Supplementary Information

Neuronal Influence on Peripheral Circadian Oscillators in Pupal *Drosophila* Prothoracic Glands

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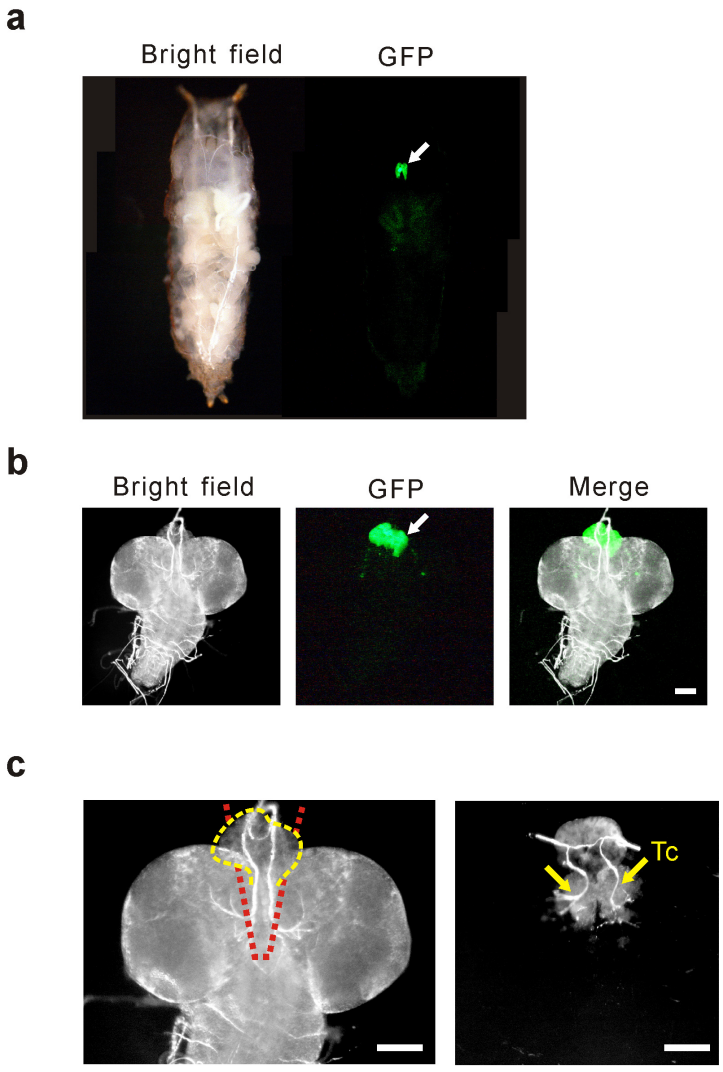
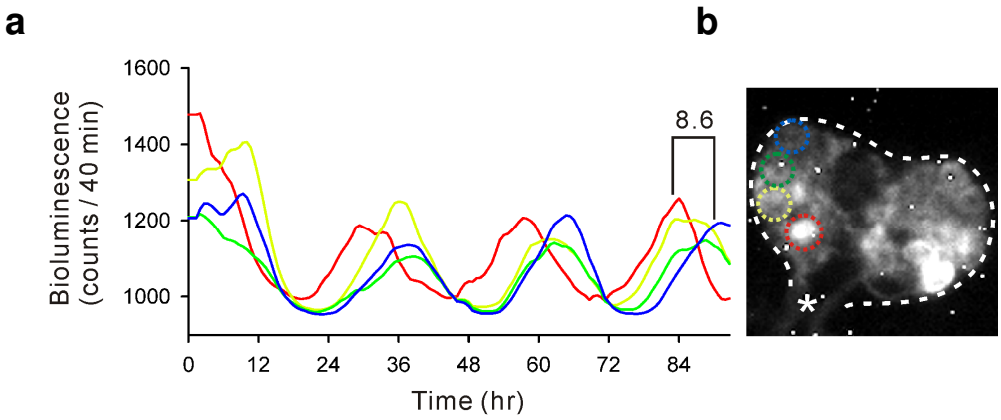
Supplementary Information contains:

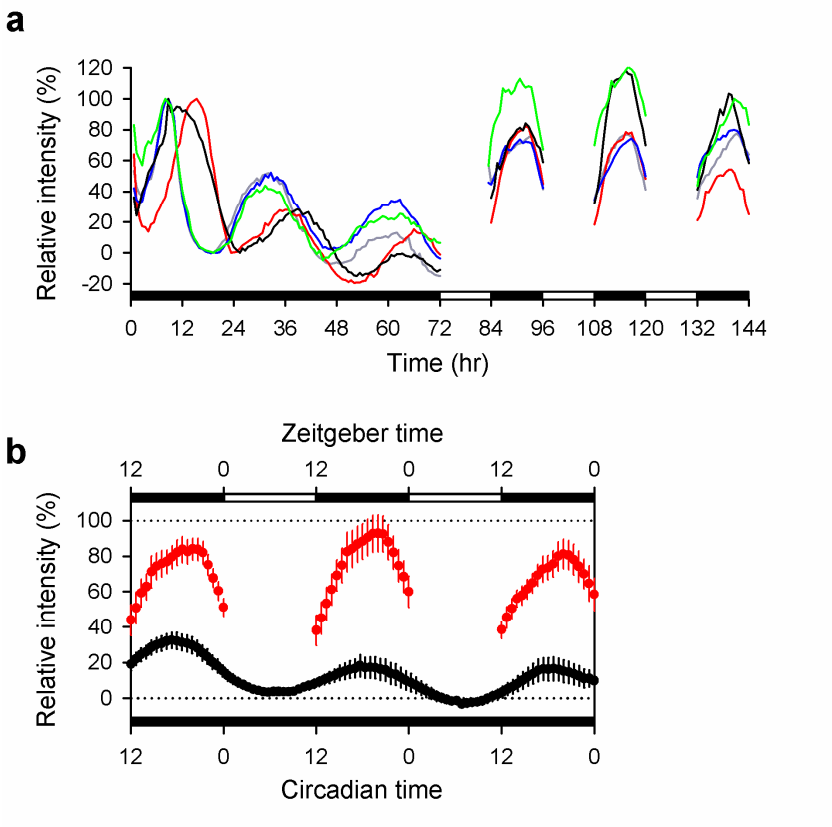
Supplementary Figures S1-S6



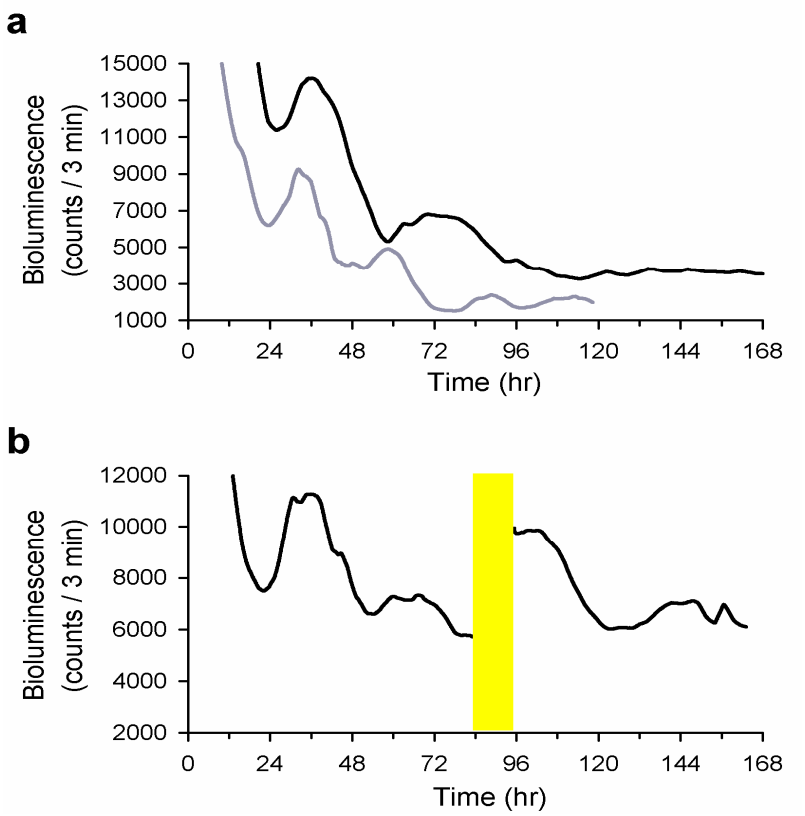
Supplementary Figure S1. Expression of *tim* gene and preparation of organotypic cultures. (**a**) A typical *Drosophila* prepupa at 1-3 hours post-pupariation, from which organotypic cultures were prepared. Cuticula and fat bodies were removed to visualize organs. Note that the RG (arrow) expressed very high levels of green fluorescent protein (GFP) in the *tim-gal4/UAS-GFPII S65T* fly. (**b**) Bright field, fluorescence (GFP), and an overlay of the two (Merge) images of an example CNS-RG complex dissected from the body of a fly. Bar = 100 µm. (**c**) Left: Approximate orientation used to dissect the isolated RG (red dotted lines) by which projecting neuronal cell bodies in the CNS were removed while retaining the connection between the RG (yellow dashed line) and the tracheal (Tc; yellow arrows) structure. Right: Isolated RG immediately after dissection. Bar = 100 µm.



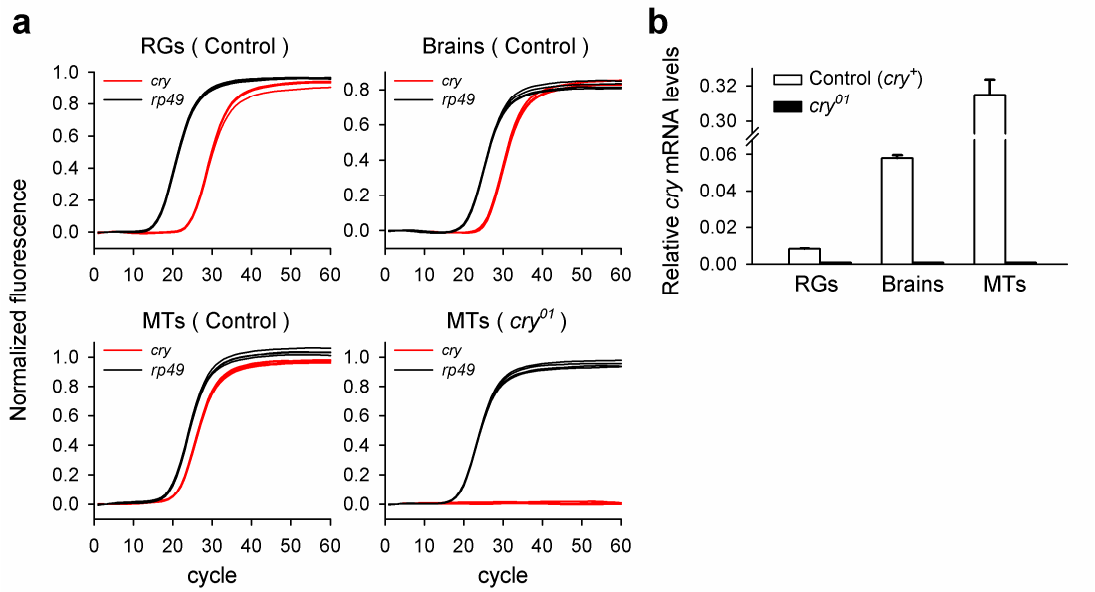
Supplementary Figure S2. Propagation of *per* transcriptional rhythms in PG cells in the CNS-RG complex. (**a**) Each trace denotes four representative *per-luc* rhythms in PG cells located in the ipsilateral RG. Warmer colors (yellow and red) indicate more proximal parts of the RG, as marked on the right panel. (**b**) The asterisk indicates the approximate location of corpus cardiacum by which the RG connects to the brain. In the CNS-RG complex, PG cell rhythms were initiated in the proximal RG and spread into the distal portion with a time lag of 8.6 hours on the fourth cycle under DD conditions.



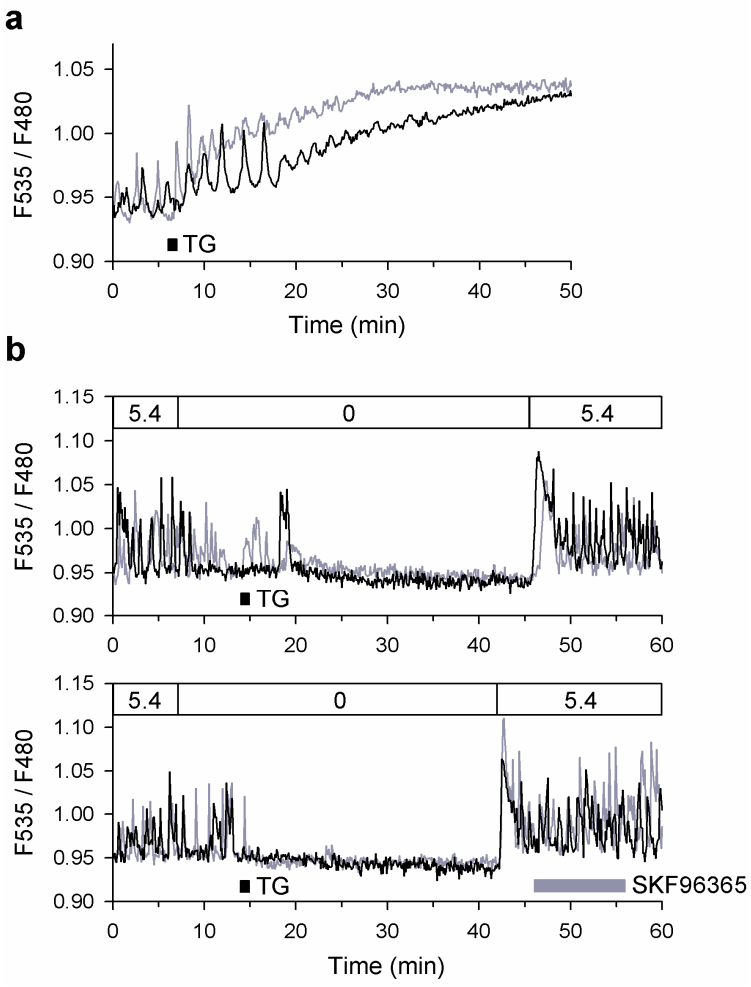
Supplementary Figure S3. *Per* transcriptional rhythms were amplified and entrained to LD cycles in PG cells in the CNS-RG complex. (**a**) Each trace denotes representative *per-luc* rhythms in PG cells located in the CNS-RG complex. The peak of the first circadian cycle in each cell was regarded as 100%. Amplitudes of *per-luc* rhythms in PG cells were reduced rapidly under DD. Exposures of cyclic 12 hour light (50 lux) regained oscillations to levels observed at the first circadian cycle. Bioluminescence measurements were conducted with culture medium containing 1 mM luciferin, which was not exchanged during the entire recording period. Black and white bars on the x-axis dark and light periods, respectively. (**b**) Averaged (±SE) *per-luc* rhythms in PG cells maintained under LD cycle conditions (red circles) or under DD (4-6 circadian cycles; black circles). Relative bioluminescence was calculated as above in **a**.



Supplementary Figure S4. *Per* transcript signal intensity changes in isolated Malpighian tubules (MTs). The MTs isolated from *per-luc/tim-gal4; UAS-GFPII S65T/+* adult flies were embedded into collagen gel and cultured under LD cycles for 28-32 hours prior to recording. Normal Schneider's medium was switched to medium supplemented with 1 mM luciferin for 1-2 hours prior to the recording. Cultures were incubated in a temperature-controlled (24±1 ^{o}C) luminometer (Turner Designs TD-20/20, Promega). The bioluminescence signals were measured at intervals of 0.2 seconds and integrated every 3 minutes. (**a**) High amplitude *per-luc* oscillations were observed at the beginning of recordings, which rapidly dampened under constant darkness. (**b**) Re-exposure to 12 hours of light (yellow bar) regained *per-luc* oscillations almost to first circadian cycle levels. Each trace denotes a *per-luc* rhythm from an individual whole tissue culture.



Supplementary Figure S5. Quantification of *Drosophila cry* mRNA levels. Control (*cry*$^{+}$) and *cry*^{01} mutant flies were entrained in 12:12 hour LD cycles and tissues from these flies were collected during the light period. RNAs were extracted from 30 prepupal RGs, 10 adult brains, and 10 adult Malpighian tubules (MTs). Isolated tissues were washed three times in PBS, pooled into 1.5 mL tubes, and then added to RNA stabilization solution (RNA *later*, Sigma-Aldrich). Total RNA was isolated using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA samples (1 µg) were reverse-transcribed using a Quantitect reverse transcription kit (Qiagen) with standard protocols. Quantitative real-time PCR was performed using the Rotor-Gene real-time PCR instrument (RG-3000A, Corbett Research, Mortlake, Australia) and a 2×Rotor-Gene SYBR Green kit (Qiagen), using the following cycling protocol: a 95 ℃ denaturation step for 5 min, followed by 60 cycles of 95 ℃ denaturation (5 sec), 60 ℃ annealing and extension (10 sec). Data were analyzed and quantified with the Rotor-Gene analysis software. *Ribosomal protein 49* (*rp49*) was used as the internal control. Sequences of primers were: *cry* forward 5′-CACCGCTGACCTACCAAA-3′, *cry* reverse 5′-GGTGGAAGCCCAATAATTTGC-3′; *rp49* forward 5′-CTGCCCACCGGATTCAAG-3′, *rp49* reverse 5′-CGATCTCGCCGCAGTAAAC-3′ (Benito et al., *J Biol Rhythms* 23: 296-307, 2008). Each sample was analyzed in duplicate in 4 separate tubes. (**a**) The real-time PCR amplification plots obtained in a single representative experiment for RGs, brains, and MTs. (**b**) Relative *cry* mRNA abundance was analyzed using the levels of *rp49* expression. The averaged *cry* expression in RGs was only 14.4% of that in brains and 2.7% of that in MTs in the *cry*$^{+}$ flies. Tissues dissected from *cry*^{01} mutants all displayed negligible levels of *cry* expressions in these assays.



Supplementary Figure S6. Intracellular Ca^{2+} dynamics Ca^{2+} from a *tim-gal4/UAS-cameleon* (*YC2.1-82*) fly. All traces are Representatives obtained from two different PG cell types. (**a**) Effects of thapsigargin (TG) on $[Ca^{2+}]_c$ dynamics in PG cells in standard extracellular buffer containing 5.4 mM $CaCl_2$ (as in Schneider's medium). A 1-minute exposure to TG (1 µM) evoked long-lasting increases in $[Ca^{2+}]_c$ and this triggered irreversible cellular swelling. Therefore, to prevent cell swelling, TG treatments were examined under 0 mM $CaCl_2$ (for depletion of internal Ca^{2+} stores) and subsequently under regular extracellular buffer containing 5.4 mM $CaCl_2$ (Fig. 6a). (**b**) Lack of an effect with the store-operated Ca^{2+} channel blocker SKF96365 on Ca^{2+} influx in PG cells in the CNS-RG complex. A Ca^{2+} rise was evoked by pretreatment with 1 µM TG in Ca^{2+}-free conditions and a subsequent switch back to the regular extracellular buffer containing 5.4 mM $CaCl_2$. A rebound increase in $[Ca^{2+}]_c$ was evoked similarly in the absence (*upper*) or presence of 50 µM SKF96365 (*lower*).