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Firing Activities of Neurosecretory Cells Producing Diapause Hormone and its Related Peptides in the Female Silkworm, *Bombyx mori*. I. Labial Cells

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ABSTRACT—There are three known clusters of neurosecretory cells expressing a gene encoding diapause hormone (DH) and four related peptides in the suboesophageal ganglion (SOG) of *Bombyx mori*. Long-term chronic recordings were made from the axonal tract (NCC-3) of a pair of cells localized in the labial (posterior) neuromere of SOG during pupal-adult development. There was a significant difference in firing activity patterns of the labial neurosecretory cells between diapause-egg and non-diapause-egg producers: labial cells in the former were active throughout pupal-adult development, whereas the same cells in the latter usually maintained an inactive state until the last quarter of pupal-adult development, a time at which a secretion of DH seems to be too late to act on the developing ovary for the induction of diapausing eggs. This observation strongly supports the notion that labial cells release DH and are responsible for determination of embryonic diapause in the silkworm.

Key words: embryonic diapause, neurosecretory cell, action potentials, diapause hormone, photoperiod

INTRODUCTION

Many species of insects have developmental arrest or diapause in their life cycles and this event enables them to adapt to seasonally changing environments and to synchronize the growth rate of populations. An embryonic diapause in a bivoltine race of the silkworm *Bombyx mori* is determined under the environmental regimen experienced during maternal embryonic and larval development: a high temperature and a long-day condition at the developmental stages lead the silkworm to become a diapause-egg producer, while a low temperature and a short day condition facilitate the destiny for a non-diapause-egg producer (Kogure, 1933; Shimizu, 1982, 1991). The embryonic diapause is induced by diapause hormone (DH) which is originated from neurosecretory cells in the suboesophageal ganglion (SOG) during pupal-adult development and acts on developing oocytes to induce diapause in the resulting embryos (Yamashita, 1996). DH isolated from pupal SOGs is an amidated peptide of 24-amino acids (Imai *et al.*, 1991; Sato *et al.*, 1992). Analysis of cDNA encoding *Bombyx* DH showed that the neuropeptide is generated along with four additional family neuropeptides from a common precursor polyprotein that is translated from a single mRNA (Kawano *et al.*, 1992;

Sato *et al.*, 1993). The four neuropeptides, including pheromone biosynthesis-activating neuropeptide (PBAN), share a conserved pentapeptide amide at the C-terminal. Six pairs of somata of neurosecretory cells expressing the gene for the precursor polyprotein are aggregated into three clusters localized at the ventral surface of the mandibular (anterior), maxillary (medial), and labial (posterior) neuromeres of the suboesophageal ganglion (SOG) (Sato *et al.*, 1994). Surgical ablation of the labial cluster of somata at an early pupal stage greatly impaired induction of diapause eggs, whereas the same event on the mandibular and maxillary clusters impaired pheromone production at the adult stage, thereby suggesting a functional differentiation of the three classes of neurosecretory cells (Ichikawa *et al.*, 1996). Immunocytochemical staining and intracellular dye injections into individual neurosecretory cells revealed that the three classes of neurosecretory cells send axons to neurohaemal sites, the corpus cardiacum (CC) and associated nerves of the CC, via different neuronal pathways: five axons from two mandibular cells and three maxillary cells enter the contralateral maxillary nerve to reach the CC, while an axon from a labial cell, passing through the brain, enters the nervi corporis cardiaci-3 (NCC-3) and spread varicose terminal branches in neurohaemal sites (Ichikawa *et al.*, 1995).

As noted with neurosecretory cells releasing PBAN in the adult moth (Ichikawa, 1998), long-term monitoring of electrical activities of identified neurosecretory cells is vital

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for elucidation of physiological functions of neurosecretory cells and their products. In a series of experiments, I made long-term chronic recordings from neurosecretory cells expressing the DH/PBAN gene in female pupae of *Bombyx mori* that had experienced different environmental regimens of diapause induction. I report here that a significant difference in the firing activity of labial cells between diapause-egg producers and non-diapause ones strongly suggests that a pair of labial cells is responsible for the secretion of DH. Another paper reports that mandibular and maxillary cells in both groups of silkworms are equally active throughout pupal-adult development, hence it is unlikely that the five pairs of cells expressing the DH/PBAN gene are involved in a secretion of DH (Ichikawa and Kamimoto, 2003).

MATERIALS AND METHODS

Eggs of F1 hybrid of bivoltine races of *Bombyx mori* (Kinshu×Showa) were purchased from a supplier. The silkworm was programmed to become a diapause-egg producer or a non-diapause egg one by incubating eggs at 27°C under conditions of continuous illumination or at 16°C in the dark, respectively. The larvae

of both groups were reared on an artificial diet at 26±1°C under a 14-hr light/10-hr dark photoperiod (0:00, light-on; 14:00, light-off). Pupae were placed at the same temperature and photoperiodic condition until use. Under these conditions, more than 95% of diapause-egg producers and all non-diapause-egg ones laid only diapause and non-diapause eggs, respectively.

A long-term recording of a neurosecretory cell was usually begun at a few hr after the onset of illumination, using a female pupa at 5–7 hr after ecdysis or on day-5. In some experiments, pupae on day 2 or 3 were used. After the ventral area of the thorax of a pupa had been fixed with paraffin to a platform, the whole body (except for head of the pupa) was placed in a plastic chamber and the chamber was filled with CO₂ gas for purposes of immobilization. A small piece of head cuticle and part of the fat body in the head capsule were removed to expose the brain and NCC-3. The lateral part of SOG localizes other neurosecretory cells which have the same axonal pathway as the labial cells (T. Ichikawa, unpublished observation). To eliminate contamination of signals from the lateral cells, the lateral part of SOG in some pupae was cauterized a few hr after pupation, using pieces of tungsten wire (0.06 mm in diameter) heated by passing an electrical current. To verify that surgical operations were successful, immunocytochemical visualization using an anti-FXPRLamide antiserum (Sato *et al.*, 1998) was done after the end of recordings, as in our previous study (Ichikawa *et al.*, 1996).

For long-term monitoring of labial neurosecretory cells' activi-

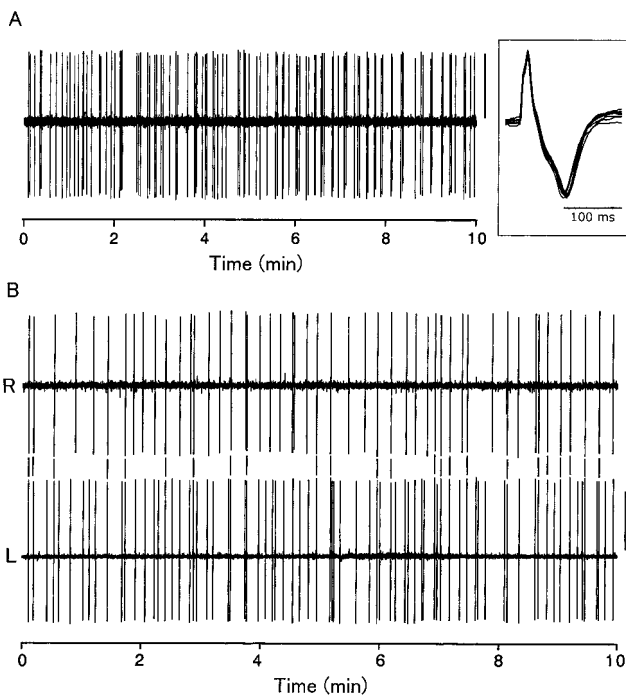


Fig. 1. Spontaneous firing activities of labial neurosecretory cells recorded from NCC-3 of diapause-egg producers on day 5. (A) A unit produces single, doublet or triplet spikes at an interval of 5–20 s. Inset shows eight overlays of spikes. The record was obtained from a pupa in which lateral neurosecretory cells had been surgically removed on day 0. Total number of spikes (N) during a 10-min recording period is 106. (B) Correlated firings of a bilateral pair of labial cells that had a soma in the right (R) and left (L) halves of SOG and send an axon to contralateral NCC-3. A pair of spikes occurring within a gap of 0.2 s is connected by a vertical line. Total number of spikes of right cell and left cell are 50 and 76, respectively. Number of synchronized spikes is 24 (48% for right cell and 32% for left cell). Scale bars: 0.2 mV.

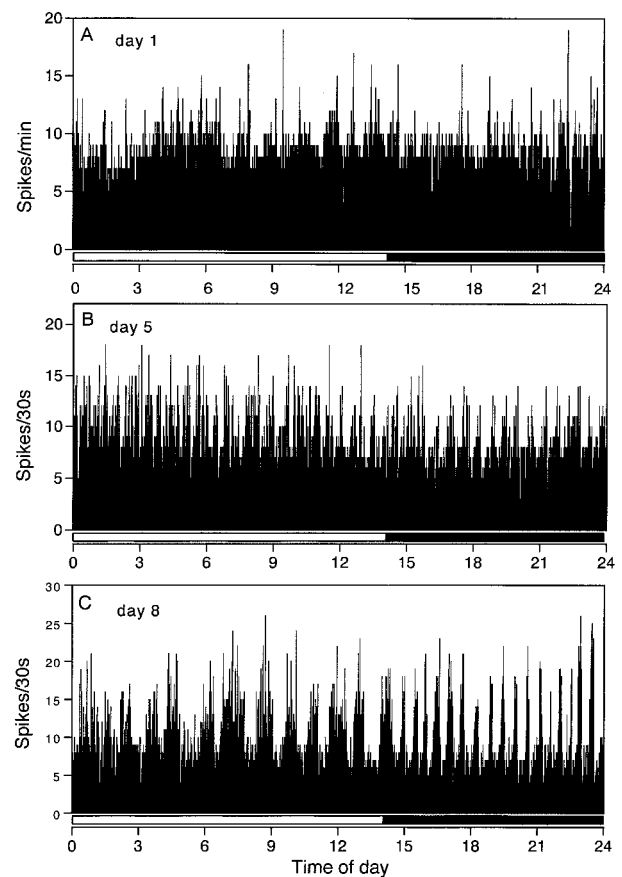


Fig. 2. Typical firing profiles of labial cells in diapause-egg producers at three different pupal stages. Rhythmic fluctuations of firing rates are evident. Photophase and scotophase are shown by white and black bars at the bottom, respectively. (B) and (C) were obtained from the same cell.

ties, NCC-3 was introduced into a suction electrode filled with physiological saline (12 mM NaCl; 32 mM KCl; 9 mM CaCl₂; 18 mM MgCl₂; 175 mM glucose) containing 0.1% streptomycin sulfate (Sigma). 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.

All electrical signals were amplified, filtered, digitized, and stored in a computer equipped with an analog/digital converter (1401plus, Cambridge Electronic Design, Cambridge). Spikes from a labial cell were discriminated using spike-sorting software (Spike 2, Cambridge Electronic Design).

The pupae was kept at 26±1°C and illuminated at the same light/dark cycles, using a fluorescent lamp. Light intensity was 50–100 lx. Under these experimental conditions, pupae usually shed the abdominal cuticle 9 or 10 days after pupation.

RESULTS

Firing activity of labial cells in diapause-egg producers

An extracellular recording from NCC-3 in a female pupa destined to lay diapause eggs started 5–7 hr (day 0) or 5 days (day 5) after pupation. A single species of large action potentials was readily distinguishable from small fluctuations (noises) of unknown origins, as shown in Fig. 1. The large spikes had a slow time course and a typical waveform of a neurosecretory cell (inset of Fig. 1A). The spikes possibly originated from another neurosecretory cell that had a soma in the lateral part of SOG and sent an axon to contralateral NCC-3 (see Discussion). But, similar large spikes could be recorded, even after the lateral neurosecretory cell had been surgically removed (Fig. 1A). Thus, it became evident that the spikes originated from an active labial (Lb) cell.

An Lb cell usually produced single or paired spikes (doublet or triplet) at an interval of 5–20 s (Fig. 1) and sometimes there was a train of several or a dozen of spikes. When spontaneous firing activities of right and left cells were recorded simultaneously, both cells often fired in near synchrony (Fig. 1B): about 48% (32%) of right (left) cell spikes fired within 0.2 s before or after the contralateral cell spikes fired. In all other five animals examined, 20–50% of spikes produced by a cell were within a range ±0.2 s and the probability of such correlated firings is significantly higher than that of a chance, thereby suggesting a weak coupling between a bilateral cell pair.

Long-term firing activities of Lb cells were recorded during early (day 0 to day 5) and late (day 5 to day 10) pupal stages, using different pupae, because a consecutive recording from a single pupa throughout an entire pupal period was quite difficult. Although amplitudes and waveforms of Lb cells' spikes gradually changed with time, significantly large spikes with a slow time course were maintained throughout a recording period, and this greatly facilitated analysis of the firing activity of Lb cells. The activity pattern of a cell was often characterized by rhythmic fluctuations of firing rates, as shown in Fig. 2. Amplitudes of the rhythmic fluctuations did vary from animal to animal. Rhythmicity varied from 10 min to 75 min at different pupal stages and a fluctuation of a long period was often observed at a later pupal stage (day 7 and day 8)(Fig. 2C).

Firing activity of a cell slowly changed during early and late pupal stages and patterns of the change varied from

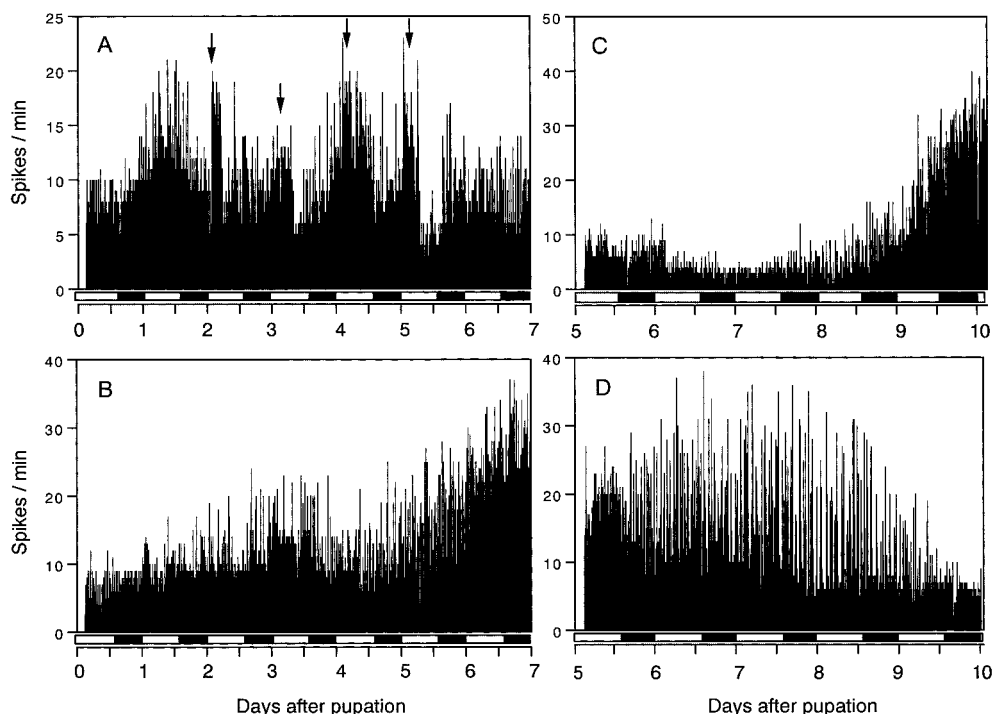


Fig. 3. Examples of long-term firing activities of labial cells in diapause-egg producers during early (A and B) and late pupal stages (C and D). Each arrow indicates a brief increase in the firing activity after an onset of illumination. Photophase and scotophase are shown by white and black bars at the bottom, respectively.

animal to animal (Fig.3). Two cells showed a diel change in the activity during an early pupal period (e.g., Fig. 3A): an increase in firing rate often occurred after an onset of illumination and this active state continued for several hours. A large diel change in firing activity was not apparent in eight other cells recorded during an early pupal period, and a few cells showed a gradual increase in the firing activity (e.g., Fig. 3B). Firing activity patterns during the late half of pupal period varied more greatly than did those during the early period. Three cells had a relatively constant firing activity throughout the late pupal period, and five became more active toward the end of pupal period (e.g., Fig. 3C). Firing activity of four other cells became maximal around day 7 and gradually declined at the last pupal period (Fig. 3D). Such a large variation in daily activity patterns among different pupae during the late half of the pupal period can be seen in the daily changes in the total number of spikes per a day, as illustrated in Fig. 4.

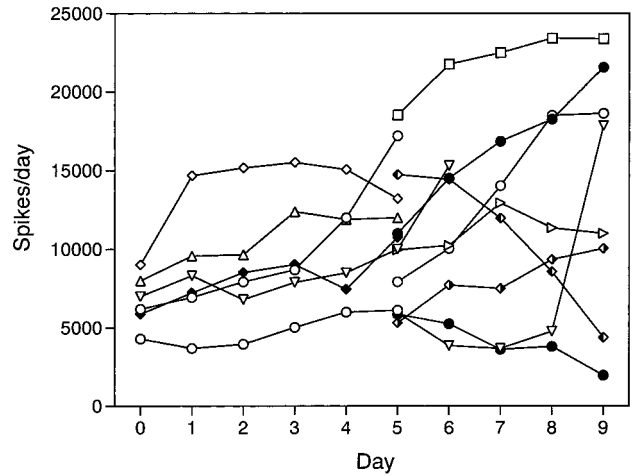


Fig. 4. Variation in daily changes in firing activities of labial cells in diapause-egg producers during the pupal period. Different symbols indicate different pupae.

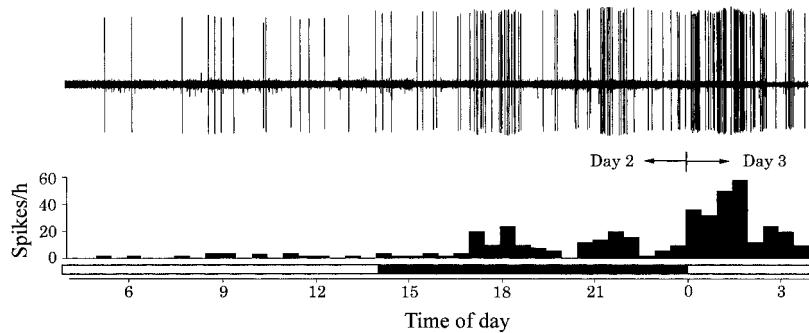


Fig. 5. Low firing activity of a labial cell recorded from NCC-3 of a non-diapause-egg producer on days 2 and 3. The histogram shows firing rates counted every one hour. N=216. Photophase and scotophase are shown by white and black bars at the bottom, respectively.

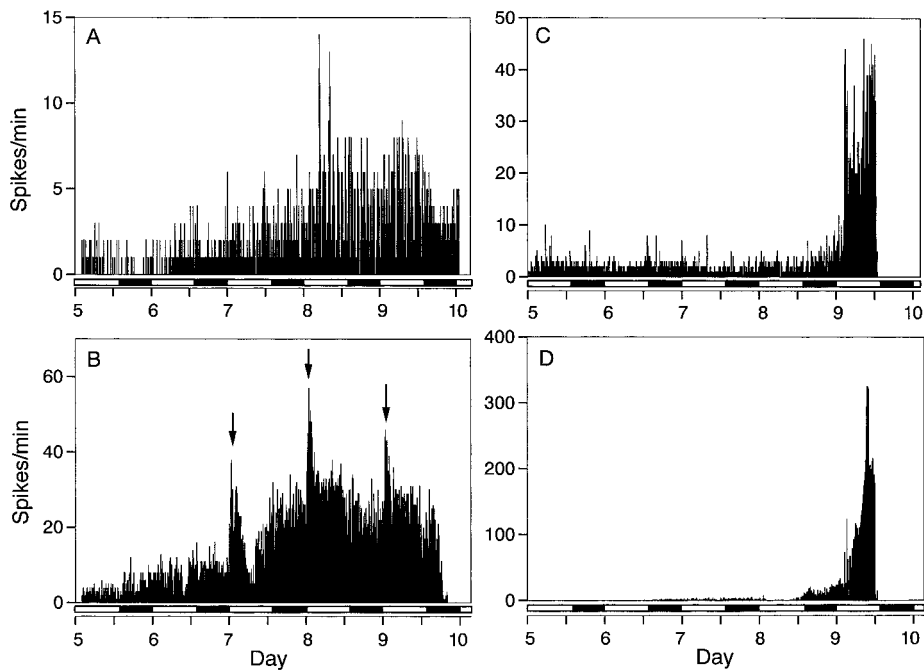


Fig. 6. Four examples of firing activity profiles of labial cells in non-diapause-egg producers during the late pupal stage. Arrows indicate rapid increases in firing rate after the onset of illumination. Note the larger scale of firing rate of a cell shown in D.

Firing activity of labial cells in non-diapause-egg producers

Extracellular recordings of Lb cells in non-diapause-egg producers were made from 30 pupae on day 0 or day 1. Few action potentials with a typical waveform of a neurosecretory cell except for two pupae could be recorded during a recording period of three consecutive days. Fig. 5 shows a 1-day record of firing activity of an Lb cell in the exceptional pupa. The cell continued to fire at a very low rate (0–20 spikes per hr) for three days after pupation and the firing rate briefly increased up to 60 spikes/hr in the morning on day 3. The other cell also showed a fluctuation of firing rate ranging from 0 to 30 spikes/hr but the fluctuation had no relationship with an onset or offset of illumination. The result indicates that a labial cell is almost completely inactive during an early pupal stage.

On the other hand, spikes were often recorded from NCC-3 of a non-diapause egg producer, when a recording started on day 5. Fig. 6 shows four examples of firing activity profiles of Lb cells during the late pupal stage. After a low rate of firing activity for two days, the firing rate of a cell increased to be maximal on day 8 (Fig. 6A). A similar pattern of firing activity was sometimes accompanied by a sharp increase in firing rate after the onset of illumination on days 7–9 (Fig. 6B). Another type of firing patterns was a marked increase in firing activity followed by a sudden stop of firing on day 9 (Fig. 6C) or day 8, and increase in an extreme case reached 300 spikes/min (Fig. 6D). Daily firing activity profiles of many cells during the late pupal period are shown in Fig. 7 and it is apparent that there is a large variability in the level of firing activity at the last pupal stage.

Comparison of daily firing activities between diapause- and non-diapause-egg producers

When averaged daily firing activity of Lb cells in D pupae was compared with that in ND pupae, the difference between them was striking, as shown in Fig. 8. An Lb cell in a D pupa usually produced more than seven thousand

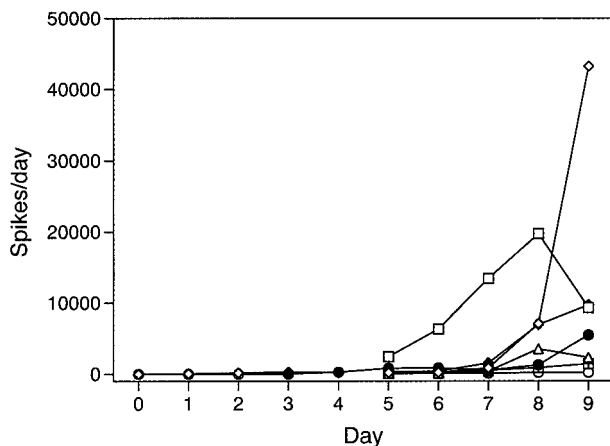


Fig. 7. Variation in daily changes in firing activity of labial cells in non-diapause-egg producers during the pupal period. Different symbols indicate different pupae.

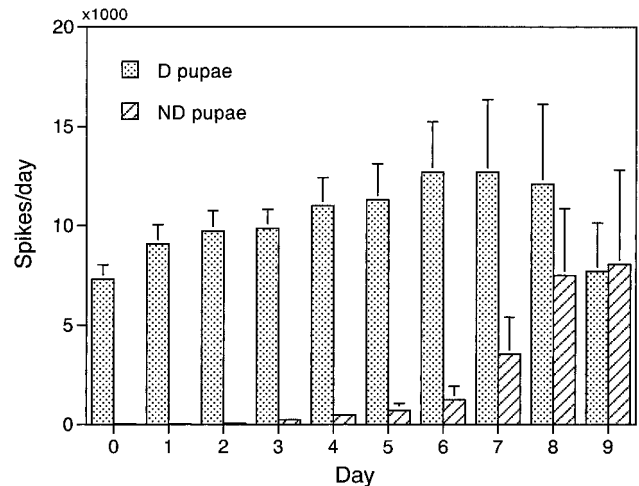


Fig. 8. Averaged daily firing activities of Lb cells in diapause- and non-diapause-egg producers during pupal period. Each bar in the histogram shows the mean and S.E.M. (N=10).

spikes every day throughout the pupal period and firing activity became maximal on days 6 and 7. On the other hand, the activity of an Lb cell in a ND pupa reached almost the same level as that in D pupa at the last stage of pupal-adult development.

DISCUSSION

In the present study, extracellular recordings were made from NCC-3 to characterize firing activity of an Lb cell. However, it is most important to determine if the spikes recorded from the nerve were from the labial cell, because the NCC-3 serves as axonal pathways for other neurosecretory cells and neurons. Although three motor neurons in the brain send an axon to the NCC-3, spikes originating from these neurons were readily eliminated because of their rapid time course (about 2 ms in duration) (Ichikawa, 1991a). There is a pair of somata of neurosecretory cells with immunoreactivity to an FXPRLamide antiserum at the lateral edge of SOG (Sato *et al.*, 1998). A similar lateral cell in SOG of *Manduca sexta* was revealed by cobalt or rhodamine-dextran backfill of NCC-3 (Copenhaver and Truman, 1986; Davis *et al.*, 2001). In a preliminary experiment using injection of a fluorescent dye (Lucifer Yellow) into the lateral cell of *Bombyx mori*, I found that the neurosecretory cell also sent an axon to the contralateral NCC-3 (T. Ichikawa, unpublished observation). The most lateral location of lateral cells' somata in *Bombyx* facilitated surgical removal of the somata (see Materials and methods), and a single species of spikes was readily recorded from the NCC-3 even after removal of the somata (Fig. 1A). Thus, it is evident that large spikes recorded from NCC-3 are from an active Lb cell. The lateral cells may be inactive during the pupal period and their function is yet unknown.

Lb cells in D pupae are fully active throughout the pupal period (Figs. 4 and 8), while the same cells in many ND

pupae becomes active only gradually after the start of the late half or the last quarter of the pupal period (Figs. 7 and 8). The different firing activity patterns of Lb cells in D and ND pupae seem to fulfill requirements for a likely candidate for a DH releasing cell. A prolonged hormonal action on developing ovaries in a D pupa may be needed for a complete production of diapause eggs, because the ovaries contain oocytes at graded stages of development (Yamashita and Hasegawa, 1966). Several lines of evidence suggest that DH may be needed at least up to day 7 in order to induce the latest oocytes to diapause (Hasegawa, 1963; Shiomi *et al.*, 1994; Shimizu *et al.*, 1997). On the other hand, an active secretion of hormone after the sensitive period in an ND pupa may no longer be ineffective for the induction of diapause eggs. We reported that mandibular and maxillary cells in D and ND pupae are fully active during the sensitive period (Ichikawa and Kamimoto, 2003). If DH were released from those cells as a secretory product, all oocytes even in an ND pupa should be induced to enter diapause. Thus, it is most likely that the Lb cell is an exclusive source responsible for releasing of DH. This notion strongly supports the results of experimental morphological-studies (Fukuda and Takeuchi, 1967; Ichikawa *et al.*, 1996).

The amount of DH in the SOG changes during the pupal period: DH content in D pupae begins to decrease just after pupation, while that in ND pupae progressively increases during the first two-thirds of pupal-adult development and then decreases rapidly to an initial, low level of pharate pupae (Sonobe *et al.*, 1977). Although a total amount of DH in the SOG may depend on biosynthesis and release of the hormone, the significant difference in the DH content profiles between two different groups of pupae, especially the timing of decline in DH content, appears to correspond to differences in the firing (=releasing) activity of Lb cells (cf. Figs. 4 and 7).

Several experimental morphological-studies have suggested that the brain controls secretion of DH in *Bombyx* (Fukuda, 1952, 1953; Morohoshi and Oshiki, 1969; Tsuchida and Yoshitake, 1983; Matsutani and Sonobe, 1987; Hasegawa and Shimizu, 1987; Shimizu *et al.*, 1997). Bilateral transection of the circumoesophageal connectives or of the protocerebrum of the brain in an ND pupa changed the pupa to a diapause-egg producer, thereby suggesting that the brain has a neuronal mechanism controlling the activity of putative DH producing cells in the SOG (Shimizu *et al.*, 1997). Firing activity patterns of Lb cells in ND pupae (Figs. 6 and 7) suggest that an inhibitory signal from such a putative neuronal mechanism may be strong enough to suppress the cell almost completely during the entire period or may become weak toward the end of pupal-adult development. The extremely higher activity on day 9 (Fig. 6C, D) is possibly a rebound induced by a release from inhibition. Because Lb cells in D pupae showed a rapid increase in firing rate toward the end of the pupal stage (Fig. 3C) and a large variability in the daily activity patterns during the late pupal period (Fig. 4), it seems likely that the inhibitory mech-

anism weakly functions even in a D pupa. The putative inhibitory signal may be graded rather than an all-or-none fashion, depending on various environmental conditions during embryonic and larval developments.

GABA is a possible candidate for a neurotransmitter mediating the putative inhibitory signal from the brain to the SOG. Shimizu *et al.* (1989) showed that injection of GABA into a D pupa led to production of non-diapause eggs and that injection of picrotoxin and bicuculline, blocking agents of GABA-mediated synaptic transmission, into an ND pupa induced production of diapause eggs, thereby suggesting that GABAergic neurons may be involved in the cerebral inhibition of DH secretion. Similar suggestions were based on an *in vitro* experiment (Hasegawa and Shimizu, 1990). Another candidate is dopamine. Noguchi and Hayakawa (2001) reported that dopamine concentrations in haemolymph and the brain-SOG complex in D-type larvae and pupae are higher than those in ND-type silkworms and that an artificial elevation in dopamine levels induced by feeding Dopa or dopamine to last-instar larvae or by injecting Dopa or dopamine into young ND pupae made the majority of them become diapause-egg producers. The putative GABAergic and dopaminergic control mechanisms of DH secretion may not be mutually exclusive with the latter possibly regulating the former, or vice versa. In a preliminary experiment, I injected picrotoxin (30 µg/10 µl) into a dozen of young ND pupae during monitoring of an inactive Lb cell and found that the cell was excited briefly (several hr) in a few pupae. A further pharmacological study of Lb cells may shed light upon the control mechanisms of DH secretion.

A significant correlation of firings was observed between bilaterally paired Lb cells (Fig. 1B), thereby suggesting a mutual coupling. Such a mutual coupling may facilitate synchronization of firing activity rhythms (Fig. 2). A similar spike-to-spike synchrony has been observed among four pairs of neurosecretory cells producing an insulin-like peptide (bombyxin) (Ichikawa, 2001a, b). The mutual couplings among bombyxin producing cells make the set of cells a weakly coupled multi-oscillator system that may generate a complex pulsatile pattern of hormone secretion, a temporal pattern which seems suitable for reducing down-regulation of a hormone receptor to maintain a biological function (Ichikawa, 2001b). Although it is unknown whether the firing activity rhythm of an Lb cell is autonomous or heteronomous, coordination of firing activity rhythms of right and left cells may be significant for the generation of a pulsatile hormone secretion.

Lb cells often showed a significant increase in firing rate in response to onset of illumination during early (Fig. 3A) and late pupal periods (Fig. 6B), but such a response to offset of illumination was usually indistinct. It is important to consider what kind of photoreceptors mediate the response to light. An old pupa or pharate adult has developing (functional) compound eyes (Eguchi *et al.*, 1962), but a young pupa has no functional eye. The pupal brain has remnants of larval eyes (stemmata) that retain a photoreceptive func-

tion (Ichikawa, 1991b). However, it is unlikely that the remnants function to receive light signals for the diel change in firing activity of Lb cells, because axons of photoreceptor cells degenerate at the earliest pupal stage (T. Ichikawa, unpublished observation), and larval visual interneurons are reorganized into adult optic lobe (Ichikawa, 1994a, b). Shimizu *et al.* (2001) found a novel visual pigment (termed Boceropsin) that was expressed in particular cells in the larval brain of *Bombyx mori* and they suggested that extraocular photoreceptors are the most likely candidates for photoperiodic receptors involving the induction of embryonic diapause in the silkworm, because an isolated, larval brain-SOG complex could respond to photoperiodic conditions to release DH (Hasegawa and Shimizu, 1987). Extraocular photoreceptors may mediate the light response of Lb cells. The sensitive period for diapause induction could be extended to a pupal period, when some bivoltine races of *Bombyx mori* had embryonic and larval life under an intermediate temperature condition (20°C) (Kogure, 1933). Hence, Lb cells in a pupa growing up under such a condition may show different firing activity patterns, including the light response, when the pupa is placed under different photoperiodic regimen. Lb cells may be a suitable model for studying how photoperiodic information is coded into neuronal activity patterns.

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