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The Compound Eye Possesses a Self-sustaining Circadian Oscillator in the Cricket *Gryllus bimaculatus*

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Many insects show daily and circadian changes in morphology and physiology in their compound eye. In this study, we investigated whether the compound eye had an intrinsic circadian rhythm in the cricket *Gryllus bimaculatus*. We found that clock genes *period* (*per*), *timeless* (*tim*), *cryptochrome 2* (*cry2*), and *cycle* (*cyc*) were rhythmically expressed in the compound eye under 12-h light/12-h dark cycles (LD 12:12) and constant darkness (DD) at a constant temperature. After the optic nerves were severed (ONX), a weak but significant rhythmic expression persisted for *per* and *tim* under LD 12:12, while under DD, *tim* and *cyc* showed rhythmic expression. We also found that more than half of the ONX compound eyes exhibited weak but significant circadian electroretinographic rhythms. These results clearly demonstrate that the cricket compound eye possesses an intrinsic circadian oscillator which can drive the circadian light sensitivity rhythm in the eye, and that the circadian clock in the optic lobe exerts its influence on the oscillator in the eye.

Key words: circadian oscillator, clock genes, compound eye, cricket, ERG rhythms

INTRODUCTION

Circadian rhythms are roughly 24-hr rhythms that play roles in various physiological and behavioral functions of insects, including antennal olfaction (Krishnan et al., 2008; Saifullah and Page, 2009), sensitivity in the visual system, cuticular deposition (Ito et al., 2008; Weber, 1995), adult eclosion (Ito and Tomioka, 2016), and locomotion (Page, 1982; Tomioka and Chiba, 1984). These rhythms are controlled by circadian clocks located in the central clock tissue and/or peripheral tissues (Tomioka and Matsumoto, 2010, 2019; Tomioka et al., 2012). In the cricket *Gryllus bimaculatus*, the central circadian clock is localized in the lamina-medulla complex of the optic lobe (Tomioka and Chiba, 1992).

The oscillatory mechanism of circadian clocks has been studied in some insects, including the fruit fly *Drosophila melanogaster* and *G. bimaculatus* (Tomioka and Matsumoto, 2015, 2019). At the molecular level, this clock is characterized by the cyclic expression of the clock genes *per* and *tim* (Tataroglu and Emery, 2015; Tomioka and Matsumoto, 2019). Their expression is thought to be transcriptionally regulated by the transcription factors CLOCK (CLK) and CYCLE (CYC). CLK and CYC form a heterodimer and bind to the E-box in the promoter region of *per* and *tim* to activate their transcription. The mRNAs are translated to PER and TIM proteins in the cytoplasm, and these proteins form a PER/TIM heterodimer, enter the nucleus, and suppress their own genes' transcription through the inactivation of CLK/CYC. In many insects, including *G. bimaculatus*, *cyc* is rhythmically expressed (Uryu et al., 2013; Tomioka and Matsumoto, 2019), while in *D. melanogaster*, not *cyc* but *Clk* is rhythmically expressed (Glossop et al., 1999). In addition to these, *cry2* and *cry1* are also involved in the clock mechanism of *G. bimaculatus*, forming an oscillatory loop separate from the *per*/*tim* loop (Tokuoka et al., 2017). Unlike their roles in crickets, *cry1* plays an essential role in photic entrainment in *D. melanogaster* and the monarch butterfly, *Danaus plexippus* (Stanewsky et al., 1998; Zhu et al., 2008), while *cry2* is an important component in the core oscillatory loop acting with *per* in the honey bee, *Apis mellifera*, and in *D. plexippus* (Rubin et al., 2006; Zhu et al., 2008; Lugena et al., 2019).

In insects, rhythmic expression of clock genes has been reported in various tissues (Plautz et al., 1997; Giebultowicz, 2000; Uryu and Tomioka, 2010). Among them, the compound eyes are the most profoundly studied. They show daily and circadian changes in their sensitivity to light (Koehler and Fleissner, 1978; Fleissner, 1982). Morphological and physiological changes probably cause this rhythm. For example, in many arthropods, the size of the rhabdom, a photoreceptive structure composed of the microvilli of photoreceptor cells, increases during night and the pigment granules in retinular cells migrate to increase light-capturing efficiency (Arikawa et al., 1988; Meyer-Rochow, 1999). It is also known that in *D. melanogaster*, the compound eye possesses an autonomous circadian clock in which clock genes are cyclically expressed (Cheng and Hardin, 1998; Damulewicz et al., 2015). However, in other insects, whether the compound eye possesses its own oscillator has not

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been examined, and more importantly, how the rhythm in the compound eye is regulated by the central clock remains largely unknown.

In this study, we examined whether the compound eye harbored a circadian oscillator and how the central clock exerted its influence on the circadian rhythm of the compound eye in the cricket *G. bimaculatus*. The compound eye of this cricket shows circadian changes in light sensitivity as measured by electroretinogram (ERG) (Tomioka and Chiba, 1982; Tomioka, 1985). With quantitative RT-PCR, we showed that the clock genes were rhythmically expressed in the compound eye under LD 12:12 and DD, and that the rhythm persisted, at least in some of the clock genes, albeit with a reduced amplitude even after the optic nerves were severed. We also demonstrated that more than half of the compound eyes of which the optic nerves were severed (ONX) maintained circadian ERG rhythms. These results suggest that the compound eye of the cricket contains a circadian oscillator, which is also governed by the central clock in the optic lobe.

MATERIALS AND METHODS

Animals

All experiments were performed with 7-10-day-old adult male crickets, *Gryllus bimaculatus*, which were taken from our laboratory colony. They were kept under controlled conditions of 12-h light and 12-h darkness (LD12:12, light: 0600-1800, Japan Standard Time) and at a constant temperature of 25°C ± 1.0°C.

Surgical manipulation

To sever the optic nerves, the dorsal side of the cuticle around the compound eye was cut with a razor knife and the compound eye was lifted with tweezers to expose the optic nerves. The nerves were severed with a pair of micro-scissors, the compound eye was placed in its original position, and the wound was sealed with hemolymph clotting. However, in some cases, especially for ERG recordings, the wound was sealed with a small amount of beeswax-colophony mixture. The dissection was performed between zeitgeber time (ZT; ZT 0 and ZT 12 correspond to lights-on and lights-off, respectively) 3 and 5 under CO_2 anesthesia using a dissecting microscope.

Measurement of RNA levels

Quantitative RT-PCR (qPCR) was used for the measurement of mRNA levels of clock genes *Gryllus bimaculatus period* (*Gb'per*, GenBank/EMBL/DDBJ Accession No. BAG48878), *timeless* (*Gb'tim*, BAJ16356), *cryptochrome 2* (*Gb'cry2*, LC202053), *cycle* (*Gb'cyc*, AB762416), and *Clock* (*Gb'Clk*, AB738083). Five untreated and five ONX compound eyes were collected for each condition, i.e., LD 12:12 and DD, and at each sampling time, i.e., ZT or circadian time (CT; CT 0 and CT 12 correspond to subjective light-on and subjective light-off, respectively) 2, 6, 10, 14, 18, and 22. A total of 60 crickets were used. The optic nerves of the crickets were unilaterally cut between ZT 3–4, and the ONX and untreated compound eyes were collected separately. Under LD 12:12, the sampling was started at ZT 6 on the day of operation. For sampling under DD, the crickets were transferred to DD at ZT 12, and the sampling was performed under dim red light, starting at CT 6.

Total RNA was extracted and purified from a single compound eye of adult male crickets using the TRIzol Reagent (Invitrogen, Carlsbad, AC, USA). Contaminating genomic DNA was removed by treating the total RNA with DNase I (Invitrogen). Approximately 450 ng of total RNA of each sample was reverse transcribed with ran-

Table 1. PCR primers used for quantitative RT-PCR.

Primers	Forward	Reverse
Gb'per	5'-AAGCAAGCAAGCATCCTCA T-3'	5'-CTGAGAAAGGAGGCCACA AG-3'
Gb'tim	5'-GATTATGAAGTCTGTGATGA TTGG-3'	5'-AGCATTGGAGAGAACTGA AGAGGT-3'
Gb'cry2	5'-AGCACCATCACACACTTCA CA-3'	5'-ACACTCAGCGCAATCCAC AC-3'
Gb'Clk	5'-AATGACCGTAGTCGAGAAA GTGAAG-3'	5'-TTGCGATGATTGAGGTTGT TG-3'
Gb'cyc	5'-GGCCGAAGCTCATAAAGTG G-3'	5'-AACCGCACAAAGGAACCA TC-3'
Gb'rpl18a	5'-GCTCCGGATTACATCGTTG C-3'	5'-GCCAAATGCCGAAGTTCTT G-3'

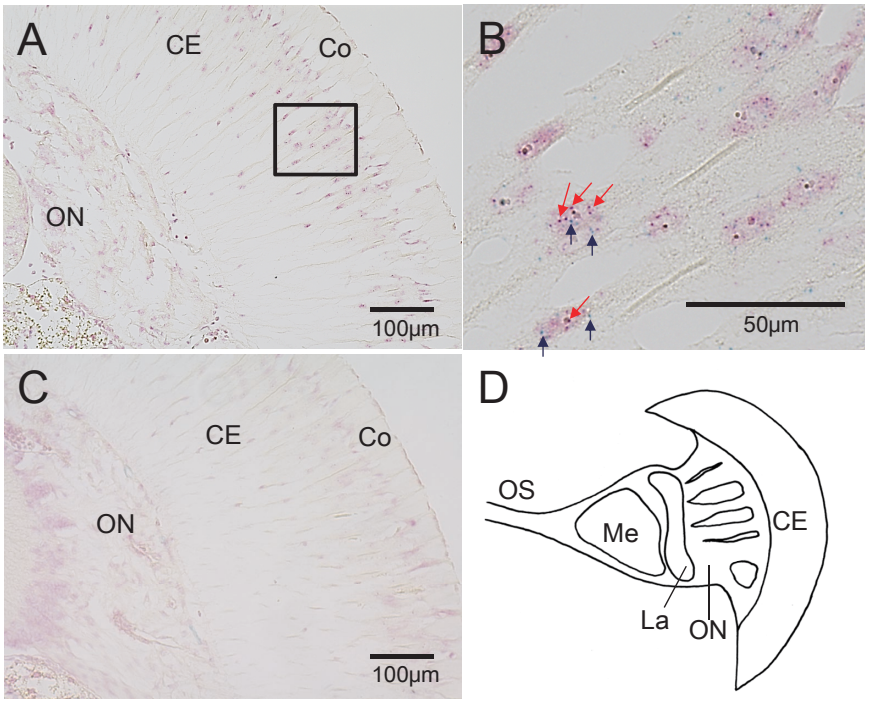

Fig. 1. In situ hybridization of *Gb'per* (red) and *Gb'cry2* (blue) in the compound eye at ZT 18 in the cricket *Gryllus bimaculatus*. **(B)** shows magnification of the area indicated by a square in **(A)**. Some of the *Gb'per* and *Gb'cry2* signals are indicated by red and blue arrows, respectively. Retinal cells co-express *Gb'per* and *Gb'cry2* and their signals are mostly located in and around the nucleus. **(C)** shows the negative control stained without probes. **(D)** illustrates the anatomical structure of the compound eye and a part of the optic lobe, including lamina and medulla, locus of the central circadian clock. CE, compound eye; Co, cornea; La, lamina; Me, medulla; ON, optic nerves; OS, optic stalk. For further explanation, see the text. Scale bars: 100 μm in **(A, C)**, 50 μm in **(B)**.

dom hexamers using the PrimeScript RT reagent kit (Takara, Otsu, Japan). qPCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and a KAPA SYBR FAST qPCR Kit (NIPPON Genetics, Tokyo, Japan), including SYBR Green, with gene-specific primers (Table 1). The qPCR conditions were as follows: 95°C for 20 s and then 40 cycles of 95°C for 3 s, and 60°C for 30 s with 0.4 μM concentration of each primer. The results were analyzed using the software associated with the instrument. The values were normalized with the values of *Gb'rpl18a*, a housekeeping gene. During these treatments, some of the samples were lost, and values obtained from 2–5 samples were pooled to calculate the mean ± SEM.

In situ hybridization

In situ hybridization (ISH) was performed according to the method described by Kutaragi et al. (2018) using 3 adult male crickets collected at ZT 18, at which the mRNA levels of the clock genes *Gb'per* and *Gb'cry2* were expected to be at or near peak levels (Moriyama et al., 2008; Tokuoka et al., 2017). Briefly, the collected cricket heads were fixed with 4% PFA solution for 24 h at 4°C, dehydrated by a series of butyl alcohol and ethanol, and embedded in paraffin. Tissues were sectioned at 6 μm thickness and mounted on slides. ISH was performed using ViewRNA ISH Tissue Assay (Affymetrix, Santa Clara, CA), following the manufacturer's instructions. To unmask the RNA targets, tissue sections were deparaffinized and incubated in pre-treatment buffer at 90–95°C for 10 min and digested with protease (Affymetrix, QVT1102) (1:100 dilution) at 40°C for 10 min, followed by fixation with 10% neutral buffered formalin at room temperature for 5 min. The unmasked tissue sections were subsequently hybridized with the ViewRNA probe set (1:50 dilution) for 2 h at 40°C, followed by a series of post-hybridization washes. The ViewRNA probes used for detecting *Gb'per* and *Gb'cry2* were designed and synthesized by Affymetrix, covering the 1027–2016 and 1548–2669 base regions, respectively. A non-probe sample was utilized as a negative control. Signal amplification was achieved via a series of sequential hybridizations and washes according to the manufacturer's instructions. Signals for *Gb'per* and *Gb'cry2* were detected with Fast Red and Fast Blue substrate, respectively. Slides were post-fixed in 10% neutral buffered formalin, mounted with Dako Ultramount mounting medium (Dako, Carpinteria, CA), observed, and photographed using light microscopy (BZ-X700, KEYENCE, Osaka, Japan).

Recording of ERG

For recording ERG, a compound electrical response generated by various retinal cells in response to a light stimulus, crickets,

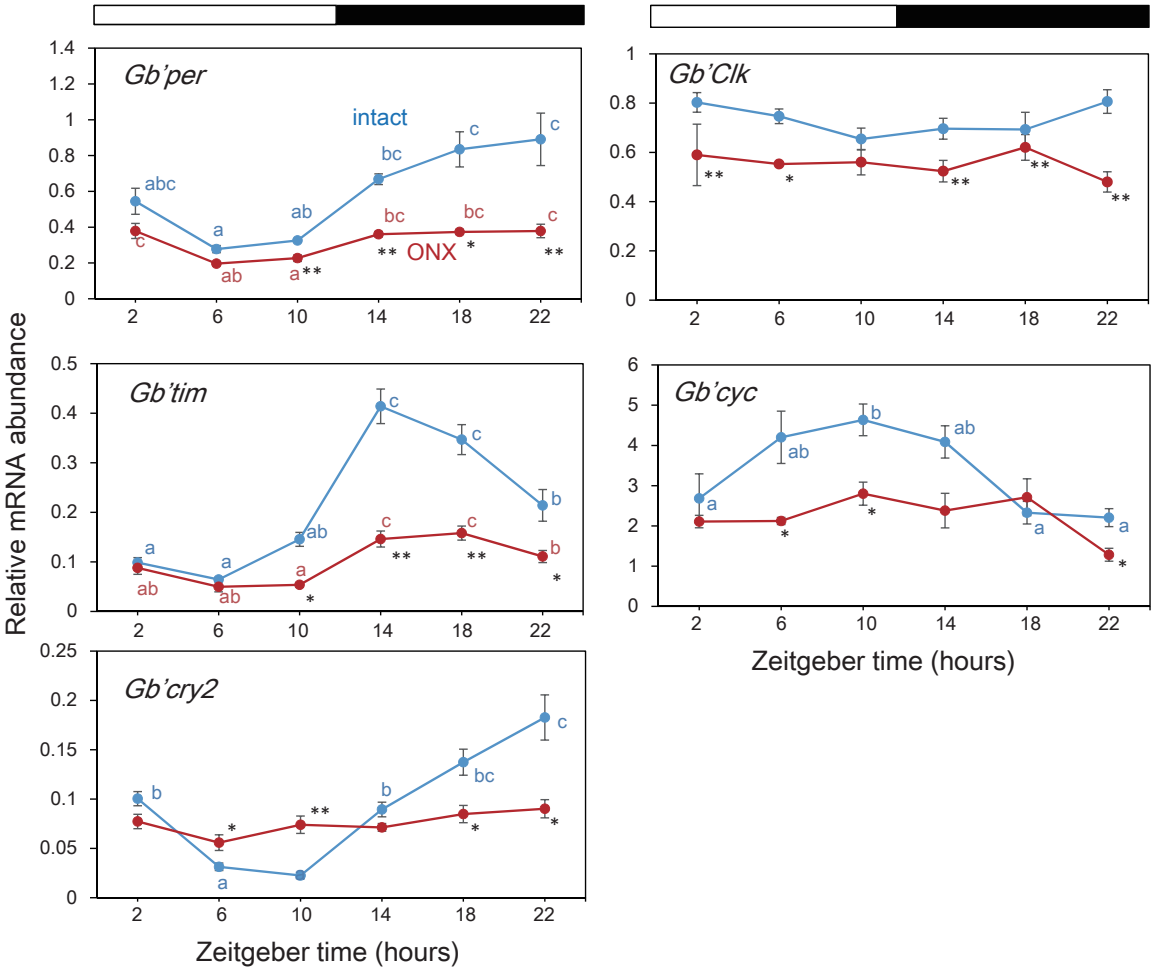

Fig. 2. Daily expression profiles of clock genes, *Gb'per*, *Gb'tim*, *Gb'cry2*, *Gb'cyc*, and *Gb'Clk*, in the compound eye of the cricket *Gryllus bimaculatus* under light-dark cycles. Blue, intact eyes; red, eyes with severed optic nerves (ONX). Error bars indicate SEM. Black and white bars above the panels indicate light (white) and dark (black) conditions. Different lower-case letters indicate that the values were significantly different from each other (Tukey-test, $P < 0.05$). Asterisks indicate that the values were significantly different from those of intact controls (*$P < 0.05$, **$P < 0.01$, *t*-test). For further explanation, see the text.

whose legs were amputated, were fixed to a supporting rod, and enamel-insulated Ag wire electrodes (ϕ 200 μm) were chronically implanted into the immediate vicinity of the receptor layer of their compound eyes. The apparatus was arranged so that the ERGs elicited by a 400 ms flash of green light (λ = 525 nm) at intervals of 1 h were recorded automatically. The flash was given by a green light emitting diode (LK-5PG, LED & Application Technologies, China) driven by an electronic stimulator (SEN-3301, Nihon Kohden, Tokyo), and the stimulus intensities were 3.35 μW/cm^2, which were below saturation for the ERG. Electrical signals were amplified by a biophysical amplifier (MEG-2100, Nihon Kohden), and monitored with an oscilloscope (2211, Tektronix, Tokyo). The signals were collected and analyzed on an IBM computer using data acquisition hardware (CED1401, Cambridge Electronic Design Ltd., Cambridge, UK) and software (Spike II, Cambridge Electronic Design Ltd.). Amplitudes of the on-component of ERGs were measured. The ERG recording was started around ZT 5–6 and the light was turned off at ZT 12 to record the ERG under DD. The recording was continued for at least 48 h and up to 120 h.

Statistical analysis

To detect daily or circadian fluctuations of mRNA levels, the one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's test was used to compare values at different time points within an identically treated group of crickets. Since ANOVA detects significant variation between time points irrespective of rhythmicity, the CircWave (ver. 1.4) program (available at https://www.euclock.org/results/item/circ-wave.html), which uses the *F*-test for significance, was used to confirm significant daily or circadian rhythmicity. Rhythmicity was considered to exist when both ANOVA and CircWave detected significant fluctuations. When the daily fluctuation was detected either by ANOVA or by CircWave, it was judged to be pseudo-rhythmic. For comparison between intact and optic nerve severed compound eyes, the *t*-test was used.

To detect rhythmicity in the ERG recordings, we removed a long-term trend estimated by linear regression and the resultant time series were analyzed with the CircWave to detect any significant circadian rhythmicity.

For all statistical tests, the significance level was set at $P < 0.05$.

RESULTS

Clock gene expression in the compound eye

To examine whether the clock genes were expressed in the compound eye, we performed an in situ hybridization (ISH) of *Gb'per* and *Gb'cry2* with the adult compound eyes sampled in the middle of the night (ZT 18). As shown in Fig. 1, the expression of both *Gb'per* and *Gb'cry2* was detected and the transcripts were most abundant in and around the nucleus. These results showed that *Gb'per* was more abundantly expressed than *Gb'cry2*.

Daily expression profiles of clock genes under LD 12:12 and DD

We examined the daily expression patterns of the clock genes *Gb'per*, *Gb'tim*, *Gb'cry2*, *Gb'Clk*, and *Gb'cyc* under LD 12:12. Except for *Gb'Clk*, they all showed clear daily expression rhythms (Fig. 2). *Gb'per*, *Gb'tim*, and *Gb'cry2* all had a trough during the day and a peak during the night, although the peak occurred slightly earlier for *Gb'tim*. *Gb'cyc* showed a peak during mid-day to late-day, while *Gb'Clk* showed a peudo-rhythmic pattern with a significant rhythm detected only by CircWave (Table 2). The expression level of *Gb'per* at ZT 18 was approximately 6-fold higher than that of *Gb'cry2*, being consistent with the above-mentioned ISH results.

Under DD, similar rhythmic profiles were observed in *Gb'per*, *Gb'tim*, *Gb'cry2*, and *Gb'cyc*, while *Gb'Clk* showed no significant rhythms (Fig. 3, Table 2). These expression profiles are similar to those found in the clock tissue, i.e., optic lobes (Moriyama et al., 2008, 2012; Danbara et al., 2010; Uryu et al., 2013).

Effects of optic nerve severance on the expression of the clock genes

To examine whether the rhythmic expression of the clock genes was intrinsic in the compound eye, we measured the mRNA expression profiles in the compound eyes which were isolated from the optic lobe by cutting the optic nerves. Under LD 12:12, for all the clock genes measured except *Gb'cry2*, mRNA levels were significantly reduced, below the lowest level in the intact control at most time points (Fig. 2). For *Gb'cry2*, the mRNA levels stayed at almost constant levels, intermediate between trough and peak, in the control. Only *Gb'per* and *Gb'tim* maintained rhythmic expressions, with a peak during the night, like in intact controls. However, the other three genes, *Gb'cry2*, *Gb'Clk*, and *Gb'cyc* were arrhythmically expressed.

Under DD, expression levels of the clock genes in the ONX compound eyes were similar to those found under LD 12:12 (Fig. 3). *Gb'cry2* mRNAs showed an intermediate level between trough and peak of the intact control eyes like in LD 12:12, while other clock genes showed significantly reduced

Table 2. Results of statistical analyses of daily clock gene expression in the compound eyes of *Gryllus bimaculatus* with intact or severed optic nerves (ONX) under 12-h light/12-h dark cycle (LD 12:12) or constant darkness (DD). R: rhythmic, PR: pseudo-rhythmic, ND: no significant rhythm was detected.

Clock genes	Treatment	Condition	ANOVA			CircWave	Rhythm
			d.f.	*F*	*P*	*P*	
Gb'per	intact	LD	5, 23	9.359	<0.001	<0.001	R
	intact	DD	5, 22	3.239	0.0243	0.0012	R
	ONX	LD	5, 19	6.097	0.0016	<0.001	R
	ONX	DD	5, 22	0.988	0.4475	>0.05	ND
Gb'tim	intact	LD	5, 22	31.966	<0.001	<0.001	R
	intact	DD	5, 20	11.990	<0.001	<0.001	R
	ONX	LD	5, 20	11.538	<0.001	<0.001	R
	ONX	DD	5, 20	3.517	0.0192	0.0085	R
Gb'cry2	intact	LD	5, 22	24.745	<0.001	<0.001	R
	intact	DD	5, 22	7.050	<0.001	<0.001	R
	ONX	LD	5, 18	1.609	0.2083	>0.05	ND
	ONX	DD	5, 21	0.837	0.5385	>0.05	ND
Gb'cyc	intact	LD	5, 23	5.983	0.0011	<0.001	R
	intact	DD	5, 17	5.739	0.0028	<0.001	R
	ONX	LD	5, 20	2.516	0.0637	>0.05	ND
	ONX	DD	5, 22	3.997	0.0099	<0.001	R
Gb'Clk	intact	LD	5, 20	1.601	0.2055	0.0370	PR
	intact	DD	5, 22	0.230	0.9456	>0.05	ND
	ONX	LD	5, 21	0.517	0.7604	>0.05	ND
	ONX	DD	5, 22	2.205	0.0903	>0.05	ND

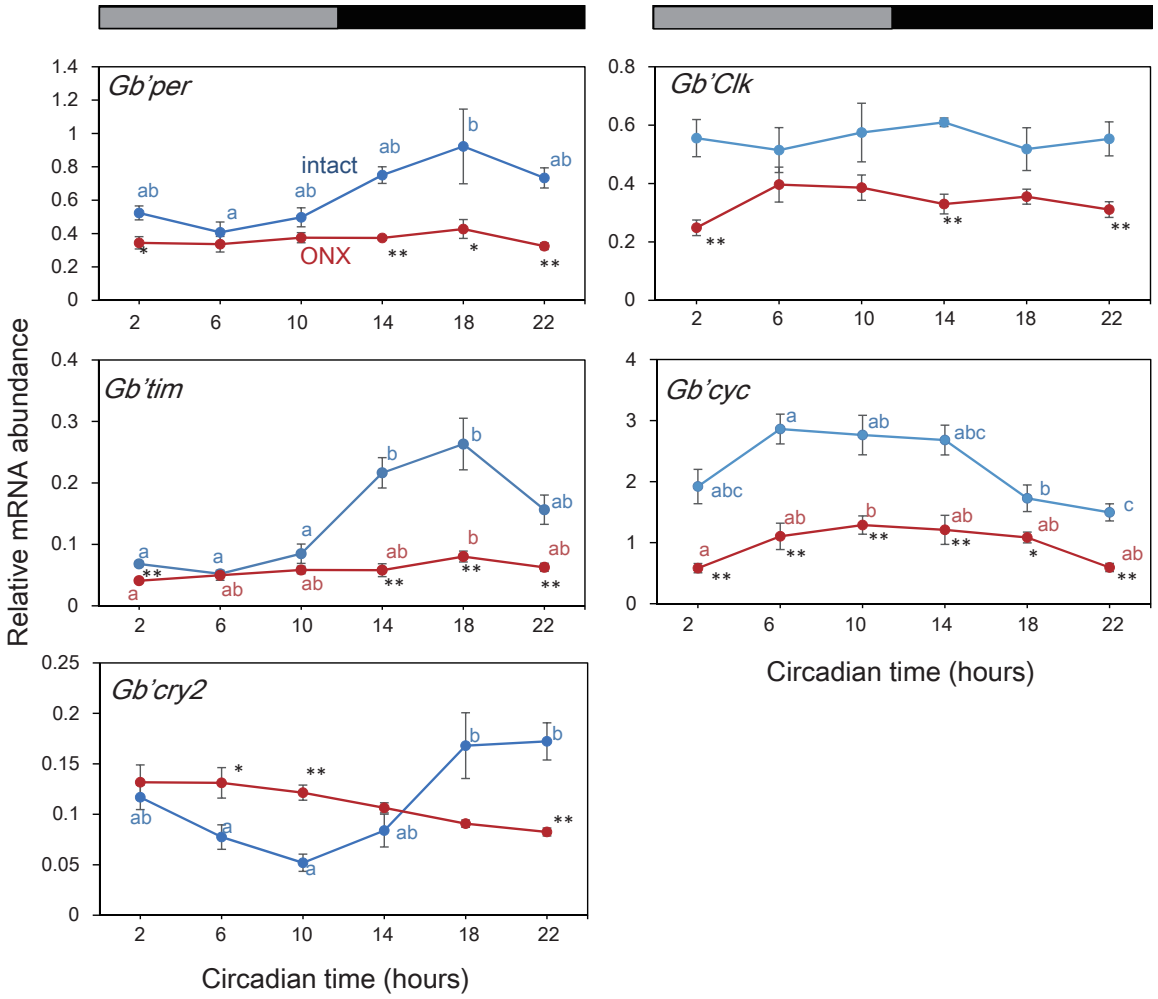

Fig. 3. Circadian expression profiles of clock genes, *Gb'per*, *Gb'tim*, *Gb'cry2*, *Gb'cyc*, and *Gb'Clk*, in the compound eye of the cricket *Gryllus bimaculatus* under constant darkness. Blue, intact eyes; red, eyes with severed optic nerves (ONX). Error bars indicate SEM. Gray and black bars above the panels indicate subjective day and night, respectively. Different lower-case letters indicate that the values were significantly different from each other (Tukey's test, $P < 0.05$). Asterisks indicate that the values were significantly different from those in the intact controls (*$P < 0.05$, **$P < 0.01$, *t*-test). For further explanation see the text.

mRNA levels, below the trough level of the control eyes. While *Gb'per* and *Gb'cry2* lost their rhythmic expression, *Gb'tim* and *Gb'cyc* showed a weak but statistically significant rhythm with a peak at the middle of the subjective night and at late subjective day, respectively (Fig. 3, Table 2).

Circadian ERG rhythms

The finding of weak but significant clock gene expression rhythms in the compound eyes neurally isolated from the optic lobe prompted us to examine whether ERG rhythms persisted after the optic nerves were severed. In *G. bimaculatus*, we have previously shown that the compound eye shows a clear circadian rhythm in amplitudes of ERG (Tomioka and Chiba, 1982; Tomioka, 1985).

We examined ERG rhythms in eight intact compound eyes subjected to DD for at least 48 h and up to 120 h. All of the eyes showed a significant circadian rhythm, with a peak during the subjective night (Fig. 4A). When the optic nerves were severed in 15 compound eyes, six lost the rhythm (exemplified in Fig. 4B) and the remaining nine showed a weak but significant rhythmicity with a peak during the subjective night, as examplified in Fig. 4C. With an additional seven crickets, we examined whether the severed optic nerves were regenerated 5 days post operation and found that there was no apparent indication of regeneration during this short period, as reported for the locust central nervous system (Boulton, 1969). Although we performed no sham operation experiment for the ONX, the weakening of ERG rhythms should be attributable to the ONX because when the optic tract was severed with a similar surgical manipulation, the operated eyes showed normal ERG rhythms (Tomioka and Chiba, 1982).

DISCUSSION

Insect compound eyes have been reported to show rhythmic changes in their morphology and physiology. In the cricket *Gryllus bimaculatus*, the compound eye shows clear circadian changes in its sensitivity, as evidenced by the amplitude of the ERG (Tomioka and Chiba, 1982; Tomioka, 1985), and by morphological changes such as rhabdom size (Sakura et al., 2003). The present study clearly demonstrated that the clock genes were rhythmically expressed in

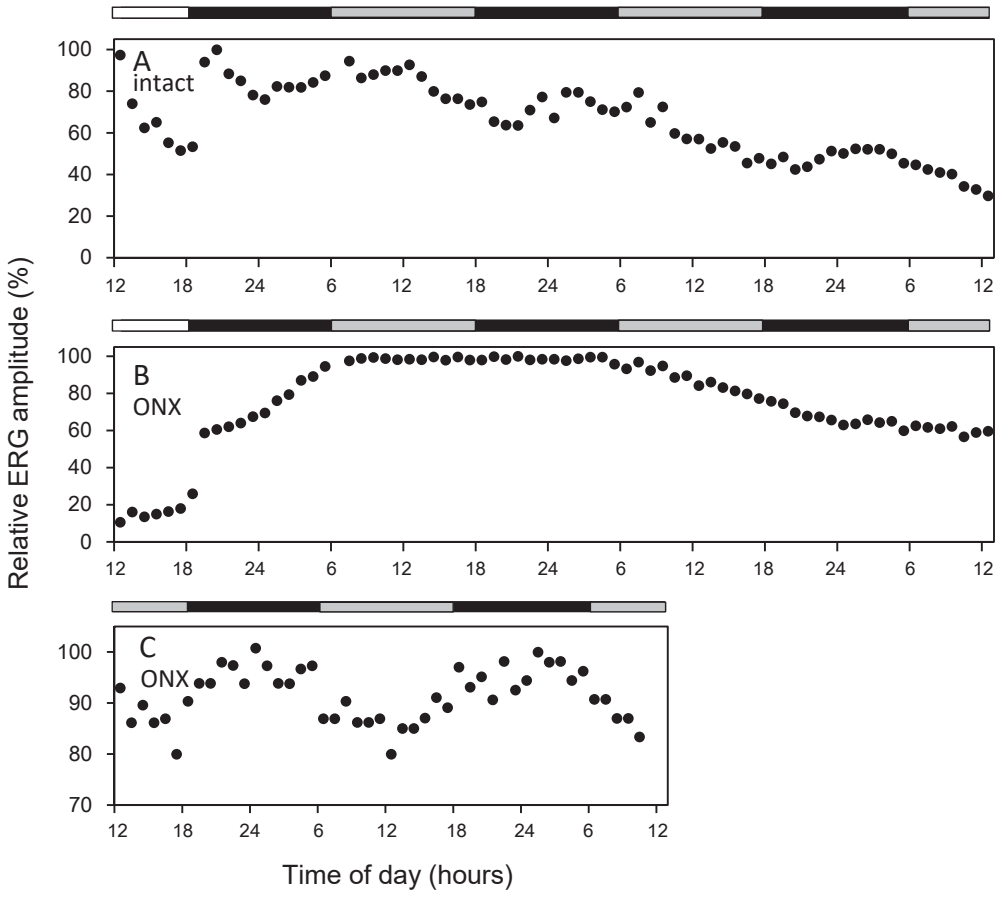

Fig. 4. ERG amplitude rhythms in an intact compound eye **(A)** or compound eyes with severed optic nerves (ONX) **(B, C)** in the cricket *Gryllus bimaculatus*. Recording began in the middle (12:00) of the last light fraction of LD 12:12 in which the animal had been held. In **(C)**, the records for 2nd and 3rd days are shown. Note that the ERG rhythm persisted in the compound eye even when the optic nerve was severed **(C)**. The white, gray, and black bars above panels indicate day, subjective day, and subjective night, respectively. For further explanation, see the text.

the compound eye under LD 12:12 and DD (Figs. 2 and 3), and that their expression patterns were quite similar to those observed in the optic lobe, the cricket's clock tissue (Danbara et al., 2010; Moriyama et al., 2008, 2012; Uryu et al., 2013). Our findings are consistent with those in other insects in that clock genes are rhythmically expressed in the compound eye (Siwicki et al., 1988; Sauman and Reppert, 1996; Damulewicz et al., 2015). This fact suggests that the morphological and physiological rhythms in the compound eye are most likely based on the circadian molecular oscillation, like the behavioral rhythms of *D. melanogaster* caused by circadian molecular oscillation in the cerebral clock neurons (Rieger et al., 2009). In fact, the morphological changes in a fly's lamina monopolar cells are known to be affected by the clock gene *period* (Pyza and Meinerzhagen, 1995).

In the cricket, the molecular oscillation seemed to be largely dependent on the efferent control from the optic lobe, since oscillation was weakened by ONX. This is reminiscent of the earlier reports in the cockroach *Leucophaea maderae* and the carabid beetle *Anthia sexguttata* (Fleissner, 1982; Wills et al., 1985): The retinal sensitivity rhythm as recorded by ERG is fully dependent on the efferent control from the circadian clock located in the optic lobe and is lost when the compound eye is separated from the optic lobe or when a putative clock site in the optic lobe is lesioned.

Our results showed that in the ONX eye, *Gb'per* and *Gb'cry2* lost their rhythmic expression, while weak but significant rhythms persisted in *Gb'tim* and *Gb'cyc* expression under DD, suggesting that there is an intrinsic self-sustaining oscillation within the compound eye. Our findings support the master (pacemaker)-slave clock hypothesis (Pittendrigh, 1981), in that the master clock in the optic lobe governs the slave clock in the compound eye, although the mechanism through which the optic lobe master clock regulates the compound eye slave clock is currently unknown. This master-slave hypothesis is reminiscent of the regulatory systems in cockroach antennal olfactory rhythms and *Drosophila* eclosion rhythms. In the cockroach *L. maderae*, antennal olfactory sensory neurons have their own rhythm, but electroantennogram rhythms are highly dependent on the central clock in the optic lobe (Saifullah and Page, 2009). In *Drosophila*, the cerebral master clock governs the slave clock in the prothoracic gland that controls eclosion (Ito and Tomioka, 2016).

One may argue that the weak rhythmic expression of clock genes in the ONX eye was the result of the desynchronization of retinal clock cells. However, this seems unlikely since the change in mRNA levels occurred soon after the

ONX, and the mRNA levels of the clock genes, except for *Gb'cry2*, were significantly reduced to a level below the trough of intact eyes (Figs. 2 and 3). If the weak rhythms were caused by desynchronization, the mRNA levels would have been at intermediate levels between the peak and the trough.

The weak oscillation in the ONX compound eye probably occurred in the retinal photoreceptor cells, because in situ hybridization revealed *Gb'per* and *Gb'cry2* signals in those cells (Fig. 1). This oscillation may be able to drive the circadian sensitivity rhythm of the photoreceptors in *G. bimaculatus*, since a weak but significant circadian ERG rhythm persisted even after the optic nerves were severed (Fig. 4C). The most important issue to be addressed in future studies is the determination of the mechanism by which the oscillation in retinal cells regulates circadian oscillation in the retinal sensitivity to light. ERG rhythms are known to be associated with morphological changes such as rhabdom size and screening pigment migration (Sakura et al., 2003). Therefore, it should also be examined whether the weak ERG rhythm in the ONX eye is associated with those morphological changes.

Another important issue is why some of the ONX eyes lost the ERG rhythm (Fig. 4B). One possible explanation is that there are inter-individual differences in the strength of the residual circadian oscillation in compound eyes. An alternative explanation may be that the residual circadian oscillation differs between locations in the compound eye, and the detection of the ERG rhythm depends on the location of the electrode. These possibilities should be examined in future studies.

The molecular oscillation in the ONX compound eye was enhanced and *Gb'per* was rhythmically expressed under LD 12:12 (Figs. 2 and 3, Table 2), suggesting that light cycles might amplify or enhance gene expression rhythms. One possible mechanism for this enhancement may be that light-evoked changes in membrane potential affect clock gene expression. Neuronal electrical activity is known to induce some immediate early genes (Watanabe et al., 2018; Takayanagi-Kiya and Kiya, 2019), and *c-fos* is the one that encodes a transcription factor and is known to be involved in the regulation of the clock in *G. bimaculatus* (Kutaragi et al., 2018). It is also notable that electrical activity plays an essential role to maintain circadian rhythmic expression of clock genes in *Drosophila* cerebral clock neurons (Nitabach et al., 2005).

Our results clearly revealed that efferent control from the optic lobe plays an important role in the persistence of normal circadian gene expression in the insect compound eye (Figs. 2 and 3). Severance of optic nerves resulted in reduced levels of *Gb'per*, *Gb'tim*, *Gb'Clk*, and *Gb'cyc* expression, while *Gb'cry2* expression was at an intermediate level between the peak and trough of the intact eyes. This fact suggests that efferent control differentially regulates *Gb'cry2* and other clock genes. This differential control is consistent with our previous finding that *Gb'cry2* forms an oscillatory loop different from the main oscillatory loop consisting of *Gb'per* and *Gb'tim* (Tokuoka et al., 2017). The mechanism through which the optic lobe master clock regulates robust circadian gene expressions should be addressed in future studies.

Finally, we should discuss why the compound eye has its own circadian oscillation. The compound eye is known to serve as the circadian photoreceptor necessary for entrainment of the central clock located in the optic lobe (Tomioka and Chiba, 1984; Tomioka et al., 1990; Komada et al., 2015). The photic information is sent to the clock via a neural pathway to reset its phase in a phase-dependent manner (Okada et al., 1991). The present study revealed that together with the central clock in the optic lobe, the circadian oscillator in the eye regulates the circadian light perception rhythm for the efficient phase-resetting or entrainment of the clock. In addition, the eye oscillator may contribute to the establishment of a stable free-running rhythm of the central circadian clock via the reciprocal regulatory pathways, i.e., the efferent control pathway from the central clock to the eye and the entrainment pathway from the eye to the central clock.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

KT conceived and designed the study. CO performed the qPCR. YM performed ISH. KT performed ERG experiments. CO and KT performed the statistical analysis. KT wrote the manuscript.

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