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ORIGINAL PAPER

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Neural pathways involved in mutual interactions between optic lobe circadian pacemakers in the cricket *Gryllus bimaculatus*

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Abstract The circadian locomotor rhythm of the cricket *Gryllus bimaculatus* is primarily generated by a pair of optic lobe circadian pacemakers. The two pacemakers mutually interact to keep a stable temporal structure in the locomotor activity. The interaction has two principal effects on the activity rhythm, i.e., phase-dependent modulation of the freerunning period and phase-dependent suppression of activity driven by the partner pacemaker. Both effects were mediated by neural pathways, since they were immediately abolished after the optic stalk connecting the optic medulla to the lobula was unilaterally severed. The neural pathways were examined by recording locomotor activity, under a 13 h light to 13 h dark cycle, after the optic nerves were unilaterally severed and the contralateral optic stalk was partially destroyed near the lobula. When the dorsal half of the optic stalk was severed, locomotor rhythm mostly split into two components: one was readily entrained to the given light-dark cycle and the other freeran with a marked fluctuation in freerunning period, where the period of the freerunning component was lengthened or shortened when the onset of the entrained component occurred during its subjective night or day, respectively. The phase-dependent modulation of activity was also observed in both components. However, severance of the ventral half of the optic stalk resulted in appearance only of the freerunning component; neither the phase-dependent modulation of its freerunning period nor the change in activity level was observed. These results suggest that neurons driving the mutual interaction and the overt activity rhythm run in the ventral half of the proximal optic stalk that includes axons of large medulla neurons projecting to the cerebral lobe and the contralateral medulla.

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Key words Circadian rhythm · cricket
interaction between pacemakers · optic lobe
neural pathways

Abbreviations *LD* light dark cycle · τ freerunning period

Introduction

Circadian rhythms of many animals are controlled by the circadian system that is generally composed of multiple pacemakers. In many cases, the system includes two coupled circadian pacemakers, which reside in bilaterally paired structures in the central nervous system. The coupled pacemakers usually keep a steady phase relationship with each other to maintain a stable temporal structure through mutual interactions (Page 1978; Wiedenmann 1983; Roberts and Block 1985; Meijer and Rietveld 1989; Tomioka et al. 1991). Several studies have focused on the mutual interactions. For example, the two pacemakers are only weakly coupled to each other in the beetle *Blaps gigas* (Koehler and Fleissner 1978) and in the cricket *Teleogryllus commodus* (Wiedenmann 1983; Wiedenmann et al. 1988). The ocular pacemakers of mollusks *Bulla gouldiana* and *Bursatella leachi plei* show a relatively strong coupling (Roberts and Block 1983; Roberts et al. 1987). In the cricket *Gryllus bimaculatus*, a pair of functionally identical circadian pacemakers reside in the optic lobes (Tomioka and Chiba 1984, 1986, 1992). They mutually interact to keep a stable temporal structure in the locomotor activity. It has been revealed that the interaction has two principal effects on the activity rhythm, i.e., phase-dependent modulation of the freerunning period and phase-dependent suppression of activity driven by the partner pacemaker (Tomioka et al. 1991; Tomioka 1993).

There are several reports on the neural pathway for the mutual interactions between the circadian

pacemakers. In the cockroach *Leucophaea maderae*, the pacemakers in the optic lobe are said to be mutually coupled through a polysynaptic pathway (Page 1978). The coupling pathway mediating mutual entrainment between ocular pacemakers in *Bulla* consists of the optic nerves, the cerebral ganglia, and the cerebral commissure (Roberts and Block 1985). In *Gryllus bimaculatus*, it has been suggested that the medulla large neurons directly connecting the bilateral medullae mediate the neural information for the mutual interaction, since the circadian rhythm in the brain efferents toward the optic lobe disappeared after the partial destruction of the contralateral optic stalk including those neurons (Tomioka et al. 1994).

The experiment reported here was designed to determine which neural pathway(s) is involved in the mutual interactions between the paired pacemakers. The mutual interaction appearing in locomotor activity was assayed after the optic stalk was partially destroyed and the lesions were analyzed by nickel chloride backfilling from the stalk. The results show that the mutual interactions are mediated through a neural pathway that runs along the ventral side of the proximal optic stalk.

Materials and methods

Experimental animal

Adult male crickets (*Gryllus bimaculatus*) reared in the laboratory were used throughout this study. They were maintained under controlled conditions of temperature of 26° ± 0.5°C with a 12 h light to 12 h dark cycle (LD 12:12, light: 06:00–18:00, Japanese standard time). They were fed laboratory chow and water via damped absorbent cotton.

Activity recording

Before recording activity, either of the forewings was removed to prevent any sound communication between individuals. Locomotor activity was monitored in an activity chamber of a transparent plastic box (20 × 7 × 6 cm) with a rocking substratum whose rocking, caused by a moving insect, was sensed by a magnetic reed switch placed on the bottom of the box. A signal from the switch was collected by a computer, which summed the signals every 6 min; the total count of every 6 min was stored on a floppy diskette. The quantitative data were later analyzed with a computer. The activity chambers were placed in an environment-controlled room in which the temperature was kept constant at 25° ± 0.5°C. The light was furnished by a cool-white fluorescent lamp connected to an electronic timer (Omron, H3CA). The light intensity within actographs varied with proximity within the environment-controlled room to the lamp and ranged from 5 to 40 lux at the animal's level.

Data analysis

Event records of locomotor activity were double plotted by a computer with a resolution of 6 min. Activity for 5 days following the surgical operation was excluded from data analysis to eliminate possible after-effects of the surgery on activity rhythms. The onset of active phase was always much sharper than the offset, and hence only the onset was used to estimate freerunning period and phase. The onset was defined as circadian time 12 for each rhythmic component. A 12 circadian hour period following the activity onset was defined as the subjective night and the rest as the subjective day. The daily freerunning period was defined as the period between the two consecutive onsets. The freerunning period was estimated by averaging the daily freerunning period. Deviation from this estimated average for a given day was determined as daily fluctuation of the freerunning period, being expressed with a + or − sign for relative lengthening or shortening, respectively. Activity level of each rhythmic component was evaluated by calculating a total amount of activity during the first 2 h period from the onset, since the activity was concentrated in this period.

Surgery

Surgical lesions were performed on animals anesthetized with CO_2. The surgical procedures for optic nerve severance, optic lobe removal, ocelli removal, or partial destruction of the optic stalk were as follows. A cricket was placed on a specially designed platform to immobilize its head. The cuticle around the compound eye was cut with a fine razor knife, and the eye was prised open with forceps to expose the lamina-medulla-retina complex. The lamina and the medulla region of the optic lobe forms an optic ganglion in this cricket; we refer to this ganglion as an optic lobe and the long nerve trunk connecting the lobe to the lobula as an optic stalk for convenience (Fig. 1). Either optic nerves alone or both the optic nerves and the optic stalk were cut with a pair of microscissors to make a blinded pacemaker or to remove an optic lobe, respectively. After placing a small square piece of thin aluminum film between the surgically separated tissues to prevent the regeneration of nervous connection, the eye capsule was replaced. The ocelli were removed after cutting the cuticle around them followed by the ocellar nerve severance. For partial destruction of the optic stalk, a small square piece of head capsule just above the stalk was removed to expose it. Either dorsal or ventral side of the stalk near the lobula was partially destroyed with a fine needle (Fig. 1). At the end of the experiment, the head was carefully dissected to verify the success of the surgical lesioning under a dissecting microscope.

Staining of neural pathways by backfilling nickel chloride

At the end of the experiment, the animals were subjected to histological examination. The animals were pinned on a polyethylene

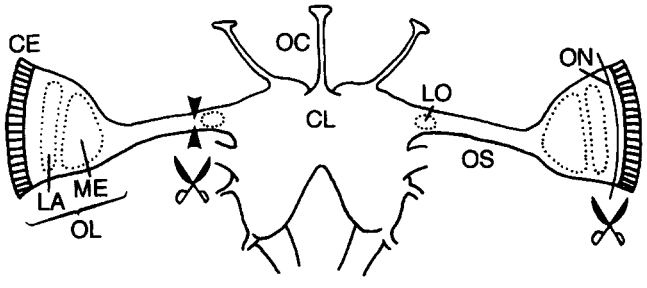

Fig. 1 Schematic diagram of cricket brain. The lamina-medulla complex forms an optic ganglion which in this paper is referred to as an optic lobe (*OL*). Either dorsal or ventral half of the optic stalk (*OS*) was destroyed near the lobula (*LO*) in addition to the contralateral optic nerve (*ON*) severance. Arrows indicate the site of the partial destruction. *CE*, compound eye; *CL*, cerebral lobe; *LA*, lamina; *ME*, medulla; *OC*, ocellar nerves

form to be immobilized, then the head capsule was partially cut to expose the optic stalk on the intact side. After cutting the optic stalk near the medulla, its proximal cut end was placed on a tip of a small capillary filled with a solution containing 0.25 *M* nickel chloride and 0.25 *M* potassium chloride. After incubating at 4°C for about 12 h, brain-optic lobe complex was dissected out and placed in an insect Ringer's solution containing 5 drops of rubeanic acid saturated in absolute ethanol. The optic stalk at the point of nickel application was uniformly labeled by the dark blue conjugate of nickel with rubeanic acid. The tissue was then fixed with 10% formaldehyde for 30 min. The staining was then intensified by Timm's silver intensification method (Tyrer and Bell 1974; Bacon and Altman 1977) to make fine branchings, fibers and cell bodies visible. After dehydration through an ethanol series, the tissue was cleared with methyl benzoate. Drawings were made from unsectioned preparations using a compound microscope equipped with a camera lucida (Olympus, BH-2).

Results

Locomotor rhythm of animals with the optic nerve unilaterally severed

Our previous experiment revealed that the coupling between the bilaterally paired pacemakers in unilaterally blinded animals can be established under light-dark cycles with periods only between 23 h and 25 h (Tomioka 1993). Without this allowable range, activity rhythm of the operated animals splits into 2 components desynchronizing with each other. In such cases, mutual interactions such as phase-dependent modulation of freerunning period and of the activity level can be clearly observed. To confirm the mutual interactions to occur, we first assayed locomotor activity rhythms of 19 unilaterally blinded animals under LD 13:13.

Sixteen (some 85%) of them exhibited two rhythmic components: one was readily entrained to the light-dark cycle and the other freeran with a marked fluctuation in its daily freerunning period (Fig. 2A). The period of the freerunning component was lengthened when the onset of the entrained component occurred during the early subjective night (CT 12–21) but was relatively shortened during the late subjective night and the subjective day (CT 21–12) (Fig. 2B). There was also marked fluctuation in activity level of both components; their activity level was significantly reduced when they occurred during the early subjective day (CT 0–8) of their partner component, gradually increased during the late subjective day (CT 8–12), and reached the highest level at the early subjective night (CT 12–16) (Fig. 2C). The remaining 3 animals exhibited a unimodal activity with an entrained component.

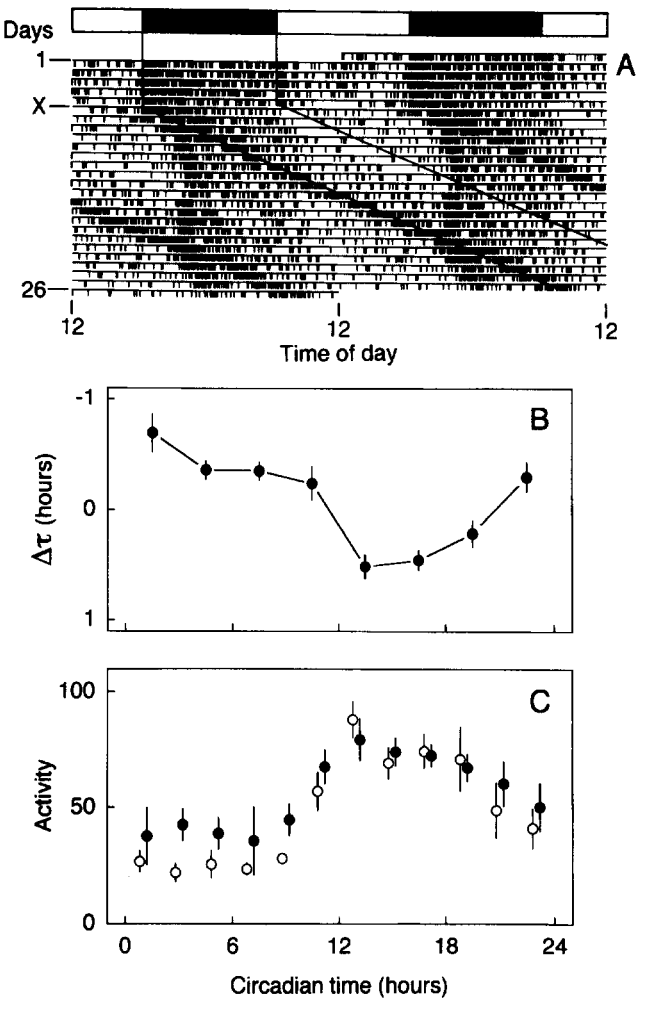

Fig. 2 A Activity rhythm of an animal that received unilateral optic nerve severance on the day of transfer from LD 12:12 to LD 13:13 (X). The animal was held in LD 12:12 for the first 5 days. Light regimes are indicated by *white* (light) and *black* (dark) bars and the solid lines on the actogram. Locomotor rhythm split into two rhythmic components on transfer to LD 13:13: one was entrained to the given LD while the other freeran with an apparent relative coordination. **B** Change of the freerunning period of the freerunning component plotted as a function of its circadian time at which the onset of the entrained component occurred. *Ordinate*, difference from the mean freerunning period. Note that the period was lengthened when the entrained component occurred during the subjective night but was relatively shortened during the subjective day. **C** Total activity during the first 2-h period from the onset of the entrained (*filled circles*) and the freerunning component (*open circles*) plotted as a function of the circadian time of the other component. *Ordinate*, activity in percentage of highest activity. In **B** and **C**, data from 13 animals were pooled. Each point represents the mean (± SE) calculated by collapsing the data in 3-h (**B**) or 2-h (**C**) bins. In both cases, the activity was reduced during the early subjective day (CT 0–8), gradually increased during the late subjective day (CT 8–12), and reached the highest level at the early subjective night (CT 12–16)

Activity rhythm of animals with ocelli removed

The previous study has reported that the photic information as well as the temporal information is involved in the modulation of freerunning period (Tomioka 1993). The photic information is believed to be provided from the compound eye, since the optic nerve severance results in a freerunning rhythm even in the light-dark cycle (Tomioka and Chiba 1984;

Tomioka et al. 1991). However, recent experiments in *Teleogryllus commodus* revealed that the photic information from the ocelli modulates the freerunning period (Rence et al. 1988). We thus assayed the locomotor rhythm of animals whose 3 ocelli were removed in addition to the unilateral optic nerve severance to examine whether the photic information from ocelli plays any role in modulation of the freerunning period.

Locomotor activity of 31 operated animals was recorded under LD 13:13. Figure 3A and B exemplify 2 of the results. Twenty-six (nearly 85%) of them exhibited a dissociation of two rhythmic components on transfer to $T = 26$: one was readily entrained to the given LD, while the other freeran with a marked fluctuation in its freerunning period. Figure 3C and D show the phase-dependent modulation curves of the period of the freerunning component and of the activity of both components, respectively. Both the shape and the amplitude of the modulation curves were very similar to those observed in control animals with the optic nerve unilaterally severed. The result indicates that the ocellar pathways have little effect on the mutual interaction.

Locomotor activity of animals with a single blinded pacemaker

To determine if the phase-dependent fluctuation in period and activity is induced only by interaction between bilaterally paired pacemakers, unilateral optic lobe removal and the contralateral optic nerve severance were performed on 29 animals. Under LD 13:13, these animals with a single blinded pacemaker exhibited a single component which freeran in almost constant period, though activity was often reduced for the first several days following the operation (Fig. 4A and B). Figure 4C and D summarize results of analysis of the period and the activity of the freerunning component, respectively. Although both curves show slight irregular fluctuations, the amplitude was significantly reduced compared with those of control animals shown in Fig. 2B and C. The results suggest that the phase-dependent modulation in period and activity occur through the mutual interaction between optic lobe pacemakers.

Effects of optic stalk severance on the interaction

To determine if the mutual interaction is mediated by the neural pathways between optic lobes, we performed an additional unilateral optic stalk severance on 22 unilaterally blinded animals showing split rhythms. Severance of the optic stalk on the intact side resulted in an immediate loss of the entrained component, leaving the other component freerunning in almost constant period (Fig. 5A), whereas that on the blinded side abolished the freerunning component (Fig. 5B). Similar results were obtained in the remaining 20 animals.

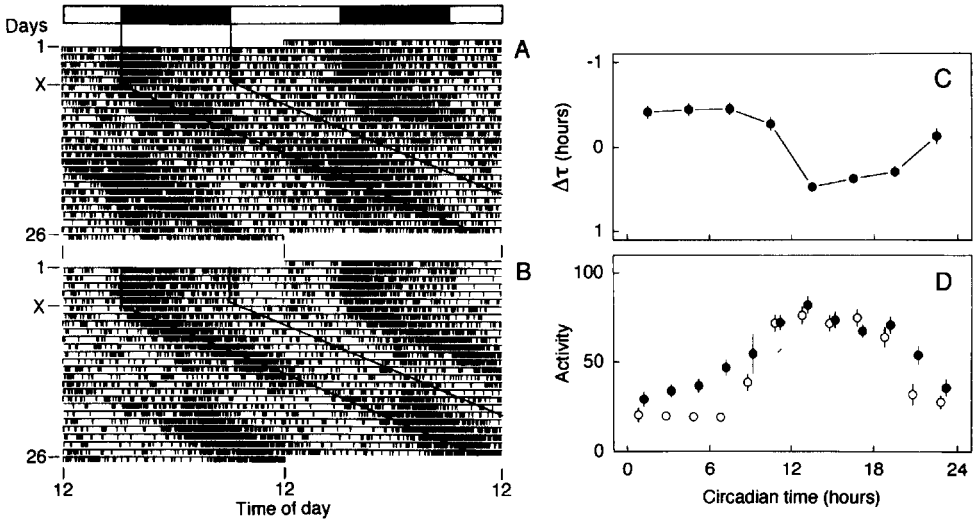

Fig. 3A, B Two examples of activity rhythms from animals that received unilateral optic nerve severance and three ocellar nerves severance on the day of transfer LD 12:12 to LD 13:13 (X). Under LD13:13, activity rhythm split into two components: one entrained to the given LD and the other freeran with a relative coordination. In both cases, the freerunning period and the activity level changed as a function of the mutual phase relationship. **C** Period modulation curve of the freerunning component plotted as a function of time of day at which the entrained component occurred. **D** Total activity during the first 2-h period from the onset of the entrained (*filled circles*) and the freerunning component (*open circles*) plotted as a function of the circadian time of the other component. *Ordinate*, activity in percentage of highest activity. In **C** and **D**, data from 16 animals were pooled. Each point represents the mean ($\pm$ SE) calculated by collapsing the data in 3-h (**C**) or 2-h (**D**) bins. For further explanations see Fig. 2

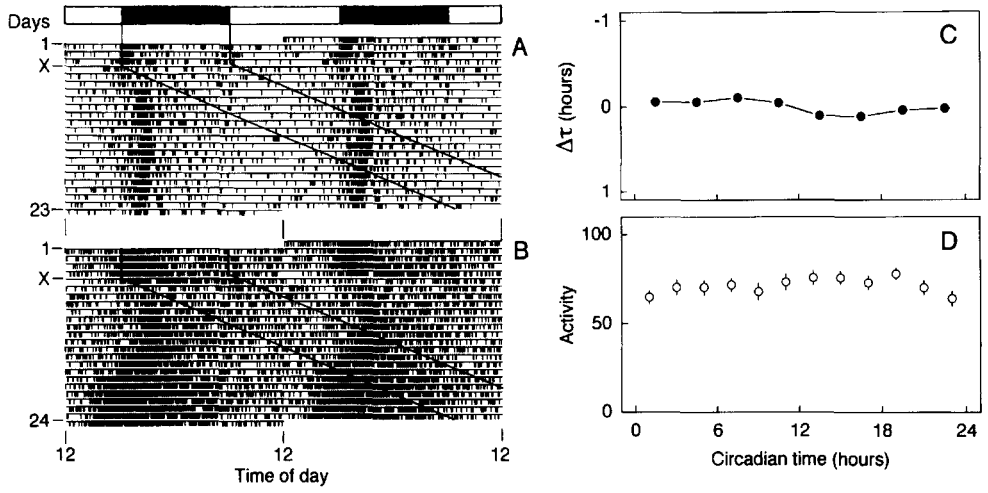

Fig. 4A, B Two examples of activity rhythms from animals with unilateral optic lobe removed and the contralateral optic nerve severed on the day of transfer to LD 13:13 (X). The operated animals showed a clear unimodal rhythm. **C** Change in freerunning period plotted as a function of circadian time at which the lights off occurred. **D** Change in activity level plotted as a function of circadian time of LD cycle at which the onset of activity fell. In **C** and **D**, data from 20 animals were pooled. Each point represents the mean (± SE) calculated by collapsing the data in 3-h (**C**) or 2-h (**D**) bins. Note that amplitudes of both curves were significantly reduced comparing with those in control animals. For further explanations see Fig. 2

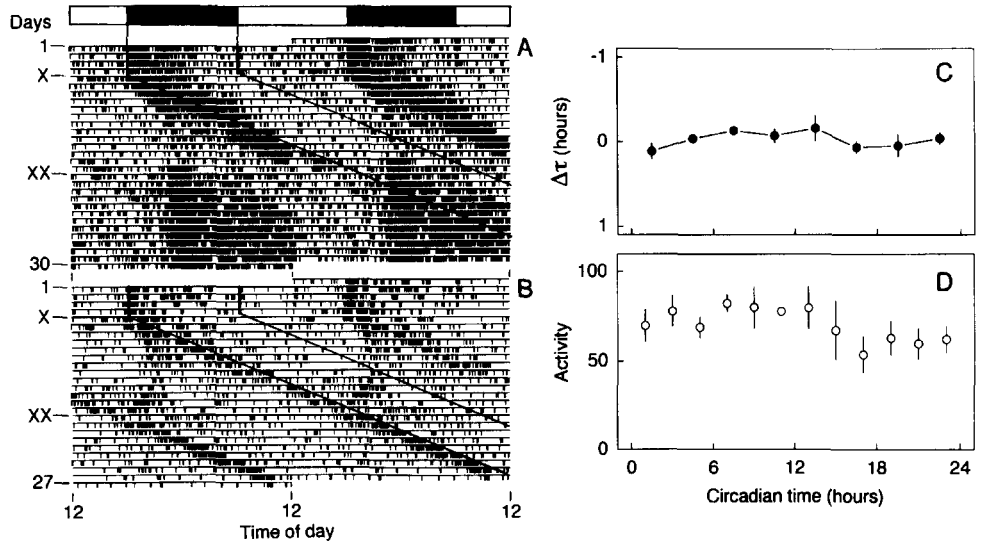

Fig. 5A, B Two examples of activity rhythms from animals that received unilateral optic nerve severance on the day of transfer to LD 13:13 (X). Both animals showed activity rhythm splitting into two components in LD 13:13. In **A**, immediately after optic stalk severance on the intact side (XX), an entrained component disappeared, and the freerunning component became enhanced. In **B**, a freerunning component disappeared immediately after optic stalk severance on the blinded side (XX). **C** Change in freerunning period plotted as a function of circadian time at which the lights off occurred. **D** Change in activity level plotted as a function of circadian time of LD cycle at which the onset of activity fell. In **C** and **D**, data from 11 animals that received optic stalk severance on the intact side were pooled. Each point represents the mean (± SE) calculated by collapsing the data in 3-h (**C**) or 2-h (**D**) bins. For further explanations see Fig. 2

Neither the period nor the activity of the freerunning component exhibited the phase-dependent modulation after the optic stalk severance (Fig. 5C and D). The result clearly indicates that the mutual interaction is mediated by a neural pathway.

To reveal the neural tracts involved in the mutual interaction, we attempted to label the neurons by backfilling nickel chloride from the cut end of the optic stalk. We could tentatively label 7 neural tracts according to Tomioka et al. (1994) (Fig. 6). The results were

nearly consistent with the data in *Gryllus campestris* (Honegger and Schürmann 1975). Three tracts (tracts 1, 3 and 5) projected into the contralateral optic lobe. Among them, tract 5 was composed of 23–26 neurons having their somata near the ventral side of the medulla, extending their branches over almost whole medulla area. Tract 6 and tracts 4 and 7 originated in the ipsilateral and contralateral hemisphere, respectively, and tract 2 terminated in the ipsilateral hemisphere.

Locomotor activity of animals receiving unilateral optic stalk partial severance in addition to unilateral optic nerve severance

The fact that the interaction occurs only through neural pathways between optic lobes suggests that some of neurons shown in Fig. 6 act as pathways for the phase-dependent modulation of period and the mutual suppression of activity during the subjective day. To elucidate the pathways, we examined the effects, on the locomotor rhythm, of destruction either of the dorsal or ventral half of the proximal optic stalk near the lobula in addition to the contralateral optic nerve severance.

In a total of 13 crickets, the dorsal half of the optic stalk was severed. Although one animal exhibited unimodal activity with an entrained component, all other operated crickets clearly showed an activity rhythm splitting into two components under LD 13:13, as was observed in the control animals with optic nerve unilaterally severed: one component was readily entrained to the LD cycle, while the other freeran with a marked fluctuation in its freerunning period (Fig. 7A). The period modulation curve of the freerunning component was very similar to that of the control, unilaterally blinded animals (Fig. 7C). The activity level of both components also exhibited the phase-dependent fluctuation (Fig. 7D). However, it is noteworthy that the suppression of the entrained component was somewhat reduced in comparison with that in the control, unilaterally blinded animals (Fig. 2C). Nickel backfilling from the partially severed optic stalk revealed that in all the operated animals tracts running dorsal part of the stalk such as tracts 1, 2 and 3 were interrupted whereas tracts 4, 5, 6 and 7 running more ventral side survived the operation (Fig. 7B). Among the surviving tracts, tract 5 is the only one including neurons originating in the optic lobe and projecting to the contralateral lobe; others seemed projecting from the cerebral lobe to the optic lobe.

In other 13 animals, the ventral half of the optic stalk was destroyed in addition to the contralateral optic nerve severance. Under LD13:13, 11 of them exhibited only a freerunning component which freeran independent of the LD cycle, but had no entrained component (Fig. 8A). The freerunning component showed neither modulation of the freerunning period nor the change in activity level (Fig. 8C and D). Nickel backfilling from the optic stalk of the destroyed side revealed that only the neural tracts projecting to the ipsilateral cerebral lobe survived the operation (Fig. 8B). The operation severed the direct neural connection to the contralateral medulla (tracts 1, 3 and 5) as well as the neural tracts originating in the ipsilateral or the contralateral hemisphere (tracts 4, 6 and 7). The remaining 2 animals exhibited two rhythmic components with phase-dependent modulation in both freerunning period and activity like the control, unilaterally blinded animals did. Nickel backfilling from the destroyed stalk in the animals labeled the same 7 neural tracts as those observed in the control. These results suggest that the neurons that run the ventral side of the optic stalk include pathways for the mutual interactions.

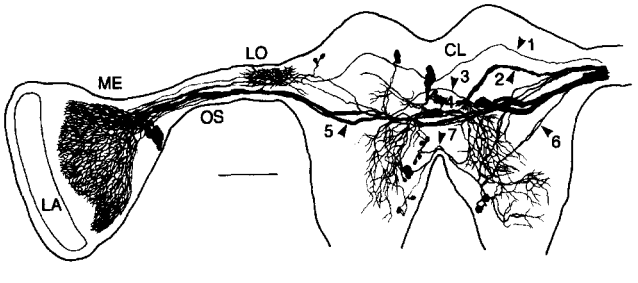

Fig. 6 Camera lucida drawing of posterior view of the neurons stained by nickel chloride backfilling from the cut end of the optic stalk on the intact side in the animal with the optic nerve unilaterally severed. Bar scale equals 300 μm. The numerals indicate seven neural tracts tentatively identified. *CL*, cerebral lobe; *LA*, lamina; *LO*, lobula; *ME*, medulla; *OS*, optic stalk

Discussion

The previous studies have shown that the optic lobe pacemakers mutually interact to maintain their synchronous movement through phase dependent modulation and to keep a stable nocturnal locomotor rhythm through a phase dependent inhibition of activity (Tomioka et al. 1991; Tomioka 1993). In the present experiment, the intact pacemaker appeared to dominate formally over the blinded pacemaker in the phase-dependent modulation of both period and activity level. As we have discussed previously (Tomioka 1993), the bilaterally distributed circadian pacemakers are functionally identical. The dominance of the intact side can be interpreted by the fact that the photic information is also involved in the mutual interaction (Tomioka 1993; Tomioka et al. 1994). This is also consistent with the assumption that light intensity influences on the coupling strength of the pacemakers (Daan and Berde 1978).

The data presented here show the following facts: First, the removal of three ocelli did not affect the

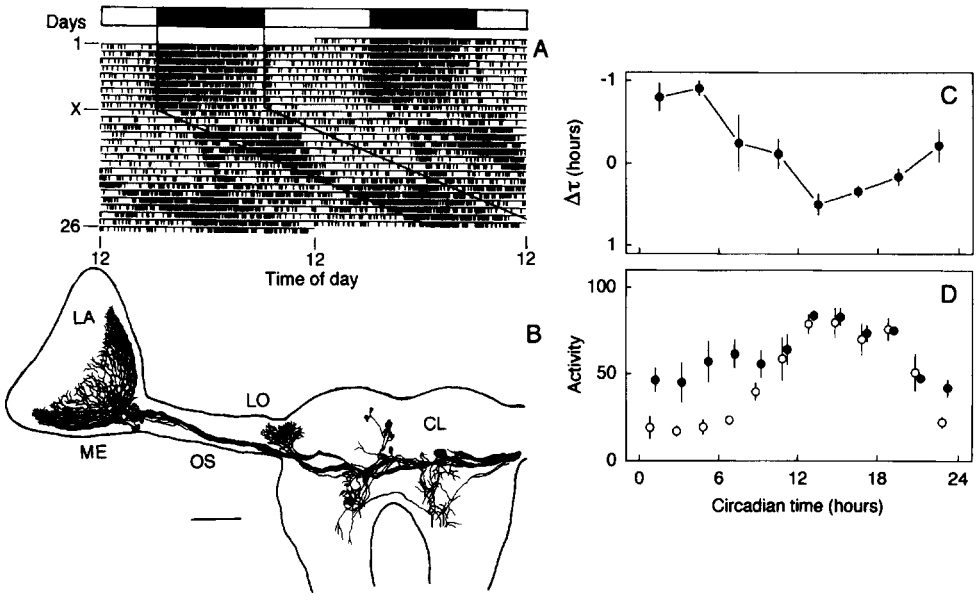

Fig. 7A Activity record of an animal that received destruction of dorsal half of unilateral optic stalk in addition to the contralateral optic nerve severance. The operation was performed on the day of transfer from LD 12:12 to LD 13:13 (X). On transfer to the new LD, activity rhythm split into two components: one was entrained to the LD cycle while the other freeran with a relative coordination. **B** Camera lucida drawing of posterior view of the nickel labeled neurons in the animal shown in **A**. Bar scale equals 300 μm. Neural tracts running ventral side of the lobula were survived the operation. **C** Period modulation curve of the freerunning component plotted as a function of time of day at which the entrained component occurred. **D** Total activity during the first 2-h period from the onset of the entrained (*filled circles*) and the freerunning component (*open circles*) plotted as a function of the circadian time of the other component. *Ordinate*, activity in percentage of highest activity. In **C** and **D**, data from 8 animals were pooled. Each point represents the mean (± SE) calculated by collapsing the data in 3-h (**C**) or 2-h (**D**) bins. For further explanations see Fig. 2

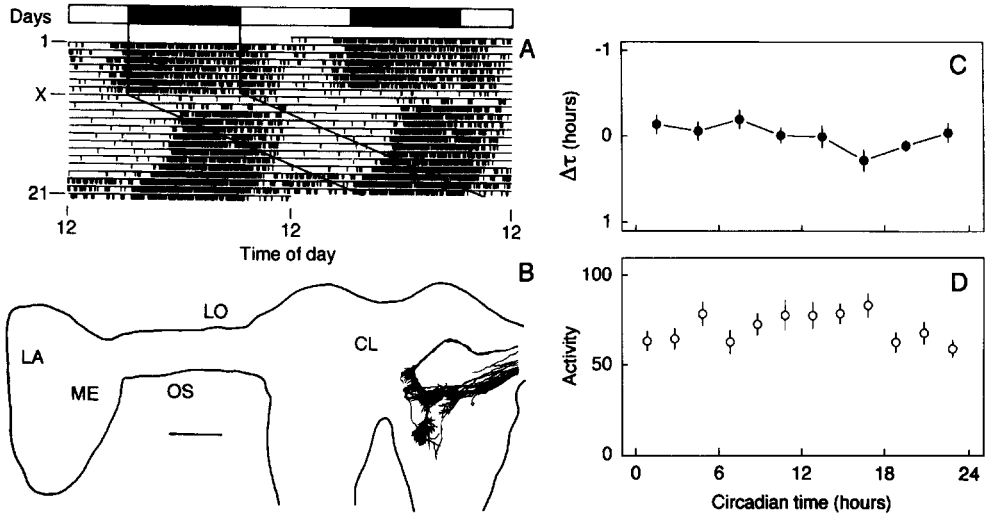

Fig. 8A Activity record of animal that received destruction of ventral half of unilateral optic stalk in addition to the contralateral optic nerve severance. The operation was performed on the day of transfer to LD 13:13 (X). Under LD13:13, the operated animal showed only a freerunning component. **B** Camera lucida drawing of posterior view of the neurons labeled by nickel chloride backfilling in the animal shown in **A**. Bar scale equals 300 μm. The operation interrupted the direct neural connection to the contralateral medulla. **C** Change in freerunning period plotted as a function of circadian time at which lights off occurred. **D** Change in activity level plotted as a function of circadian time of LD cycle at which the onset of activity fell. In **C** and **D**, data from 9 animals were pooled. Each point represents the mean (± SE) calculated by collapsing the data in 3-h (**C**) or 2-h (**D**) bins. Neither phase-dependent modulation of the period nor the activity level were observed. For further explanations see Fig. 2

mutual interaction appeared in both freerunning period and activity level (Fig. 3). Second, animals with a single blinded pacemaker did not show any significant fluctuation in freerunning period and activity level (Fig. 4). Third, severance of the optic stalk immediately eliminated the interaction (Fig. 5). Finally, the ventral

part of the proximal optic stalk includes neruons necessary for the interaction (Figs. 7 and 8). These facts exclude the possibility of involvement of the photic information from the ocelli or humoral pathways in the mutual interaction and clearly confirm the previous view that the interaction is mediated by neural pathways between the pacemaker tissues, i.e. optic lobes (Tomioka et al. 1991, 1994; Tomioka 1993).

Pathway for modulation of freerunning period

Phase-dependent modulation of freerunning period represents an internal relative coordination between 2 optic lobe pacemakers, indicating that the coupling force is weak (relative to the light entrainment pathway). It is suggested that the period modulation occurs mutually in intact animals and contributes to a synchronous movement of the pacemakers (Tomioka et al. 1991). There are two possible mechanisms underlying the phase dependency of the period modulation. First, some circadian information derived from the contralateral pacemaker may produce the phase-dependent phase shifts of the pacemaker. Second, the pacemaker phase-shifts in a phase-dependent manner even if the information from the contralateral pacemaker is not circadian but constant. Our recent data obtained by electrophysiological experiments support the former possibility: electrical activity toward the optic lobe clearly shows a circadian rhythm with higher firing frequency during the subjective night, and the light induced response is also under circadian control, being higher also during the subjective night (Tomioka et al. 1994). These kinds of circadian neural information probably somehow cooperate to phase-shift (hence modulate the freerunning period of) the pacemaker located in the optic lobe.

The present results revealed that the modulation of the period is mediated by a neural pathway, since an additional severance of the intact optic stalk resulted in disappearance of the modulation (Fig. 5A). Partial destruction of the stalk further clarified that the pathway necessary for the modulation runs along ventral side of the stalk near the lobula. This fact is consistent with our recent finding that some neurons running in the same portion of the stalk encode circadian information as well as photic information that is also modulated in circadian manner (Tomioka et al. 1994). The modulation occurs when the tracts 4, 5, 6, and 7 survived the operation, suggesting one or more of these neural tracts mediate the signals necessary for the period modulation. Among them, tract 5 composed of about 26 large medulla neurons seems to be the most likely pathway, since it is the only one originating from the optic lobe and directly connecting the bilateral pacemaker loci. Our electrophysiological data also suggest that the large medulla neurons encode the circadian information necessary for the mutual interaction (Tomioka et al. 1994).

In cockroaches, similar large medulla neurons have been suggested to be involved in the mutual interaction of the circadian pacemakers (Roth and Sokolove 1975). However, Page (1978) excluded this possibility by showing that lesions in the optic lobe which should have destroyed the axons of those cells had no effect on the interaction. He suggested that the interaction pathway must be polysynaptic and presumably involves synaptic connections in the midbrain and/or the lobula (possible pacemaker site of the cockroach). The discrepancy between crickets and cockroaches should be addressed to further study.

The hypothesis that the mutual coupling is achieved by mutual exchange of the circadian information between pacemakers has been stressed in several opisthobranch molluscan species (Roberts and Block 1985). Pacemakers in such animals are located in the retina and send circadian information coded into the impulse frequency not only to the brain but also to the contralateral retina. Therefore the mutual exchange of circadian information seems to be a common way to keep a stable temporal relationship between pacemakers in invertebrate species.

Pathway for mutual inhibition of activity

Locomotor activity driven by a pacemaker decreased when it occurs during the subjective day of its partner pacemaker (Fig. 2C). This reduced activity is thought to be caused by a phase-dependent inhibitory effect from the partner pacemaker (Tomioka et al. 1991). Although the suppression occurred in both components, quantitative analysis revealed that it was greater in the component driven by the blinded pacemaker (Fig. 2C). This domination of the intact side suggests that some photic information as well as the circadian temporal information is involved in the inhibitory effect.

The results of the present experiment demonstrated that the phase-dependent suppression of activity is also caused by some neurons running the ventral side of the stalk like that for freerunning period modulation: The phase-dependent change in activity level was eliminated in animals with the ventral half of the optic stalk severed (Fig. 8D), while it was clearly observed in those with the dorsal half severed (Fig. 7D).

Although the site of mutual suppression of activity is still largely unknown, some observations allow speculation. The animal with bilateral optic lobe removed exhibits an increased activity level (Tomioka and Chiba 1989), suggesting that somewhere in the central brain may be a possible site of the activity suppression. Our present results suggest the optic lobe to be another possible site. Although animals with the dorsal half of

the optic stalk severed showed a marked activity inhibition when the two pacemakers are out of phase, inhibition of the entrained component during the subjective day was not as strong as was that in unilaterally blinded animals (Fig. 7D). A possible explanation for this is that partial interruption of the inhibitory pathway reduced suppressing signal from the blinded pacemaker to the intact pacemaker. Although it is likely that the suppression of activity partly occurs in the optic lobe, further critical study is required for identification of the site for the mutual suppression of activity.

The suppression of activity by the optic lobe pacemakers has also been observed in the cockroach. Experiments with unilateral optic lobe transplantation revealed that the intact optic lobe perfectly inhibits the activity rhythm driven by the transplanted optic lobe (Page 1983). It is said that in intact animals the suppression of activity occurs mutually through a neural pathway. Mutual suppression of activity is also known in some mammalian species such as *Tupaia* (Meijer et al. 1990) and hamsters (Menaker and Vogelbaum 1993). Taking these facts together, suppression of activity may commonly contribute to keep a stable temporal structure in various animals.

Pathway for driving the locomotor rhythm

It is generally thought that locomotor rhythms of cockroaches and crickets are driven by the optic lobe pacemaker by a neural pathway (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Loher 1972). According to this hypothesis, there are some output neurons of circadian pacemaker originating in the optic lobe and projecting to the cerebral lobe. In the present study, we have demonstrated that destruction of ventral half of the proximal optic stalk near the lobula totally abolished not only the mutual interaction but also the locomotor rhythm driven by the ipsilateral optic lobe (Fig. 8). The result suggests that circadian output for driving the overt activity is also mediated by some neurons running the ventral side of the proximal optic stalk. The fact opens an important question whether common neurons mediate the circadian information necessary for the mutual interaction between pacemakers and driving the overt activity. We still do not have any answer to this question. However, our recent experiments with intracellular staining revealed that many of the large medulla neurons, in tract 5, connecting bilateral medullae regions have collaterals, possible output sites, in both or either of the ipsilateral and the contralateral brain hemispheres, and that they show a circadian rhythm in both spontaneous and light evoked activities (Tomioka and Yukizane in preparation). On the basis of this anatomical and physiological data, it may be possible that the information necessary for both functions is mediated by these neurons.

Our results would contribute to identification of output pathway of the circadian pacemaker that regulates locomotor rhythms. The optic lobe has been recognized as a circadian pacemaker tissue not only in hemimetabolous insects such as crickets and cockroaches (see Page 1988 for review) but also in some holometabolous insects such as beetles (Balkenohl and Weber 1981; Fleissner 1982). However, in no case, has the output pathway for driving the locomotor rhythm been elucidated. Further critical study with a combination of partial destruction of the neural tracts and nickel backfilling will reveal the pathway.

Our present results provide an anatomical base to identify the pacemaker cell(s) in the optic lobe. It has been long time since the optic lobe has reported for the pacemaker locus in the cockroach and the cricket (Nishiitsutsuji-Uwo and Pittendrigh 1968; Loher 1972). Although many works have been performed on this critical issue, no answer has been given so far. The fact that the some neurons in the ventral side of the proximal optic stalk encode the circadian rhythm implies that there may be a circadian pacemaker cell(s) upstream of those neurons. Anatomical and physiological identification of those neurons would lead to elucidation of the pacemaker cell(s).

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