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Circadian oscillations outside the optic lobe in the cricket *Gryllus bimaculatus*

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ABSTRACT

Although circadian rhythms are found in many peripheral tissues in insects, the control mechanism is still to be elucidated. To investigate the central and peripheral relationships in the circadian organization, circadian rhythms outside the optic lobes were examined in the cricket *Gryllus bimaculatus* by measuring mRNA levels of *period* (*per*) and *timeless* (*tim*) genes in the brain, terminal abdominal ganglion (TAG), anterior stomach, mid-gut, testis, and Malpighian tubules. Except for Malpighian tubules and testis, the tissues showed a daily rhythmic expression in either both *per* and *tim* or *tim* alone in LD. Under constant darkness, however, the tested tissues exhibited rhythmic expression of *per* and *tim* mRNAs, suggesting that they include a circadian oscillator. The amplitude and the levels of the mRNA rhythms varied among those rhythmic tissues. Removal of the optic lobe, the central clock tissue, differentially affected the rhythms: the anterior stomach lost the rhythm of both *per* and *tim*; in the mid-gut and TAG, *tim* expression became arrhythmic but *per* maintained rhythmic expression; a persistent rhythm with a shifted phase was observed for both *per* and *tim* mRNA rhythms in the brain. These data suggest that rhythms outside the optic lobe receive control from the optic lobe to different degrees, and that the oscillatory mechanism may be different from that of *Drosophila*.

1. Introduction

Insects show rhythmic lives to adapt daily cyclic environment (Saunders, 2002). Those daily rhythms are controlled by an endogenous mechanism called the circadian clock. The locus of the clock has been identified in some insects. For example, it is the optic lobe in crickets (Tomioka and Chiba, 1992), cockroaches (Page, 1982), and beetles (Balkenohl and Weber, 1981), but it is within the central brain in flies (Handler and Konopka, 1979), mosquitoes (Kasai and Chiba, 1987) and moths (Truman, 1974). In addition to these central clock tissues regulating activity rhythms, peripheral tissues also show some rhythms. The compound eyes have a circadian rhythm in their responsiveness to light in crickets (Tomioka and Chiba, 1982a) and cockroaches (Wills et al., 1985), and sperm release from testis to the vas deferens in moths occurs in a circadian rhythmic manner (Giebultowicz et al., 1989). Antennal odor sensitivity is also under regulation of the circadian clock in cockroaches and flies (Krishnan et al., 2001; Page and Koelling, 2003). Cuticle secretion also occurs in a rhythmic manner in cockroaches (Wiedenmann et al., 1986) and flies (Ito et al., 2008).

The relationship between the central and peripheral clocks has been investigated in only limited species, however. In *Drosophila*, the peripheral oscillators are almost independent from the central clock, and they show a persistent rhythm even after being isolated and cultured *in vitro* (Plautz et al., 1997). *Drosophila* Malpighian tubules transplanted to a host, which has a rhythm with an antiphase relationship to the donor, maintained its own phase for a long period (Giebultowicz et al., 2000). On the other hand, the cockroach antennal response to odors and compound eye sensitivity to light are apparently controlled by the central clock (Wills et al., 1985; Page and Koelling, 2003). However, the mechanisms through which the peripheral rhythm is regulated by the central clock are still largely unknown.

In this study, we have investigated circadian rhythms in tissues beyond the optic lobe in the cricket using mRNA expression of the clock genes, *period* (*Gryllus bimaculatus period*, *Gb'per*) and *timeless* (*G. bimaculatus timeless*, *Gb'tim*), which are major players in the oscillatory mechanism of *Drosophila* (cf. Stanewsky, 2002; Hardin, 2006). We have also examined the relationship between rhythms outside the optic lobe and the central clock in the optic lobe. We found that some tissues show rhythmic expression of the clock genes both under a light–dark cycle and under constant darkness. After optic lobe removal, the brain showed a persistent rhythm of both *Gb'per* and *Gb'tim* mRNAs, while mid-gut and terminal abdominal ganglion (TAG) maintained only *Gb'per* mRNA rhythm and anterior stomach lost both rhythms. These data suggest that the rhythms outside the optic lobe are controlled by the optic lobe to different degrees, and that the oscillatory mechanism may be different from that of *Drosophila*.

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2. Materials and methods

2.1. Animals

Adult male crickets, *G. bimaculatus*, were used. They were purchased or obtained from a laboratory colony maintained under standard environmental conditions with a light dark (LD) cycle of 12 h light to12 h dark (light: 06:00–18:00 h; Japanese standard time) at a constant temperature of 25 ± 0.5 °C. They were fed laboratory chow and water.

2.2. RNA preparation and reverse transcription

Total RNA was extracted and purified from 5 adult male brains, anterior stomachs, mid-guts, Malpighian tubules, testes and 10 adult male terminal abdominal ganglia (TAG) with ISOGEN (Nippon Gene, Tokyo, Japan) or TRIzol (Invitrogen, Carlsbad, CA). To remove contaminating genomic DNA, total RNA was treated with DNase I. About 500 ng of total RNA of each sample was reverse transcribed with random 6mers using Primescript RT reagent kit (Takara, Ohtsu, Japan).

2.3. Detection of mRNAs in the neural and peripheral tissues

RT-PCR was used to investigate whether clock genes were expressed in the male brain, anterior stomach, mid-gut, Malpighian tubule, testis and TAG. PCR was performed with Gb-*per*-F1 (5′-AAGCAAGCAAGCATCCTCAT-3′) and Gb-*per*-R1 (5′-CTGAGAAAGGAGGCCACAAG-3′) primers for *Gb'per* (GenBank/EMBL/DDBJ accession no. AB375516), Gb-*tim*-F1 (5′-GCGGTATTTAGAAGGCCACACAGTAA-3′) and Gb-*tim*-A2048 (5′-CAGCTCCAATTCCAGTTGTG-3′) for *Gb'tim* (GenBank/EMBL/DDBJ accession no. AB548625; Danbara, Sakamoto, Uryu and Tomioka, submitted), and Gb-*Rpl18a*-195F (5′-GCTCCGGATTACATCGTTGC-3′) and Gb-*Rpl18a*-339R (5′-GCCAAATGCCGAAGTTCTTG-3′) for *rpl18a* (GenBank/EMBL/DDBJ accession no. DC448653), a housekeeping gene. PCR amplifications of the synthesized cDNA were performed for 30 cycles according to the following schedule: 94 °C for 30 s, 63.8 °C for 30 s, and 72 °C for 1 min for *Gb'per*, 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 2 min for *Gb'tim*, and 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 1 min for *rpl18a*. The PCR products were analyzed by electrophoresis. The amounts of PCR products detected were quantified by ImageJ (http://rsb.info.nih.gov/nih-image/).

2.4. Measurement of mRNA levels

Quantitative real-time RT-PCR (qPCR) was performed to measure mRNA levels by Mx3000P real-time PCR System (Stratagene, La Jolla, CA) using FastStart Universal SYBR Green Master (Roche, Tokyo, Japan) including SYBR Green with Gb-*per*-F1 and Gb-*per*-R1 primers for *Gb'per*, Gb-*tim*-A1 (5′-CATCCGTTGACCCTATTTGC-3′) and Gb-*tim*-S1 (5′-GACGAATGCATTTGGTTGTG-3′) for *Gb'tim*, Gb-*Rpl18a*-195F and Gb-*Rpl18a*-339R for *rpl18a*. Standard curves for the transcripts were generated by serial (10×) dilutions of amplified cDNAs and included in each real-time PCR run. After 40 cycles, samples were run for melting curve analysis, and in every case, a single expected amplicon was confirmed. The results were analyzed using software associated with the instrument. The values were normalized with the values of *rpl18a* at each time point as described previously (Moriyama et al., 2008, 2009). Four independent experiments were used to calculate the mean ± SEM.

2.5. Surgical operation

Animals were collected from the colony chamber between 10:00 (Zeitgeber time (ZT) 4; ZT0 corresponds to light-on and ZT12 corresponds to light-off) and 14:00 (ZT8) for operation. We surgically removed the outer two neuropils of the optic lobe, the lamina and medulla (hereafter referred to as the "optic lobe"), as follows. An incision was made along the margin of the compound eye, and the eye capsule was pried open so that the optic lobe and optic nerves were exposed. Then the optic stalk and optic nerves were cut with a pair of fine scissors to remove the optic lobe. The eye capsule was then put back into the original position. The operation was made under CO_2 anesthesia. The whole procedure took about 5 min. The post-operative animal was kept in a plastic container and placed in the rearing room under LD 12:12 at 25 °C until use.

2.6. Statistical analysis

Significance of the daily and circadian rhythmicity in mRNA expression was examined and the acrophase was determined by the single cosiner method (Nelson et al., 1979) fitting a cosine curve of 24 h period using Time Series Analysis Serial Cosiner 6.3 (Expert Soft Technologie Inc.). For DD experiments, data starting at CT2 on the first day of DD were used for analysis.

3. Results

3.1. Expression of Gb'per and Gb'tim mRNA in tissues outside the optic lobe

We first examined expression of *Gb'per* and *Gb'tim* mRNA in tissues outside the optic lobe. Total RNAs were extracted from the

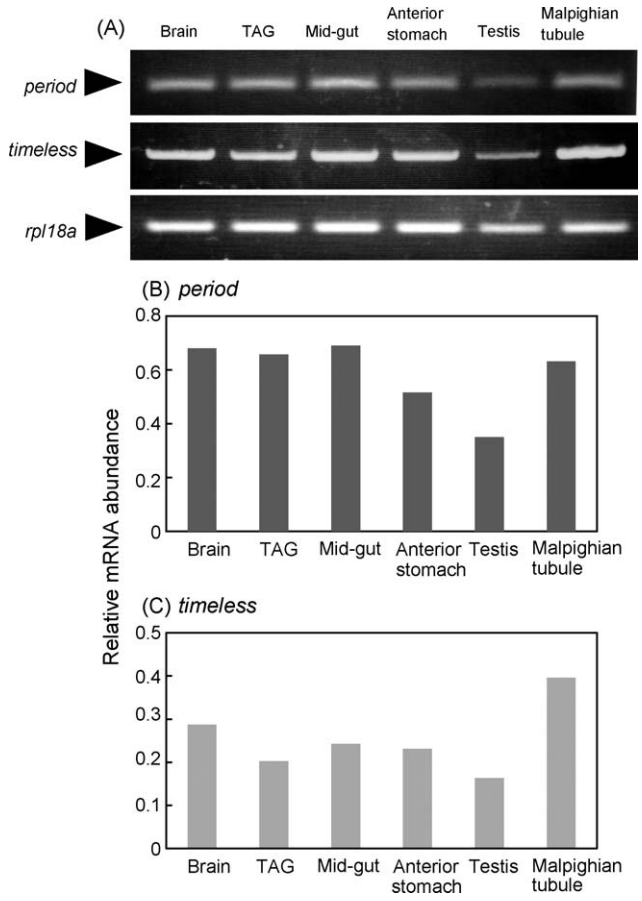

Fig. 1. Expression of *Gb'per* and *Gb'tim* mRNA in the adult brain, terminal abdominal ganglion (TAG), testis, Malpighian tubules, anterior stomach, and mid-gut of the cricket *Gryllus bimaculatus* kept under LD 12:12, 25 °C. (A) RT-PCR was performed with 22.5 ng total RNA extracted from the tissues at ZT14. (B) and (C) show relative amount of *Gb'per* (B) and *Gb'tim* (C) mRNA in each tissue to that of *rpl18a* mRNA.