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Circadian Firing Activities of Neurosecretory Cells Releasing Pheromonotropic Neuropeptides in the Silkmoth, *Bombyx mori*

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ABSTRACT—Neurosecretory cells releasing the pheromone biosynthesis-activating neuropeptide (PBAN) in *Bombyx mori* exhibit diurnal firing activity. Diel changes in the firing activity of the PBAN producing cells persisted in both constant dark and constant dim light (0.1 lx) at mean periods of 23.0±1.6 hr and 22.6±0.8 hr, respectively, thereby suggesting that the neurosecretory cell system is under the control of a circadian pacemaker. The circadian firing rhythm was greatly modified by background illumination: (1) the period of free-running activity rhythm was significantly short (18.5±1.6 hr) under continuous illumination of a moderate intensity (100 lx) and (2) the duration of a firing period of cells elongated by 2.1±0.7 hr, when light intensity during a photophase was lowered to 0.01 lx. The suppressive effect of light on the firing activity may induce a nocturnal component of a daily activity pattern by releasing PBAN cells from suppression after termination of illumination.

INTRODUCTION

Many physiological and endocrinological processes are under the control of endogenous circadian oscillators that entrain to daily light/dark cycles. Female moths extrude the pheromone gland to attract males during specific times of the day and the production and emission of sex pheromone appears to operate on a circadian cycle (Delisle and McNeil, 1987; Raina *et al*., 1991; Webster and Yin, 1997; Choi *et al*., 1998b). Sex pheromone production is regulated by pheromonotropic neuropeptides, such as pheromone biosynthesis-activating neuropeptide (PBAN) and PBAN-like factors (Raina, 1993; Teal *et al*., 1996; Ma *et al*., 2000). PBAN is generated along with four additional family peptides from a common precursor polyprotein translated from a single mRNA (Kawano *et al*., 1992; Sato *et al*., 1993; Ma *et al*., 1994, Choi *et al*., 1998a). There are three known clusters of neurosecretory cells expressing the gene for the precursor protein in the suboesophageal ganglion (SOG) of *Bombyx mori* (Sato *et al*., 1994). A surgical ablation experiment revealed that two anterior clusters of cells are functionally specialized for the secretion of PBAN in an adult female moth, whereas the remaining posterior one is largely involved in secretion of another hormone (diapause hormone) during adult development (Ichikawa *et al*., 1996b). The former neurosecretory cells project an axon to the contralateral corpus cardiacum (CC) after passing through a branch of the maxillary nerve and release PBAN into the haemolymph (Ichikawa *et al*., 1995).

Long-term chronic recordings from the maxillary nerve branch revealed complex firing activity rhythms of PBAN producing cells that are closely related to calling behavior, circulation of haemolymph, and diurnal changes in pheromone titers in a light/dark cycle (Ichikawa, 1998). Because electrical signals from neurosecretory cells can be sampled from a single female moth with a higher temporal resolution, analyses of the diurnal firing rhythms of PBAN cells may facilitate understanding of circadian control of pheromone production of individual moths and the nature of a circadian oscillator of the adult silkmoth. We now report that firing activities of PBAN cells showed a circadian oscillation. Periodicity of this oscillation was strongly modified by background illumination.

MATERIAL AND METHODS

Commercially available F1 hybrid of *Bombyx mori*, Kinshu ×Showa, was used. Pupae were purchased from a supplier and placed at 26±1°C under a 16-hr light/8-hr dark photoperiod until eclosion.

After removing all legs of a 1-day-old female moth, the ventral part of the thorax and wings were fixed to a platform at an angle of 60° with paraffin. The head was immobilized with paraffin and part of the cuticle over the SOG was removed to exposed a branch of maxillary nerve, nervus corporis cardiaci ventralis (NCC-V). The NCC-V was cut and its proximal stamp was introduced into a suction electrode. A piece of silver wire serving as an indifferent electrode was made to contact the haemolymph, and the cuticular window was sealed with melted paraffin. Electrical signals were amplified, digitized at 400 Hz, and stored on a computer equipped with an analog/digital converter [1401 plus (Cambridge Electronic Design, Cambridge, UK)]. Spike-sorting software (Spike 2, Cambridge Electronic Design) was used to discriminate compound action potentials originating from syn-

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chronously active cells (Ichikawa *et al.*, 1999). The number of action potentials was counted every one minute to draw a firing activity pattern of a set of PBAN cells.

During chronic recordings, each animal was placed in a small box (20×20×25 cm) with a window (6×9 cm) in the ceiling. The window was covered with a piece of tracing paper serving as a diffuser. The animal in the box was illuminated through the window, using a fluorescent lamp. The intensity of illumination in the box was measured, using an illuminometer (IM-3, Tokyo Optical Co., Tokyo), and adjusted to 100 lx as a standard intensity. Further attenuation of illumination in the box was made by covering the window with calibrated neutral density filters made of electron microscopic film.

To calculate (free-running) periods of the firing activity rhythm of PBAN cells, the phase reference point of the rhythm was usually defined as the time at which the moving average of firing rates for 7 min exceeded 50% maximal firing rate.

RESULTS

Daily activity patterns

Firing activities of PBAN producing cells were recorded from NCC-V in 21 virgin females kept under 16-hr light/8-hr dark cycles at the light intensity of 100 lx. Firing rates of the cells increased rapidly 5–30 min after the onset of light and the increased level of firing activity continued for 8–14 hr before there was a rapid decline in the rates to near zero. Duration of the active period determined at about 20% of maximal activity during earlier 3 days of recordings was 11.8±1.6 hr (mean±SD), though amplitudes and durations of active periods often gradually decreased. Firing activity patterns were somewhat complex and a minor component was often observed after onset and/or offset of light (Fig. 1). There was a large variation in amplitude and duration of the minor components among different animals: a distinct initial component after the onset of illumination was found in five females, a nocturnal component after the offset of illumination was found in seven animals, and both were found in three females.

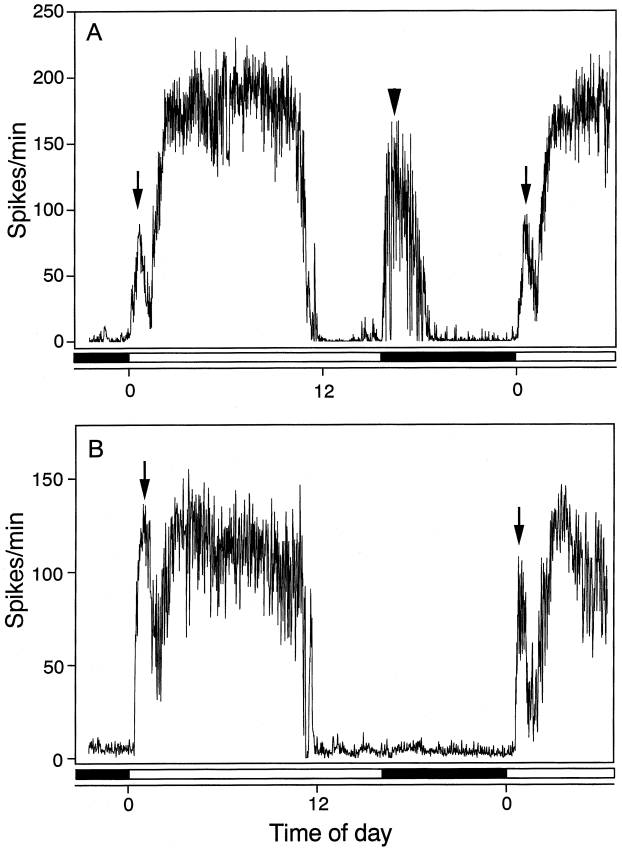

Fig. 1. Two examples of daily firing activity patterns of PBAN cells under a 16-hr light/8-hr dark condition. Light (100 lx in intensity) and dark periods are indicated by open and solid bars, respectively. Note a minor component appearing at the beginning of the photophase (arrows) and the scotophase (arrowhead).

Free running rhythms under constant conditions

Twenty females were held in continuous darkness (DD) after completing two LD cycles. Daily activity rhythms of PBAN cells in only two females persisted in DD (Fig. 2), though there

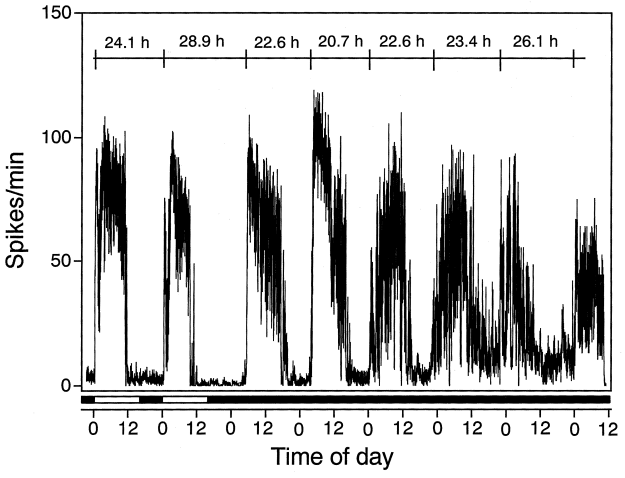

Fig. 2. Circadian rhythm in the firing activity of PBAN cells transferred from light/dark cycles to continuous darkness. Light intensity of the photophase was 100 lx.

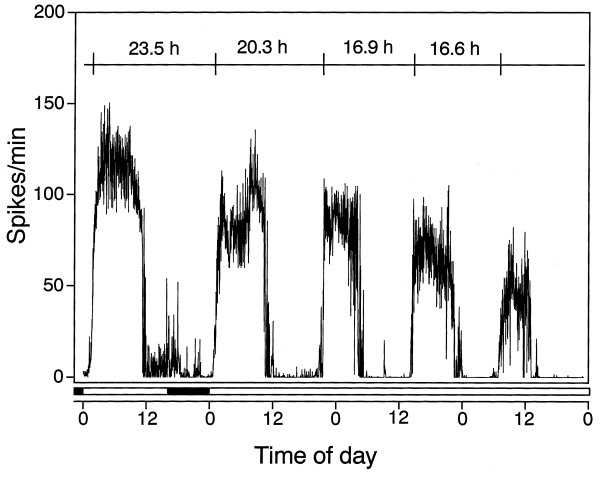

Fig. 3. Example of a free running activity rhythm of PBAN cells persisting under a constant light condition. The intensity of light was 100 lx. Note short periods of the rhythm.

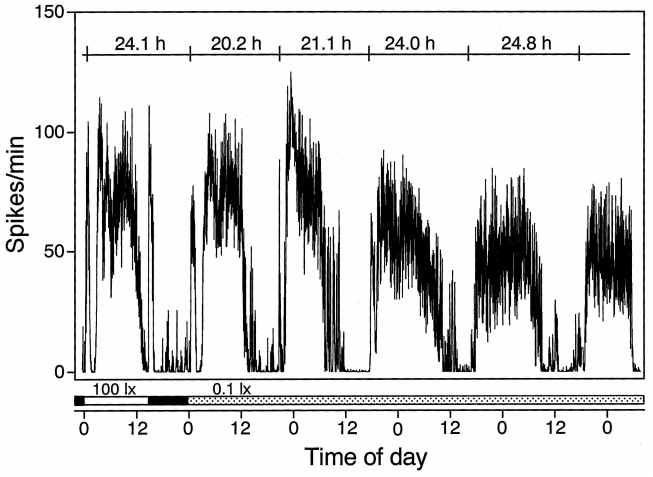

Fig. 4. Free running activity rhythm of PBAN cells persisting under a constant dim light condition (shaded bar). When the intensity of constant light was low (0.1 lx), the rhythm periods became circadian (about 24 hr). The animal died at the end of the record.

was a delay of an active period at the day of transfer to DD condition. The rising and falling of firing activity became slower, as the DD period extended. Mean periods of the free-running rhythm were 22.5 hr (Fig. 2) and 23.6 hr. Firings of PBAN cells in the remaining 18 females stopped in the complete darkness throughout recording periods of 3–5 days.

Free-running activities of the neurosecretory cells were also recorded under constant illumination (LL) of 100 lx. PBAN cells in many females exhibited a distinct firing rhythm but periods of the rhythm were usually shorter than 20 hr (Fig. 3). The mean period of the rhythms was 18.5±1.6 hr (N=8).

To reveal effects of light on the rhythmicity, the intensity of background illumination was then reduced to 0.1 lx. A free running period was elongated under such a dim LL condition (Fig. 4). The mean period of the rhythms was 22.6±0.8 hr (n=11). Duration of a bout of the firing period lengthened (13.9±1.6 hr) and often exceeded 16 hr (Fig. 4).

Influence of light on activity patterns

To examine effects of light intensity on the duration of active periods in LD cycles, light intensity of the photophase on day-2 and day-3 was reduced at a step of 1 log unit. A higher rate of firings of PBAN cells in most females continued until near the end of the photoperiod at 0.01 lx (Fig. 5), but such an elongation of active periods was not usually significant at >0.1 lx. When the start and end of an active period were defined as the time at which the firing rate become 20% of maximum firing rate, a mean increment of duration after the transfer to the dim light condition (0.01 lx) was 2.1±0.7 hr (n=11). A transfer from the dim LD to the complete darkness induced a further elongation of the duration in a few animals (Fig. 5A). A transfer back from the dim LD to the standard LD cycle shortened the active periods (data not shown).

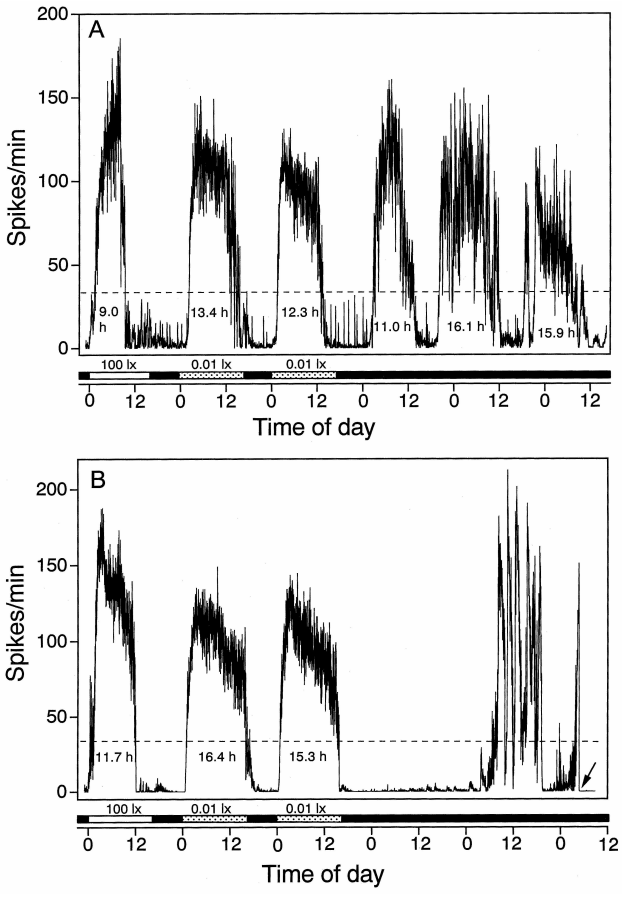

Fig. 5. Two examples of the elongation of the duration of firing activity of PBAN cells induced by transfers to a dim light/dark condition and to continuous darkness. A female (B) lacks the first bout of firing activity of cells in DD. A dim light period is indicated by a shaded bar. A dotted line indicates the level of firing rate at which the duration of a firing period was determined. Arrow, death of the animal.

DISCUSSION

Firing activity rhythm of PBAN cells free-ran at a period of 22–24 hr in DD and dim LL conditions (Figs. 2 and 4), thereby indicating that sex pheromone production is under the control of a circadian pacemaker. It is often difficult to find a robust circadian rhythm in pheromone production by measuring pheromone contents in the gland because of a slow decline of pheromone titers after a stop of PBAN secretion (decapitation or mating) (Ichikawa, 1995, 1998). It should be noted that the proportion of females expressing a circadian oscillation of PBAN cells in DD was significantly low (10%); one reason being that complete darkness may be repelled by many female moths of *Bombyx mori* to perform calling behavior, because an abrupt termination of illumination of 100 lx at a mid-photophase stopped firings of PBAN cells and such a silent state often continued for as long as the darkness continued (unpublished observation). A short stay in a dim LD condition before a transfer to DD appears to mitigate the repellency (see Fig. 5). Another reason may be that a restraint condition, especially a fixation of the head, strongly suppressed

initiation of calling behavior: only a half the number of restrained females began to call even in the next photophase while >90% of the females with a mobile head displayed calling behavior. When the latter females were transferred from LD to DD, 35% revealed calling behavior in DD, with a mean free-running period of 23.5±1.2 hr (n=17), a value which did not significantly differ from that in the restrained females. Thus, a free-running activity rhythms of PBAN cells in a few restrained females (Fig. 2) may represent an innate rhythmicity of a circadian pacemaker in *Bombyx mori*.

In arthropods, the period of the free-running rhythms in DD is generally shorter than that in LL (Aschoff, 1979). The free-running period of PBAN cells of *Bombyx* was greatly shortened under constant illumination of 100 lx and it was often shorter than the lower limit of "circadian" period (20 h) (Fig. 3). Prolonged active periods of PBAN cells occurred during DD (Fig. 2) or dim light conditions (Figs. 4 and 5) rather than in LL of 100 lx. The results in the present study suggest that a female moth of *Bombyx* somewhat prefers a dim light condition for calling rather than a bright light condition or complete darkness. A mated female of the moth lays eggs mainly around dusk (a dim light condition) under a natural light regime (Yamaoka *et al*., 1976).

The active period in DD or a dim light condition often exceeded the length of a photophase (16 hr) (Figs.2, 4 and 5), while there was no marked change in the amplitude of firing activity. The result suggests that light has a strong suppressive effect on the firing activity of cells during the late photophase. The nocturnal component found at the early scotophase (Fig. 1A) may be a re-appearance of a remaining firing activity of PBAN cells released from suppression in the dark. Daily activity patterns of many PBAN cells had no nocturnal component (Fig. 1B). An innate activity pattern in those cells may have a relatively short duration (<16 hr) of firing period. Shortening of the duration may be due to shortening of circadian periods by light, though the relationship between them remains to be examined.

Shimizu and Miura (1987) reported similar short periods in the eclosion rhythm of *Bombyx mori* and dependency of the period shortening on light intensity within the range of 3–3000 lx. Physiological and behavioral events associated with eclosion are triggered by secretion of the eclosion hormone from cerebral neurosecretory cells (Gammie and Truman, 1999; Ichikawa, 1992). These findings suggest that both neurosecretory cell systems controlling pheromone production and eclosion may be under the control of a common circadian pacemaker with a strong sensitivity to light.

Because a neurosecretory cell, like a motoneuron, is an output neuron that governs peripheral target organs, it may be under the control of a higher neuronal mechanism. Preliminary experiments revealed that firing activity of PBAN cells was abolished by a transection between right and left hemispheres of the brain or a bilateral transection of connectives between the SOG and the brain, thereby suggesting that each brain hemisphere has a putative neuronal mechanism (or a pattern generator) that regulates temporal firing patterns of contralateral PBAN cells. The neuronal mechanism may activate bursting activities of PBAN cells and motor neurons involved in rhythmic abdominal movement for calling by a virgin female (Ichikawa, 1998), and it appears to be suppressed permanently after mating with a fertile male (Ichikawa *et al*., 1996a). It is likely that a circadian pacemaker controls the brain neuronal mechanism to regulate rhythmicity of the neurosecretory cell system.

Identification of pacemaker cells and their input and output pathways is vital for understanding circadian clock functions. In *Drosophila*, products of clock genes, *period* and *timeless*, cycle in and out of nuclei in several types of brain cells to generate a molecular circadian oscillation (Dunlap, 1999; Giebultowicz, 2000). Among those clock cells, specific neurons in a lateral part of the brain release neuropeptides that appear to modulate daily activity patterns of neurons controlling locomotion (Helfrich-Föster, 1998; Renn *et al*., 1999). Although rhythmic expression of similar clock genes was observed in four lateral neurosecretory cells in each brain hemisphere of the silkmoth *Antheraea pernyi*, gene products did not move into the nucleus and a different oscillatory mechanism was considered (Sauman and Reppert, 1996). The sequence of the period gene of *Bombyx mori* has been partially determined (Regier *et al*., 1998). Identification and physiological characterization of neurosecretory cells expressing the *Bombyx* clock genes may facilitate understanding of the nature of a clock cell, couplings between clock cells, pathways of photic signals, and output pathways mediating physiological and behavioral rhythms.

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