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[REVIEW]

Circadian Organization in Hemimetabolous Insects

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ABSTRACT—The circadian system of hemimetabolous insects is reviewed in respect to the locus of the circadian clock and multioscillatory organization. Because of relatively easy access to the nervous system, the neuronal organization of the clock system in hemimetabolous insects has been studied, yielding identification of the compound eye as the major photoreceptor for entrainment and the optic lobe for the circadian clock locus. The clock site within the optic lobe is inconsistent among reported species; in cockroaches the lobula was previously thought to be a most likely clock locus but accessory medulla is recently stressed to be a clock center, while more distal part of the optic lobe including the lamina and the outer medulla area for the cricket. Identification of the clock cells needs further critical studies. Although each optic lobe clock seems functionally identical, in respect to photic entrainment and generation of the rhythm, the bilaterally paired clocks form a functional unit. They interact to produce a stable time structure within individual insects by exchanging photic and temporal information through neural pathways, in which serotonin and pigment-dispersing factor (PDF) are involved as chemical messengers. The mutual interaction also plays an important role in seasonal adaptation of the rhythm.

Key words: circadian system, clock tissue, hemimetabolous insects, optic lobe

INTRODUCTION

Circadian rhythms are about 24 hour endogenous oscillations that can be observed in most animals. They have several special properties (Edmunds Jr., 1988). They persist in the constant conditions, running with a period slightly different from 24 hr, but synchronize to environmental cycles such as daily light-dark and temperature cycles; the light is the most powerful entraining agent. Their free-running period in constant conditions is nearly unaffected by changes in temperature. They are driven by an endogenous mechanism called a circadian system that consists of three major constituents, i.e. circadian clock that generates ca. 24 hr oscillation, receptors for entraining stimuli and driven system that regulates various physiological functions under the regulation of the clock (Fig. 1; Eskin, 1979). This general scheme led studies for elucidating the localization of the constituents and their interaction to understand the underlying neural mechanism. Hemimetabolous insects such as cockroaches and crickets have been served as good experimental animals to analyze those physiological mechanisms at a neural level (Page, 1985a; Helfrich-Förster *et al.*, 1998;

Tomioka *et al.*, 2001; Homberg *et al.*, 2003). Their relatively large nervous system with identifiable neurons allowed the search for constituents of the circadian system in the CNS. Efforts have resulted in the localization of the circadian clock in each of a bilateral pair of the optic lobe and the major circadian photoreceptor in the compound eye. Subsequent studies have brought about extensive analysis of the neural organization of the circadian system in crickets and cockroaches. However, reported putative locus of the circadian clock is not consistent among species. In cockroaches, the accessory medulla is now recognized as the clock site and its neuronal organization is extensively studied (Loesel and Homberg, 1996; Homberg *et al.*, 2003; Reischig and Stengl, 2003). However, the tissue is not likely clock locus in the cricket *Gryllus bimaculatus*, since removal of the distal part of the optic lobe without damaging the tissue makes the cricket arrhythmic (Okamoto *et al.*, 2001).

The aim of this review is to make an overview of the neural organization of the circadian system in hemimetabolous insects. The locus of the circadian clock is discussed and the role and the biological significance of the bilaterally paired circadian clocks are considered.

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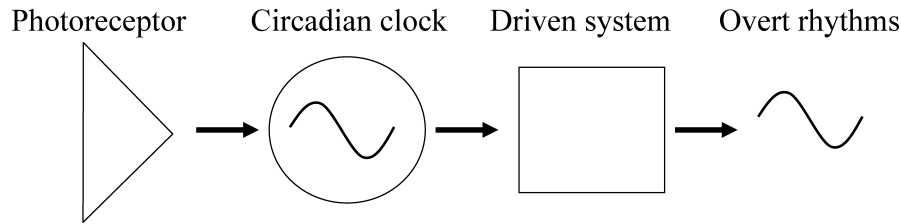


Fig. 1. Hypothetical diagram of the circadian system. Arrows indicate pathways between the components for the direction indicated.

STRUCTURE OF THE CIRCADIAN SYSTEM

Photoreceptor

Entrainment to environmental cycles is a general property of circadian rhythms. Light is the most powerful environmental factor (zeitgeber) for entrainment. The compound eyes are the only photoreceptor for the photic entrainment in cockroaches and most crickets (Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Loher, 1972; Page, 1978; Tomioka and Chiba, 1984): removal of the compound eyes or sectioning of the optic nerves results in a loss of entrainment even in the light-dark cycles. In the cricket *G. bimaculatus*, all parts of the compound eye contribute for the photic entrainment so that the partial reduction of the compound eye leads to weaker entrainment (Tomioka *et al.*, 1990). Green light is most effective for the photic entrainment in cockroaches (Mote and Black, 1981), suggesting the green receptor may be the circadian photoreceptor. The compound eye is the circadian photoreceptor also in a hemipteran insect, *Graphosoma lineatum* (Nakamura and Hodkova, 1998). However, there are some reports suggesting other receptors might be involved in the photic entrainment. The cricket *Teleogryllus commodus* shows light-dependent changes in free-running period after removal of the compound eyes, and ocelli are thought to be the source of the light information for this period modulation (Rence *et al.*, 1988). In species of *Ephippiger*, photic entrainment still occurs after removal of the compound eyes and ocelli by electrocoagulation (Dumortier, 1972). Similar weak photic entrainment can be observed after removal or occlusion of the compound eyes in the New Zealand weta *Hemideina thoracica* (Waddel *et al.*, 1990) and the ground cricket *Dianemobius nigrofasciatus* (Shiga *et al.*, 1999). In these insects, extraocular photoreception might be involved in the photic entrainment. Cryptochrome may be a candidate molecule for the extraretinal circadian photoreception considering its involvement in the *Drosophila* circadian entrainment (Emery *et al.*, 1998). Putative extraocular photoreceptor organs were recently reported for cockroach (Fleissner *et al.*, 2001). They are located distal to the outer chiasma and on the anterior distal surface of the lobula, showing some structural characteristic for a photoreceptor with immunoreactivity to antibody against *Arabidopsis* Cryptochrome 2. However, there is no physiological evidence suggesting its involvement in the photic entrainment.

Recent studies demonstrated that GABA and Mas-allatotropin are the neurotransmitters involved in the photic entrainment pathway in the cockroach *Leucophaea maderae* (Petri *et al.*, 2002). GABA-immunoreactive neurons innervate the optic lobe including medulla and accessory medulla, while Mas-allatotropin-immunoreactive neurons are intrinsic and innervate the accessory medulla. Injection of these substances into the optic lobe induces phase dependent phase-shifts and yielded phase response curves that are quite similar to that for light. Although PDF and serotonin also elicit phase shifts of the locomotor rhythm in phase-dependent manner, they mimic the action of light only in a part of the circadian day (Page, 1987; Petri and Stengl, 1997). These findings suggest that the circadian clock receives photic information through different channels.

Interestingly, the sensitivity of the compound eye, a principal circadian photoreceptor, is controlled by the circadian clock (Tomioka and Chiba, 1982a; Wills *et al.*, 1985). Thus through the entrainment pathway the clock receives cyclical entraining signal even in constant conditions. This oscillatory input might contribute to stabilize the movement of the circadian clock.

Locus of the clock

Circadian clocks have been localized in several insect species. For locomotor rhythms, the optic lobe has been recognized as the locus of the principal clock in cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968a), crickets (Tomioka and Chiba, 1984; Abe *et al.*, 1997) and New Zealand weta (Waddel *et al.*, 1990). It is also true in some holometabolous insects such as beetles (Balkenohl and Weber, 1981). This is mostly based on the finding that the removal of the tissue resulted in arrhythmicity in their locomotor activity. In cockroaches, transplantation of the optic lobe to an animal that had been arrhythmic by bilateral optic lobe removal restored circadian rhythm with a period close to that of the donor (Page, 1982). The optic lobe of the hemimetabolous insects consists of three neuropils, i.e., lamina, medulla and lobula from distal to proximal in this order (Fig. 2). With precise surgical lesions within the optic lobe, the locus of the clock was further restricted to the ventral side of the lobula region (Sokolove, 1975; Page, 1978). Experiments with measurement of electroretinogram (ERG) of the compound eye also supported the view: ERG rhythms persisted after cutting the optic tract but were eliminated after

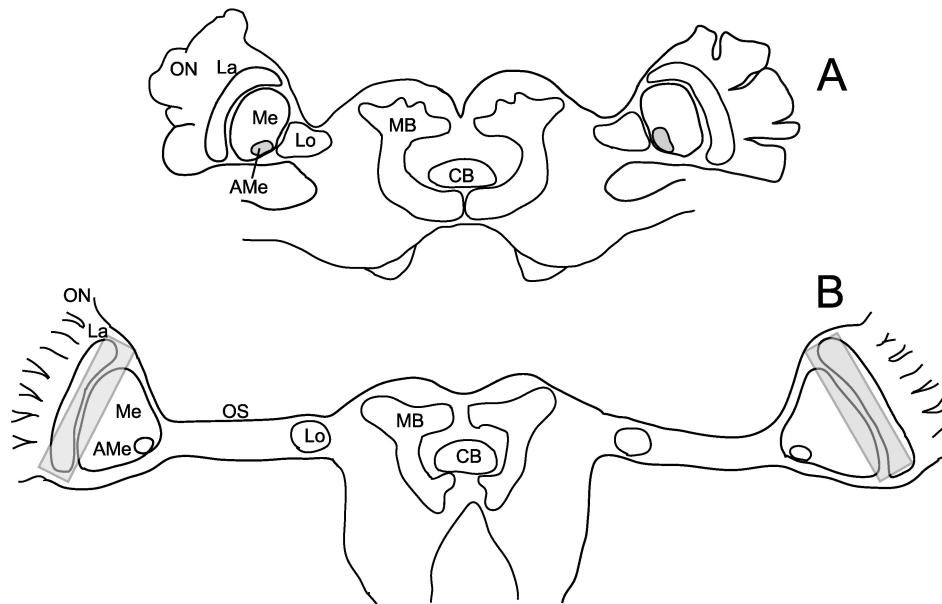


Fig. 2. Schematic drawing of cerebral lobes of the cockroach (**A**) and the cricket (**B**). Shaded areas indicate the plausible loci of the circadian clock. AMe, accessory medulla; CB, central body; La, lamina; Lo, lobula; MB, mushroom body; Me, medulla; ON, optic nerve; OS, optic stalk. **A:** modified from Reischig and Stengl (2002).

removal of the lobula (Wills *et al.*, 1985). The importance of the lobula in the ERG rhythm was stressed also in the beetle, *Anthia sexguttata* (Fleissner, 1982). In crickets, the lobula region is not the locus of the clock because removal of the outer two neuropils, i.e., the lamina and medulla, leads to arrhythmia (Tomioka and Chiba, 1984), and the neural activity of the lamina-medulla-compound eye complex shows circadian rhythms (Tomioka and Chiba, 1992).

Stengl and her coworkers suggested that the accessory medulla area with associated PDF-immunoreactive neurons lying at the ventromedial edge of the medulla is the most likely locus of the cockroach circadian clock (Fig. 2A). This small neuropil was first found in holometabolous insects as a remnant of the larval optic neuropil (Pflugfelder, 1936), but also exist in hemimetabolous insects (Homborg *et al.*, 1991). The most convincing evidence for its importance is that transplantation of the tissue into optic lobeless cockroaches restored locomotor rhythms in *L. maderae* (Reischig and Stengl, 2003). Importance of the neuropil is further stressed in recent review by Helfrich-Förster (2004). However, our partial lesioning experiments revealed that the removal of the lamina and the outer medulla area resulted in arrhythmic activity in the cricket (Okamoto *et al.*, 2001), suggesting this area, not the accessory medulla, to be the likely locus of the clock at least in the cricket, *G. bimaculatus* (Fig. 2B).

Since PDF is expressed in a group of *period* gene-expressing neurons located laterally in the protocerebrum of *Drosophila* (Helfrich-Förster, 1995), the PDF-immunoreactive neurons have notified as putative clock neurons. Fig. 3 shows PDF-immunoreactive neurons in the optic lobe of the cricket *G. bimaculatus*. There are three clusters of PDF-

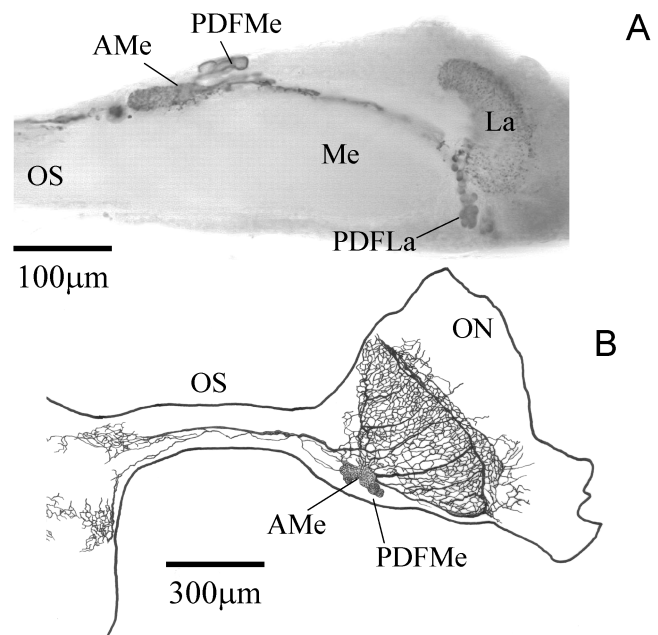


Fig. 3. PDF-immunoreactive neurons in the optic lobe of the cricket *G. bimaculatus*. **A:** Horizontal section of an optic lobe. Photograph is oriented with the anterior region at the top. **B:** Camera lucida drawing of PDF immunoreactive neurons in an optic lobe. Drawing is oriented with the dorsal side at the top. AMe, accessory medulla; La, lamina; Lo, lobula; Me, medulla; ON, optic nerve; OS, optic stalk; PDFMe, PDF-immunoreactive neurons located at the proximal medulla; PDFLa, PDF-immunoreactive neurons located near the outer chiasma.

immunoreactive neurons in the optic lobe of crickets and cockroaches, i.e. neurons located near proximal medulla with innervation of accessory medulla (PDFMe) and those

located ventrally and dorsally near the outer chiasma (PDFLa). Stengl and coworkers stress the PDFMe to be the likely candidate of the circadian clock (Stengl and Homberg, 1994; Reischig and Stengl, 2003). The rather indirect evidence for their argument includes that reappearance of the rhythm in the optic stalk severed cockroaches correlates with the regeneration of PDF-immunoreactive fibers from the accessory medulla to the median protocerebrum (Stengl and Homberg, 1994) and that a similar correlation between the rhythm restoration and the PDF innervation was found when an AMe from a rhythmic donor was transplanted to an arrhythmic host cockroach (Reischig and Stengl, 2003). Results of immunostainings with anti-*Periplaneta* PER antibody in *Periplaneta americana* are not perfectly consistent with their view: PER-immunoreactivity was observed in two clusters of small sized cells located dorsal and ventral to the outer optic chiasma in addition to the neurons in the pars intercerebralis and pars lateralis in the central brain (Takeda *et al.*, 2000). Recent findings in two cricket species *Teleogryllus commodus* and *T. oceanicus* (Lupien *et al.*, 2003) using antibodies against *Drosophila* PER might support their view, however. Anti-PER-immunoreactive neurons located near the medulla send dens tufts of small arborizations into the accessory medulla, processes to the medulla and the proximal lamina area, and project through the optic stalk. Some of them are also immunoreactive to anti-PDF antibody. However, our careful examination of the role of PDF neurons in the cricket *G. bimaculatus* in combination of partial removal of optic lobe with immunohistochemistry using anti-PDF antibody revealed that the PDFMe neurons located near the accessory medulla are not the clock neurons at least in the cricket *G. bimaculatus* (Okamoto *et al.*, 2001): The destruction of the distal area of the optic lobe eliminates the circadian locomotor rhythm without affecting PDFMe neurons.

In the cricket *Acheta domesticus*, on the other hand, the pars intercerebralis was reported to be the locus of the circadian clock. The neurosecretory cells in this area show circadian rhythm in RNA and protein synthesis (Cymborowski and Dutkowski, 1969, 1970). Destruction of the neurosecretory cells in this area results in arrhythmic activity and the implantation of the brain restores rhythmicity with a period close to that of the donor (Cymborowski, 1981). Unfortunately, the potential importance of the optic lobe has not been examined yet in this particular species.

An important point to be noted is that the circadian clock may be formed by many clock neurons. PER-immunostainings have revealed that in many insect species, several clusters of PER-immunoreactive neurons are located in the optic lobe as well as in the central brain (Lupien *et al.*, 2003; Zavodska *et al.*, 2003). Their number of clusters and localization vary from species to species. The multicellular organization of the circadian clock is true for *Drosophila*: There are at least 6 groups of neurons expressing clock genes (Kaneko and Hall, 2000). The role of each group of neurons are now under investigation and it becomes evident

that neurons located ventrally near the optic lobe play principal role in generating the locomotor rhythm (Helfrich-Förster, 2003). Likewise the role of each neural group may be different and which group takes the principal role might differ depending on species. For further dissection of the circadian organization in hemimetabolous insects, the localization of the clock neurons is prerequisite and awaits further critical study.

Secondary oscillator

Both in crickets and cockroaches, lines of evidence suggest the existence of a secondary oscillator for the locomotor rhythm in the central brain. After bilateral removal of the optic lobe, some crickets showed residual rhythms that gradually disappeared within several days (Tomioka, 1985). Optic lobeless crickets showed locomotor rhythms in light dark cycles when the neural connection between the retina and the central brain was regenerated (Tomioka and Chiba, 1989a). In response to temperature cycles, optic lobeless crickets and cockroaches exhibited locomotor rhythms (Rence and Loher, 1975; Page, 1985b). Phase of the rhythm changed dependently on the period (T) of temperature cycles (Page, 1985b) or the rhythm appeared only when T was close to 24 hr (Rence and Loher, 1975). The oscillator seemed highly damping since the activity quickly became arrhythmic when transferred to constant conditions. In accordance with the empirical view of master-slave organization, the secondary oscillator is hypothesized to be driven by the circadian clock in the optic lobe (Page, 1985b; Tomioka and Chiba, 1989a).

Output pathway of the circadian clock

The output pathway for driving the locomotor rhythm still remains to be uncovered. The electrical activity of the optic lobe efferent neurons toward the central brain apparently plays important roles, since transection of the optic stalk or optic tract leads to a loss of circadian locomotor rhythm. The projection from the optic lobe to the central brain has been examined in detail in crickets (Honegger and Schürmann, 1975; Tomioka *et al.*, 1994). They are distributed in the wide range both in the ipsilateral and the contralateral hemisphere. A cluster of about 25 neurons called medulla bilateral neurons (MBNs) are likely candidate of the output neurons (Yukizane *et al.*, 2002). MBNs are visually responding interneurons directly connecting two optic medulla areas (Fig. 4). They have somata near ventral edge of proximal medulla, dendrites extending over the medulla and axons running toward the contralateral optic lobe. They also have some collaterals in the posterior area of the ipsilateral and/or contralateral lateral protocerebral lobe. They show circadian rhythms both in spontaneous and light induced responses (Yukizane *et al.*, 2002). Partial destruction of the optic stalk including the MBNs abolishes the circadian locomotor rhythm driven by the optic lobe clock (Yukizane and Tomioka, 1995). Thus the lateral protocerebral areas are the likely output area of the clock signal for

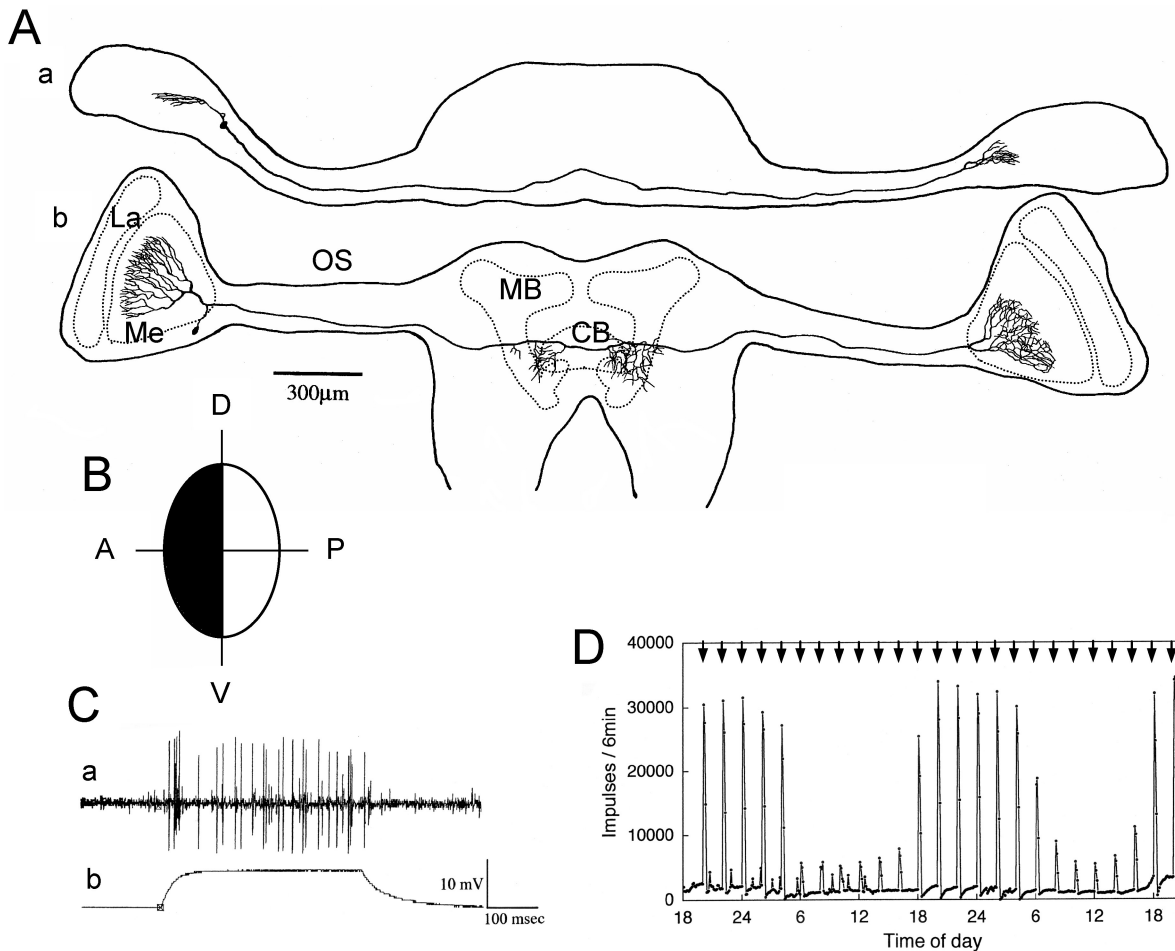


Fig. 4. Morphology and photo-responsiveness of a medulla bilateral neuron (MBN) in the cricket *G. bimaculatus* that have a receptive field in the anterior region of the compound eye. **A:** Dorsal (**a**) and posterior views (**b**) of a MBN that was stained by intracellular injection of Lucifer Yellow CH. CB, central body; La, lamina; MB, mushroom body; Me, medulla; ON, optic nerve; OS, optic stalk. **B:** Schematic representation of its receptive field. A, D, P, and V indicate anterior, dorsal, posterior and ventral, respectively. **C:** Extracellular recording of its electrical response given to the compound eye. **a:** Electrical response of the neuron. **b:** Light stimulus. **D:** Circadian rhythm of the responsiveness to 15-min light pulses given every 2 hr in constant darkness. Arrows indicate the time of light pulse was given. After Yukizane *et al.* (2002).

driving the locomotor activity. They lack innervations to the accessory medulla and this fact is consistent with our view that the accessory medulla is not a circadian clock locus in the cricket. Cockroaches also have similar neurons although their physiological properties are not clear (Roth and Sokolove, 1975; Reischig and Stengl, 2002).

PDF neurons might be another candidate of output neurons. In *Drosophila*, PDF is expressed in so-called ventral lateral neurons (LNvs), putative clock neurons (Helfrich-Förster, 1995), and *pdf⁰* mutant flies lacking PDF show aberrant rhythm in constant darkness (Renn *et al.*, 1999). In crickets and cockroaches, PDF has phase-shifting effects on the circadian clock (Petri and Stengl, 1997; Singaravel *et al.*, 2003). PDF-immunoreactive neurons are thought to be the likely candidate of clock neurons in cockroaches, as has been mentioned. Transplantation of the accessory medulla graft including PDF neurons occasionally restores the locomotor rhythm in optic lobe ablated arrhythmic cockroaches (Reischig and Stengl, 2003); the target area of reinnervation

of transplanted PDF fibers, the superior median and lateral protocerebrum, are thought to be important for the coupling between circadian clock and the locomotor activity.

Some neurosecretory factor might be involved in the generation of the locomotor rhythm. Restoration of the *Acheta domesticus* locomotor rhythm by implantation of the brain into the abdomen of arrhythmic recipients, of which neurosecretory cells in the pars intercerebralis had been destroyed, suggests that the implanted brain released some secretory factor to regain the rhythmic activity (Cymborowski, 1981). Overall, the output channel for driving the rhythm may be multiple.

In *G. bimaculatus*, the locomotor rhythm reverses from nymphal diurnal to adult nocturnal pattern 3~5 days after the imaginal molt (Tomioka and Chiba, 1982b). This reversal is associated with the increase of serotonin level in the brain (Nishinokubi and Tomioka, 2000). The peak activity in nymphal rhythms occurred in the middle of the subjective day, while in the adult at the early subjective night at 25°C

(Tomioka and Chiba, 1982b). When temperature was lowered to 20°C, adult crickets often show an additional peak at the middle of the subjective night (Ikeda and Tomioka, 1993; Akashi and Tomioka, unpublished). This suggests that the optic lobe pacemaker outputs signals at two phases, at the early subjective night and mid subjective day, to drive the activity. There seems a temperature sensitive switching mechanism in the output pathway.

COUPLING BETWEEN OSCILLATORS

To keep a temporal order within a single animal, the constituent oscillators must maintain an appropriate phase angle relationship. There are several model systems to dissect the mechanism through which the constituent oscillators to keep a stable phase relationship. In cockroaches, the locomotor rhythm is regulated with tightly coupled two optic

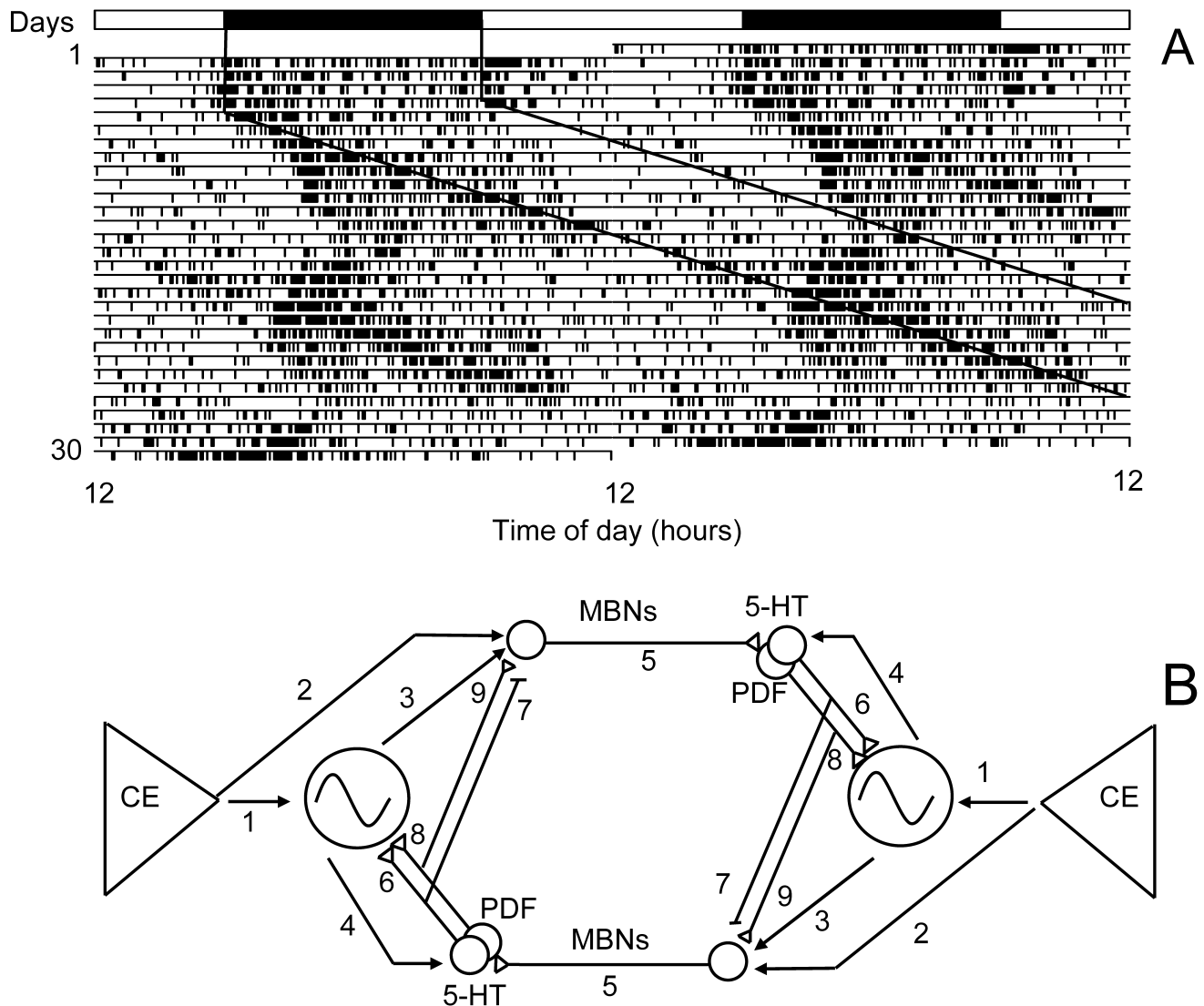


Fig. 5. Mutual interaction between the bilateral optic lobe circadian clocks that appeared in the locomotor rhythm (**A**) and its underlying neural mechanism (**B**). **A:** Locomotor rhythm of an adult male cricket, *G. bimaculatus*. White and black bars above actogram indicate the initial light (white) and dark (black) cycle. For the LD13: 13, the dark portion was indicated by the black oblique lines. The cricket was kept under LD12: 12 for the first 4 days and transferred to LD13: 13 on day 5 after unilateral severance of the optic nerve. Then two rhythmic components appeared: one was readily entrained to the given LD and the other ran free but with the marked fluctuation of its free-running period. The period was lengthened when the onset of the entrained component occurred at its subjective night but was shortened when it occurred at its subjective day. **B:** Schematic diagram of the neural circuit for the mutual interaction. CE, compound eye; 5-HT, serotonergic neurons; PDF, PDF neurons; MBNs, medulla bilateral neurons. The circadian clock in the optic lobe synchronizes not only to the environmental light dark cycle through the photic information from the compound eye (1) but also to their contralateral partner. MBNs are the major component of the coupling system and receive photic information from the photoreceptor (2) and the circadian information from the clock (3) on their own side. Serotonin and PDF are probably released under the regulation by the circadian pacemaker (4) and the contralateral MBNs (5). The released serotonin and PDF shift the phase of the pacemaker (6, 8) in a phase-dependent manner, and at the same time, they reduce or increase the coupling signals by suppressing (7) or enhancing (9) the activity of MBNs.

lobe circadian clocks (Page, 1983): When one optic nerve was severed, the blinded clock always synchronized to the contralateral intact side. When the optic tract was severed the coupling pathway never regenerated. There is a pathway through which the clock modulates the activity. The coupling makes the free-running period shorter than that of each circadian clock: The free-running period in cockroaches with a single optic lobe was slightly but significantly longer than in intact animals (Page *et al.*, 1977).

In crickets, the coupling between the optic lobe clocks is much weaker than in cockroaches. The circadian stridulatory rhythms of the cricket *T. commodus* sometimes spontaneously split into two activity components in constant light or after a low temperature pulse (Wiedenmann, 1983). The splitting was more frequently induced by cutting the optic nerve unilaterally. Similar splitting was reported for locomotor rhythms in other species of crickets, *G. bimaculatus* and *Gryllobates sigillatus* (Tomioka *et al.*, 1991; Ushirogawa *et al.*, 1997). When the optic nerve was unilaterally severed and the animal was kept in constant light, there occurred two rhythmic components: one ran with a period longer than 24 hr and the other shorter than 24 hr. When light cycle was given, the rhythm driven by the intact clock synchronized to the given light cycle and the other ran free but with marked fluctuation in its free-running period (Fig. 5A). The period was slightly shortened when the entrained component occurred during the subjective day of the free-running component, while lengthened during the subjective night (Tomioka, 1993).

The coupling is achieved by exchanging the clock information between the bilateral optic lobes in crickets. The information is electrical and conducted by MBNs. Their spontaneous and light-evoked activity has a circadian rhythm with greater responses during the subjective night (Tomioka *et al.*, 1994; Yukizane *et al.*, 2002). The electrical information may cause phase-dependent phase shifts in the contralateral circadian clock through serotonergic system (Tomioka, 1999). As schematized in Figure 5B, serotonergic and PDF systems play important roles in regulating the circadian rhythm of MBNs (Saifullah and Tomioka, 2002, 2003a, b): Electrophysiological and biochemical data suggest that serotonin is released during the day and reduces the responsiveness of MBNs probably through 5HT₇-like receptors to set the day state, while PDF is released during the late day to early night to increase the responsiveness, setting the night state. Involvement of the serotonergic and PDF systems is also suggested in the coupling system in the cockroach (Page, 1987; Stengl and Homberg, 1994; Petri and Stengl, 1997).

SIGNIFICANCE OF MULTI-OSCILLATOR SYSTEM

Central vs peripheral clocks

Animals generally show circadian rhythms in many physiological functions and one general idea on the underlying system is that it is composed of many circadian oscillators

lie in different tissues and some specific tissues serve as a driving oscillator or a master clock to regulate all other clocks in the hierarchical structure (Pittendrigh, 1974). The idea is supported by findings from behavioral and physiological experiments (Pittendrigh *et al.*, 1958; Hardeland, 1982) and more recently by results of molecular studies of clock gene expression not only in *Drosophila* but also some vertebrate species (Plautz *et al.*, 1997; Sakamoto *et al.*, 1998; Giebultowicz, 1999). In flies, most of the tissues show rhythmic expression of a clock gene, *period*, that independently entrained by light cycles when cultured (Plautz *et al.*, 1997). In a hemimetabolous insect, *Rhodnius prolixus*, a peripheral circadian clock has been found in the prothoracic gland for synthesis of ecdysteroids (Vafopoulou and Steel, 1998).

In crickets and cockroaches, however, the optic lobe contains a more centralized clock that regulates a variety of physiological functions. For crickets, the optic lobe circadian clock drives circadian rhythms of locomotor and stridulatory activity (Loher, 1972; Tomioka and Chiba, 1984), spermatophore production (Loher, 1974), ERG amplitude of the compound eye (Tomioka and Chiba, 1982a), and responsiveness of the visual interneurons (Tomioka and Chiba, 1986). Cockroaches also show similar rhythmicity except for stridulatory rhythm. In addition to these, the antennal sensitivity rhythm to odor stimuli was recently shown to be driven by the optic lobe clock (Page and Koelling, 2003). The cuticular formation rhythm is the only exception so far. It persists even in isolated cultured conditions, thus may be driven by the oscillator in epidermal cells (Wiedenmann *et al.*, 1986).

Significance of paired clocks

The optic lobe is bilaterally paired structure; hence, crickets and cockroaches have two circadian clocks. Unilateral removal of the optic lobe revealed that a single optic lobe clock can regulate the locomotor rhythm (Page, 1978; Okada *et al.*, 1991). Then why these insects have two redundant bilaterally paired circadian clocks? One parsimonious explanation may be that the bilaterally symmetrical structure of the nervous system entails the paired redundant clock structures. More functional reasons may be that novel physiological properties are yielded by the coupling.

One is the stability of the output of the circadian system. Both in crickets and cockroaches, the two optic lobe clocks not only drive the locomotor activity during their subjective night but have some inhibition on the activity (Page, 1983; Tomioka *et al.*, 1991; Ushirogawa *et al.*, 1997). In crickets, the inhibitory action occurs only during the subjective day of the clock so that the activity is confined within the period where the subjective nights of the two oscillators overlap (Tomioka *et al.*, 1991, 1993). This assures stability of the rhythm not only in entrained conditions but also in free-running state.

Changes in free-running period are the other notable property of the coupled clock system. It is slightly longer when coupled in the crickets *G. bimaculatus* (Okada *et al.*, 1991), but shorter in cockroaches (Page *et al.*, 1977). The

changes may be caused through the mutual phase shifting (Tomioka, 1993) and probably contribute toward the stability of the frequency (Pittendrigh, 1974) and entrainability of the system (Eskin, 1971), although detailed analysis has not been performed yet in these insects.

Seasonal adaptation

One important functional role of the clock system is to time physiological functions appropriately in seasonally changing environment. For example the circadian locomotor rhythm of the cricket *G. bimaculatus* is known to change in response to given photoperiods (Tomioka and Chiba, 1989b). To adapt seasonal changes in photoperiod, it is hypothesized that two consisting clocks change their properties by synchronizing to seasonally changing lights-on and -off, respectively, to adapt the active and rest phases to specific photoperiods (Tomioka and Chiba, 1989b). The modulated circadian waveform is maintained in constant conditions. Although this change is at least partly attributable to the modulation of circadian rhythm in the optic lobe circadian clock (Tomioka and Chiba, 1989c), its complete maintenance requires the coupling between the right and left circadian clocks, since when one optic lobe is removed the waveform changes significantly (Tomioka and Koga, unpublished).

It is noteworthy that the stridulatory rhythm of the cricket *Teleogryllus commodus* is hypothesized to be governed by weakly coupled two circadian clocks with differential entrainability to light dark cycles (Wiedenmann and Loher, 1984). The cricket shows a nocturnal singing rhythm and the clocks govern the early part and the latter part of the subjective night, respectively. The clocks are most likely located in each of the two optic lobes, respectively, considering a loss of the singing rhythm after removal of the optic lobe (Loher, 1972; Wiedenmann, 1983). Therefore, it is plausible that the two circadian clocks are not only bilaterally redundant but also have functional differentiation. This dual clock system is reminiscent of mammalian two oscillator model with a morning and an evening oscillator (Pittendrigh and Daan, 1976). The dual clock system seems suitable for seasonal adaptation of the activity rhythm with changing day length.

CONCLUSION

Hemimetabolous insects have provided a useful model to investigate the neural mechanism of the circadian clock system at a cellular level, especially for functional analysis of multi-oscillator structure. The circadian system comprised of bilaterally paired optic lobe clocks can be easily manipulated both surgically and pharmacologically. Some of the output neurons, such as PDF neurons and MBNs, have been characterized. The most important issue to be addressed is the identification of the clock neuron. Pulse and chronic treatments with a translation inhibitor, cycloheximide, suggested that the circadian clock in crickets is also based on the molecular cycling (Tomioka, 2000). Probably

using appropriate probes, the clock cells would be finally identified. Then, the study for understanding the whole circadian system would be promoted at the cellular level and contribute toward understanding the neural mechanism of the circadian timekeeping.

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