Relationships between Bilateral Circadian Pacemakers in Intact and Neurally Separated Optic-Lobes of a Carabid Beetle

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The mutual interactions between the bilaterally-paired optic-lobe circadian pacemakers in the carabid beetle, *Carabus insulicola insulicola*, were investigated by recording electroretinograms (ERGs) under constant darkness. The ERG amplitudes of both right and left compound eyes exhibited clear circadian rhythms due to direct efferent controls from the ipsilateral optic-lobe pacemakers. In the free-running state, the circadian period of the two pacemakers in each beetle ran parallel to each other. The phase-angle difference between the two pacemakers was generally close to 0° but we found that the two-oscillator system also stabilizes with phase-angles of 90°, 180° and 270°. Unilateral severance of the optic tract resulted in increased free-running period lengths of both the ipsilateral and contralateral pacemakers, indicating that the mutual neural interactions shorten the period.

Key words: circadian rhythm, pacemaker coupling, electroretinogram, phase-angle difference, *Carabus*

INTRODUCTION

The electroretinogram (ERG) amplitude of compound eyes exposed to repetitive light stimulation exhibits circadian rhythmicity in several insect species kept under continuous darkness (DD) (cockroach: WILLS et al., 1985; COLWELL and PAGE, 1989; cricket: TOMIOKA and CHIBA, 1982, 1985; honeybee: MILDE, 1985; ant: MENZI, 1987; moth: BENNETT, 1983; beetle: JAHN and CRESCITELLI, 1940; KOEHLER and FLEISSNER, 1978; FLEISSNER, 1982; FLEISSNER and FLEISSNER, 1986). The ERG rhythms of compound eyes are known to be under efferent control from ipsilateral optic-lobe pacemakers that control the circadian locomotor activity rhythm (TOMIOKA and CHIBA, 1982; WILLS et al., 1985). Most previous studies on possible interactions between circadian pacemakers have analyzed the locomotor activity rhythms where the outputs are usually dealt with as single, interacting outputs (PAGE et al., 1977; TOMIOKA et al., 1991; TOMIOKA and CHIBA, 1992; WADDELL et al., 1990; WIEDENMANN, 1983). However, analysis of the ERG rhythms has a clear advantage because it enables evaluation of two outputs monitored independently from bilateral pacemakers. This paper reports the long-term coupling mechanism of bilateral pacemakers in a carabid beetle using computer-based automatic recording of their ERGs to clarify the physiological bases of circadian organization.

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MATERIALS AND METHODS

Field-collected carabid beetles, *Carabus insulicola insulicola*, entrained under 12 h light, 12 h darkness and a constant temperature of 20°C, were used for long-term ERG recordings except #3202 (Fig. 3) whose recording was started immediately after collecting. The beetles, whose mandibles were gagged by a small brass plate, were fixed on a plastic platform. Platinum wires were implanted as indifferent electrodes adjacent to each compound eye. Electrically-sharpened tungsten wires were inserted into each cornea as recording electrodes. The ERGs were evoked at 10 min intervals by a flashing green light ($\lambda_{\max} = 555$ nm, $\Delta\lambda_{1/2} = 30$ nm), of 50 ms duration and $1 \mu\text{W}/\text{cm}^2\cdot\text{s}$ power emitted from LEDs 1 mm from both the right and left compound eyes. Sequential amplified signals of the corneal negative responses were fed to a personal computer (NEC: PC-9801) through an A/D converter and stored on microfloppy disks. The amplitude of each ERG was calculated as the difference between the average potential 0.5 s before the flash and the peak response value. The change in the amplitude for the two compound eyes showed the typical sine-curve circadian oscillation (Fig. 1). Acrophases of the ERG rhythms were calculated daily from these curves by the least-squares cosine fitting method (HALBERG et al., 1971). The free-running periods were determined by regression lines drawn from the acrophases during at least six successive days.

A surgical operation was used to isolate the bilateral pacemakers in six animals after determining the circadian period. The cuticle above the brain was removed and the optic tract was unilaterally severed between the protocerebrum and optic-lobe. The wound was covered with the original detached cuticle and sealed with molten wax. Sham operation was done by making appropriate handling instead of severing. Surgery was done quickly in less than 10 min between circadian time 2 (CT, the acrophase in the middle of subjective night was/is designated as CT 18) and CT 14. The brief light exposure during surgery has been confirmed to have no effect on the phase and period by analyzing the phase response curves (unpublished data).

All experiments were done under constant temperature of $20 \pm 0.5^\circ\text{C}$ in DD. The start of experiments (light-off) was timed to coincide with the light-off time of the preceding rearing condition to prevent phase shifts of the rhythm.

RESULTS AND DISCUSSION

Phase

The ERG amplitude was high in the middle of subjective night and low in the middle of subjective day in both left and right eyes (Fig. 1). In all intact animals, the two pacemakers ran with identical periods in DD. The phase-angle differences between right and left pacemakers was often around 0° (Fig. 2, see also Fig. 1 and before operation in Fig. 4). This stable phase angle at around 0° is interpreted as a coupled condition of bilateral pacemakers because the phase relationship recovers from light-forced desynchronization to the previous steady state one (YAMAZAKI and SASAKI, unpublished). The small phase-angle differences distributed around 0° (Fig. 2) are real differences and are not due to measurement or calculation errors. This has been evidenced by the corresponding magnitude or direction of the different phase shifts in response to the light pulse(s) given to bilateral pacemakers (YAMAZAKI and SASAKI, unpublished). As indicated in the frequency distribution (Fig. 2), however, other stable angles were also found at 90° in one animal, 180° in two animals, and

Fig. 1. Typical example of circadian changes in ERG response amplitude recorded from both right and left compound eyes under constant darkness (DD). The similar inphase state (phase-angle around 0°) continued for more than ten days.

Fig. 2. Frequency distribution of Ψ_{RL} (phase-angle difference between left and right oscillator).

Fig. 3. Example of an individual with synchronized bilateral ERG rhythms but with a marked difference in phase-angle relationship. Shaded box represents the LD (light : dark) cycle 12 : 12 from days 14–19. At other times, the animal was in DD. In this case, the average Ψ_{RL} is 5.4 h or 80.3° . After LD, the phases were synchronized to each other. Time axis (x) represents time of peak acrophase.

Table 1. Comparison of free-running period (τ) of ERG rhythms of bilateral compound eyes before and after severance of left (or right) optic tract

Individual no.	Severed side	Free-running period before and after surgery in DD ^a		
		τ_c	τ_L	τ_R
#3301	Left	23.8	26.0 (+9.2) ^b	> 24.9 (+4.6) ^b
#3306	Left	23.9	25.2 (+5.4)	> 24.8 (+3.8)
#3322	Left	23.2	24.6 (+6.0)	> 24.4 (+5.2)
#3312	Right	23.4	24.5 (+4.7)	< 24.7 (+5.6)
#3318	Right	22.4	23.0 (+2.7)	< 23.4 (+4.5)
#3319	Right	22.7	26.2 (+15.4)	< 26.4 (+16.3)

^a τ_c : free-running period (h) before surgery, right and left oscillator synchronized, τ_L : free running period (h) of left oscillator after surgery, τ_R : free-running period (h) of right oscillator after surgery.

^b % of period changed by optic tract severance: $\frac{\tau_L \text{ or } \tau_R - \tau_c}{\tau_c} \times 100$.

270° in one animal out of 26 animals. An example at 90° is shown in Fig. 3. The two pacemakers exhibited the same period with a phase-angle difference of 80.3° (5.4 h). After recording this split state, we applied 12 h light and 12 h dark conditions to this animal on day 14 to check the capacity for entrainment to light and the interaction of the two oscillators. The split rhythms were entrained (fused) with the acrophases in the middle of night after a transient interval of three days where both periods differed. Thus, external light entrainment dominates each pacemaker and the mutual internal entrainment between the two pacemakers is subordinate.

In a similar experiment using ERGs, FLEISSNER and FLEISSNER (1986) observed that the pacemakers of a carabid beetle, *Pachymorpha sexguttata*, coupled at inphase (0°) or antiphase (180°). Splitting of free-running activity rhythms into two distinct components suggests that there are two pacemakers locked into a stable relationship of 180° antiphase to each other. From the theoretical view point, this 'bistability' has been explained as "Hopf bifurcation" (KAWATO and SUZUKI, 1980). However, our work shows that the two oscillators can desynchronize and stabilize at two other points (90° and 270°). It should be difficult to distinguish the present phenomenon from a simple prolongation/shortening of the active period as long as the behavioral output was used as an index. This might be the reason why "tetrastability" phenomenon has not been recognized so far. The evidence that the circadian system composed of the two oscillators stabilizes with phase-angles of 90° or 270° in addition to known 0° and 180° requires a new logical (mathematical) explanation.

Period

To investigate the coupling between the two pacemakers, the bilateral optic lobes were isolated by cutting one optic tract of six beetles. No effect on the free-running period of the ERG rhythms was found in sham-operated animals. In contrast, operated animals showed distinct lengthening (2.7–16.3%, 7.8% on an average) of periods in both pacemakers after surgery (Table 1). This suggests that neural coupling between the bilateral pacemakers reduces the period. A comparison of the amount of period lengthening between right and left sides shows that it was always larger in the operated side. Thus, isolation of the pacemaker from the central brain may result in the increase of the period. A detailed example of the changes in phase relationships between bilateral oscillators is shown in Fig. 4. Since

Fig. 4. Effect of optic-tract severance to eliminate coupling between bilateral eyes on circadian period (τ) and phase of two ERG rhythms. The experiment was done in DD except during the severance (< 10 min) at 20:00 (CT) on day 6. Time axis (x) represents time of peak acrophase.

the augmentation in the severed side was larger than in the intact side, the associated acrophases were dissociated after surgery. A phase jump in the operated side was also seen in Fig. 4, but it was sporadic (occurred in #3301, #3306, #3318, and not in #3312, #3319) and seemed due to the operation itself.

The consequence of pacemaker coupling in insects has been investigated by recording the locomotor activity rhythms. In the cockroach (PAGE et al., 1977) and the orthopteran *Hemideina* (WADDELL et al., 1990), the coupling reduces the period, but it increases the period in the cricket (OKADA et al., 1991). A similar period increase has been reported in the marine mollusks *Bulla* (ROBERTS and BLOCK, 1985) and *Bursatella* (ROBERTS et al., 1987) by recording optic-nerve compound action potentials. The period change ratio due to neural uncoupling (footnote of Table 1) was +1.0% in the cockroach, +3.0% in *Hemideina* (calculated by assuming a free-running period of 24 h because free-running period was not shown in the paper), -0.5% in the cricket, -2.9% in *Bulla*, and -5.7% in *Bursatella*. The ratio for *Carabus* was +7.8% on average, which is similar to that of mollusks in magnitude, but opposite in direction.

The physiological meaning of the possible tetrastability in phase-angle differences is unclear because it was detected only under the DD condition and it may not be present in natural conditions. However, the evidence suggests a need for further analysis of the circadian system in general.

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