was determined to cause a ca. 2 h delay of CS flies (Figure S2), similar to heat and about half the response to a full intensity light pulse (Figure 1A and 1B). The group activity

actograms were generated using a signal-processing toolbox (Levine et al., 2002) implemented in MATLAB (MathWorks). Phase-response curves and heat pulses were generated as described (Kaushik et al., 2007).

Whole-mount Immunocytochemistry

Adult flies were entrained for at least 3 days in12:12 LD before light/heat treatment and dissection. For light pulses at ZT15 or ZT21, flies were pulsed for 10 min and returned to darkness for 1 h before dissection. For heat pulses at ZT15, flies were incubated at 37°C for 30 min and dissected immediately. Immunostaining was performed as described (Yoshii et al., 2008). Briefly, fly heads were removed and fixed in 4% paraformaldehyde for 45 min at 4°C in darkness. Fixed fly heads were rinsed and dissected in PBS. The whole brains were blocked in 5% normal goat serum (Jackson Immunoresearch, PA) and subsequently incubated with primary antibodies at 4°C overnight. After incubation with Alexa Fluor 633 or Alexa Fluor 488-conjugated secondary antibodies (1:200 dilution; Molecular Probes, CA), brains were mounted in Vectashield Mounting Medium (Vector Laboratories, CA) and viewed on a Leica confocal microscope.

A polyclonal rat anti-TIM antibody was generated against the recombinant *Drosophila* TIM protein (amino acids 581 to 1390). Antibody was characterized by western blotting from wild type and tim^{01} fly heads extract (Figure S8). It was used at 1:200 dilution. For PDF staining, a mouse anti-PDF antibody developed by Justin Blau was obtained from the Development Studies Hybridoma (University of Iowa). It was used at 1:10 dilution. Rabbit anti-CRY antibody was a generous gift from Charlotte Helfrich-Förster and was used at 1:1000 dilution. All experiments

were performed at least three times with very similar or identical results. TIM-stained neurons were counted in each pacemaker cell group manually throughout the rostrocaudal (coronal section) Z series. DN3s contain large numbers of smaller neurons, with more than 15 neurons on each side and significant difference after light or heat pulses. The images presented in the figures are overlays of several confocal stacks (1 μm). Quantitation of the signal strength at s-LNv and l-LNv was done by using the quantify function of Leica Confocal Software (Version 2.61, Leica Microsystems Heidelberg GmbH). TIM staining signals from s-LNv and l-LNv were measured and background signals were subtracted before normalized to the PDF staining signals.

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