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Firing Activity of "Diapause Hormone" Producing Cells in the Male Silkmoth, *Bombyx mori*

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ABSTRACT—Diapause hormone (DH) originally identified to be a factor originating from neurosecretory cells in the suboesophageal ganglion acts on developing ovaries to produce diapause eggs in a female silkmoth, *Bombyx mori*. A male silkmoth has homologous neurosecretory cells, but little is known of the physiological nature of the cells and actions of their products. We examined the long-term firing activity of putative DH-producing neurosecretory cells and hormonal activity of their products in male pupae that had been experienced different environmental regimens for diapause induction. Firing activity patterns of male labial cells strongly depended on diapause types of pupae: cells in a diapause-type male were active throughout the pupal period, whereas the same cells in a non-diapause-type male were usually inactive during the early two-thirds of the pupal period. A male pupa with electrically active labial cells could induce diapause eggs in a female pupa connected parabiotically to that male. The firing activity of male neurosecretory cells and hormonal action of their products are qualitatively the same as in the female previously examined. We suggest that there is no evident sexual dimorphism in the physiological and biochemical nature of neurosecretory cells producing DH and the amidated peptide DH has different functions in a male.

Key words: pupa, diapause, neurosecretory cell, action potentials, neuropeptide

INTRODUCTION

A large number of neurohormones or neuropeptides have been identified and mapped in central and peripheral nervous systems in many species of insects (Nässel, 1993; Nässel *et al*., 1994). Many of these peptides were first isolated as a hormonal factor involved in the regulation of a specific physiological, developmental or behavioral event at a specific life stage, but it has become apparent that they have more actions than those ascribed to them at the time of isolation (Gäde, 1997).

Diapause hormone (DH) is one of insect neurohormones whose biochemical nature, hormonal actions, gene structure and expression, and distribution in the nervous systems have been extensively studied in *Bombyx mori* (Yamashita, 1996; Gäde, 1997). DH is an amidated peptide of 24-amino acids that is produced in neurosecretory cells in the suboesophageal ganglion (SOG) (Yamashita, 1996), transported to neurohaemal sites and released to act on the developing oocytes for production of diapause eggs (Ichikawa *et al*., 1995; Shimizu *et al*., 1997). The hormone is encoded by a single gene, together with the pheromone biosynthesis-activating neuropeptide (PBAN) and three additional peptides (Sato *et al*., 1993). Six pairs of neurosecretory cells were identified to express the DH/PBAN gene and classed into three groups (mandibular, maxillary and labial), according to the location of somata in presumptive neuromeres of SOG (Sato *et al*., 1994). They have different axonal tracts for transportation of neurosecretory products to common neurohaemal sites, the corpora cardiaca (CC) and its associated nerve: the maxillary nerve and its branch leads axons of mandibular and maxillary cells to CC, and the circumoesophageal connective to the brain and the nervi corporis cardiaci 3 (NCC-3) makes the way for labial cells (Ichikawa *et al*., 1996; Sato *et al*., 1998).

DH is not a female-specific hormone and is also present in male *Bombyx mori* (Hasegawa, 1964), and millions of heads of male moths were used as a source material for isolation and purification of DH (Imai *et al*., 1991). Male silkworms have neurosecretory cells with immunoreactivity to DH and PBAN (Ichikawa *et al*., 1995). Little is known of physiological functions of the cells and actions of DH. Yamanaka *et al*. (2000) suggested that male DH in Daizo race of *Bombyx* acts on a wing disk at the earliest pupal

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stage and produces wings of an autumn morph with a dark-colored pattern.

In a series of experiments, we obtained long-term recordings from axonal tracts of neurosecretory cells expressing DH/PBAN gene in female pupae of *Bombyx mori* that had experienced different environmental regimens of diapause induction. We found that firing activity of labial cells exclusively depended on environmental regimens during embryonic development (Ichikawa, 2003; Ichikawa and Kamimoto, 2003). We report here that labial neurosecretory cells in male pupae show similar firing activity profiles and that a male pupa with active labial cells has a DH-active substance in the haemolymph.

MATERIALS AND METHODS

Eggs of F1 hybrid of bivoltine races of *Bombyx mori* (Kinshu×Showa) was incubated at 27°C under conditions of continuous illumination or at 16°C in the dark, respectively. Female silkworms experiencing the former and latter environmental regimens became diapause-egg and non-diapause-egg producers, respectively. Male silkworms growing up under the same environmental conditions for the diapause-egg and non-diapause-egg producers in female system were named D-type and ND-type males, respectively. The larvae of both types were reared on an artificial diet at 26°C under a 14-hr light/10-hr dark photoperiod. Pupae were placed at the same temperature and photoperiodic conditions until use. Methods for a long-term recording of electrical signals of neurosecretory cells were as described elsewhere (Ichikawa, 2003).

A parabiotic method was used to test whether a male pupa had DH-active peptides in the haemolymph. The SOG was removed from a female pupa serving as a recipient on day 0. Dorsal cuticle of the thorax of a male pupa (donor) and the same area of the recipient pupa on day 1 were removed using a razor blade after the moth had been immobilized on crushed ice; dorsal parts of the thorax of both pupae were brought into contact with each other and this position was maintained with melted paraffin. For a control experiment, a female pupa with an intact SOG served as a donor. After maintaining at 16°C overnight, pupae were placed at 26°C under 14L:10D photoperiod. DH activity was expressed as the amount of 3-hydroxykynurenine in the recipient ovaries, because diapause eggs accumulate this substance (Sonobe and Ohnishi, 1970). 3-hydroxykynurenine was extracted and diazo-oxidized as described by Inagami (1954) and the amount was calculated from absorbance at 410 nm.

RESULTS

Firing activity of labial cells

An extracellular recording from NCC-3 in a male pupa was usually began 5–7 hr (day 0) or 5 days (day 5) after pupation. A labial (Lb) cell in a D pupa was electrophysiologically active throughout the pupal period, while the same cell in an ND pupa was usually inactive during early half or two-thirds of the pupal period.

Fig. 1 shows spontaneous firing activity of an Lb cell in a D pupa on day 1 of the pupal period. A single species of

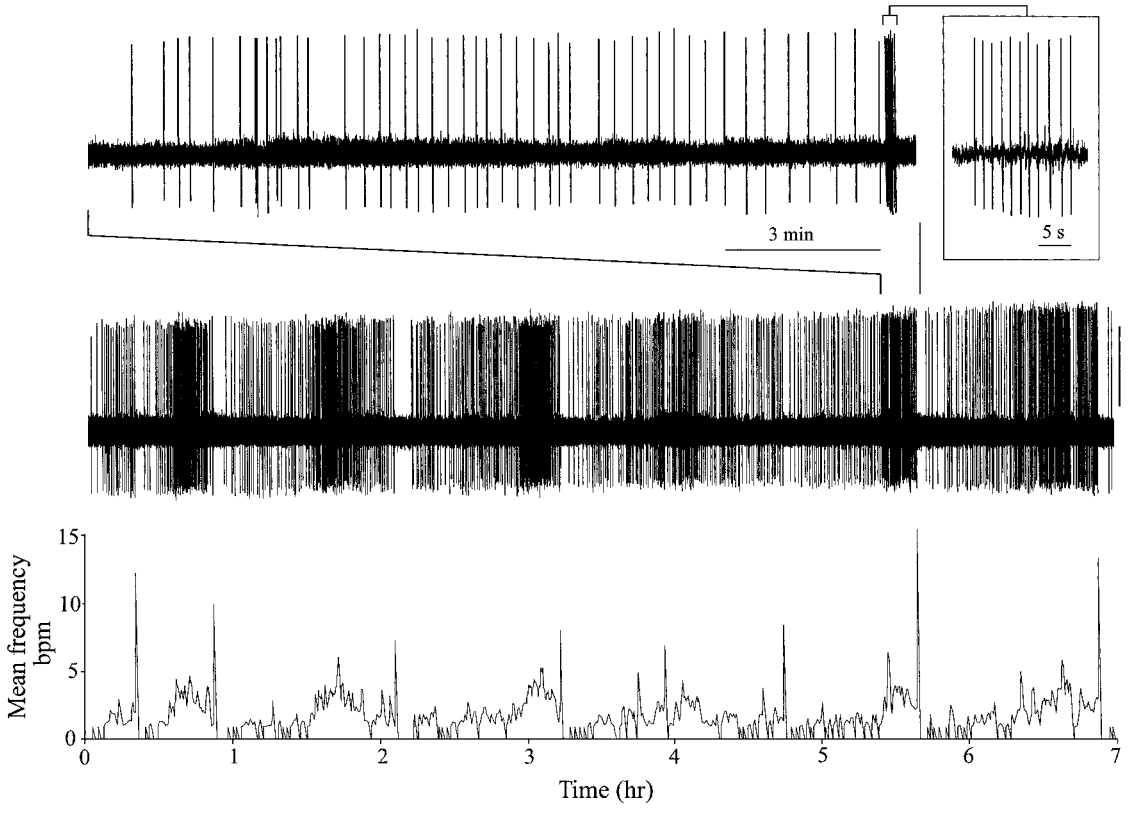

Fig. 1. Typical, rhythmic firing activity of labial neurosecretory cells recorded from NCC-3 of a D-type male *Bombyx* pupa on day 1. Part of a 7-hr recording is shown in the upper trace on an expanded time scale, and a brief train of spikes is shown in inset on a further expanded scale. Mean frequencies calculated at an interval of 1 min are shown at the bottom. Scale bar: 0.2 mV.

large action potentials had a typical waveform of a neurosecretory cell and was readily distinguishable from small and fast fluctuations of unknown origins throughout the entire period of recording. An Lb cell usually produced single or paired spikes (doublet or triplet) at an interval of 5-30 s and the firing rate often showed a rhythmic fluctuation with a period of 15–80 min (Fig. 1, bottom). The cell often discharged a brief train of several or a dozen spikes at the end of an active phase (Fig. 1, inset). Periods and amplitudes of the rhythmic fluctuations varied from animal to animal and a fluctuation of a long period was often observed at a later pupal stage.

Long-term firing activity of a cell in a D pupa slowly changed during the early pupal period and patterns of the change showed a considerable variability in different pupae (Fig. 2). Four cells showed a relatively constant, low or moderate firing activity for 4 or 5 days, while six other cells showed a progressive increase or a diel change in firing activity, after a lower firing activity for a few days (e.g., Fig. 2A, B). Firing activity during late half of the pupal period varied more so than seen during the early period. Firing activity of five cells became maximal around day 7 and gradually declined at the last pupal period (Fig. 2C). Five other cells characterized by a small decrease in firing activity before a gradual or rapid increase toward the end of pupal period (e.g., Fig. 2D). A large variation in daily activity patterns among different pupae was seen in the daily changes in the total number of spikes per a day (see Fig. 4A).

Extracellular recordings of Lb cells of ND-type males were made from more than 20 pupae on day 0 or day 1. Few action potentials with a typical waveform of a neurosecretory cell except for one pupa could be recorded during a recording period of three consecutive days. An Lb cell in the exceptional pupa had a lower firing activity and fluctuation of firing rate ranged from 10 to 70 spikes/hr (data not shown). Thus, a labial cell in an ND pupa is almost completely inactive during the early pupal stage.

On the other hand, Lb cells in ND pupae often became active during the late pupal period. Fig. 3 shows three examples of firing activity profiles of active Lb cells. After a low rate of firing activity for one or two days, the firing rate of cells became maximum on day 8 (Fig. 3A, B). Another type of activity pattern was a progressive increase in firing activity toward the end of pupal period (Fig. 3C). Daily changes in the activity of many cells during the late pupal period are shown in Fig. 4B, and a large variability in the firing activity was apparent at the last pupal stage.

When averaged daily firing activity of Lb cells in D pupae was compared with that in ND pupae, the difference between them was evident, as shown in Fig. 5. Lb cells in D pupae usually produced more than three thousand spikes daily throughout the pupal period and firing activity became maximal on day 7. On the other hand, the activity of Lb cells in ND pupae finally reached the half level of activity of D pupae at the last stage of pupal-adult development. A dotted line in Fig. 5 indicates averaged daily firing activity of Lb cells in D-type female pupae. Daily activity profile of D-type males is qualitatively similar to that of females, though firing activity of male neurosecretory cells is about 50–70% of the activity seen in females throughout the pupal period.

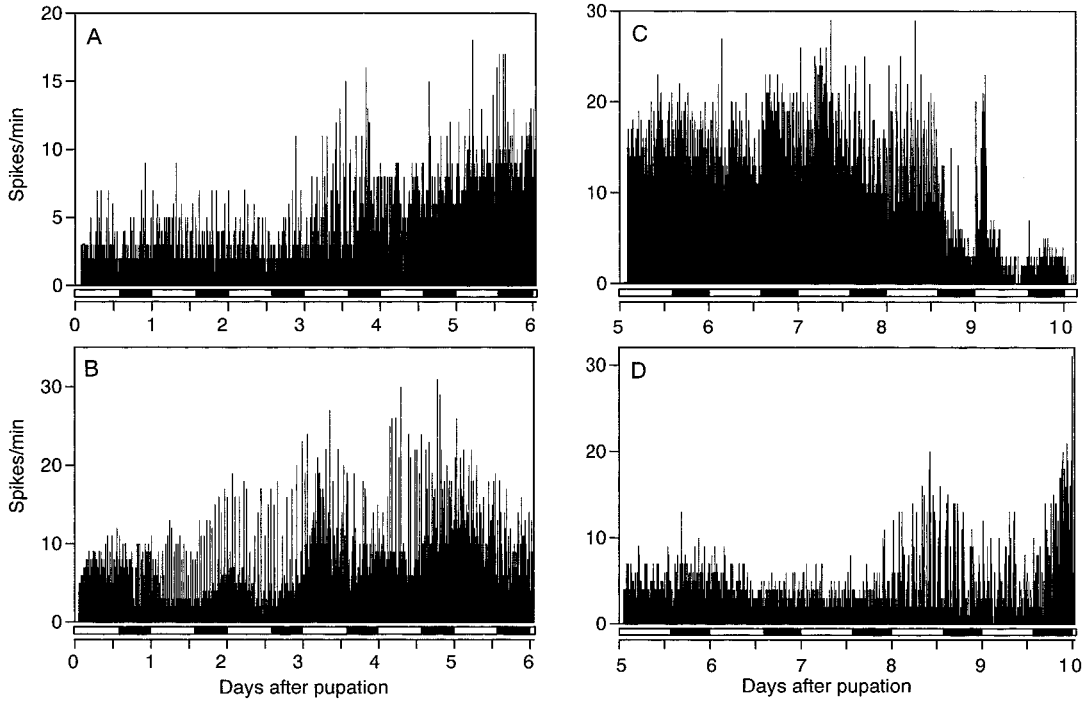

Fig. 2. Examples of long-term firing activities of labial cells in D-type males during the early (A and B) and late pupal periods (C and D). Photophase and scotophase are shown by white and black bars at the bottom, respectively.

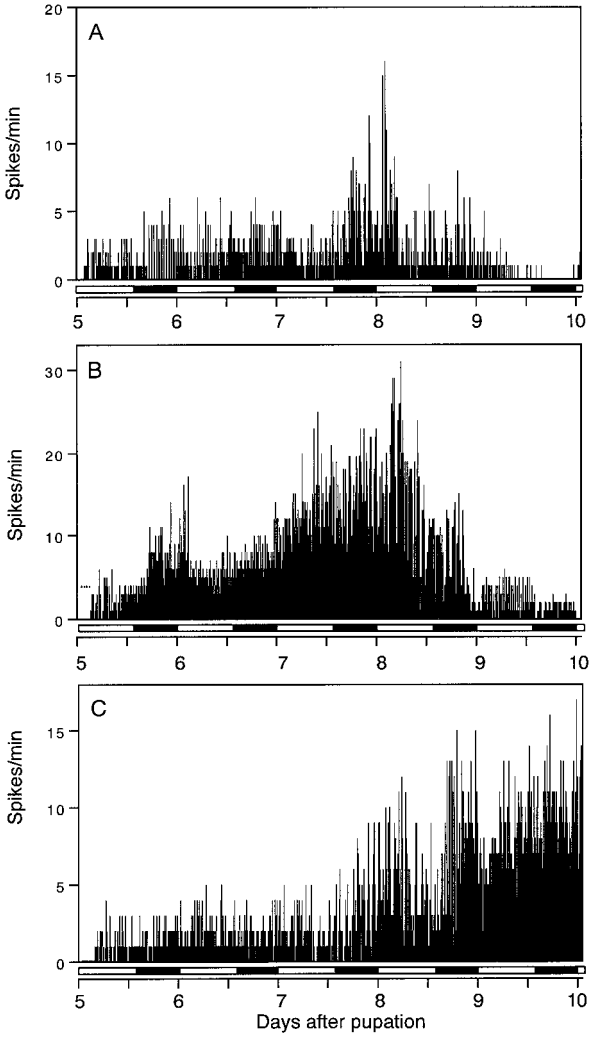

Fig. 3. Three example of firing activity patterns of labial cells in ND-type males during the late pupal period. Photophase and scotophase are shown by white and black bars at the bottom, respectively.

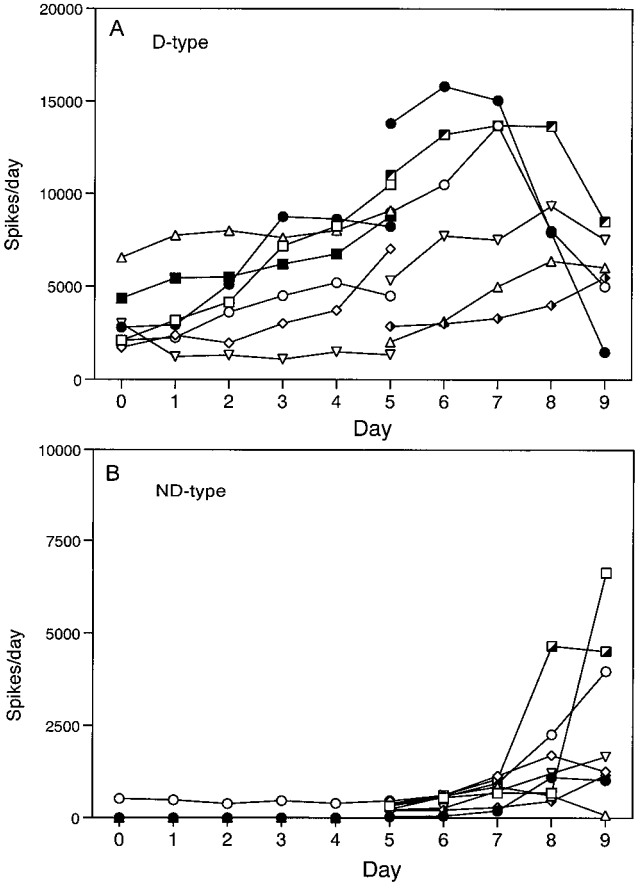

Fig. 4. Variation in daily firing activity of labial cells in D-type (A) and ND-type (B) male *Bombyx* pupae. Different symbols indicate different pupae.

DH activity in male pupae

To determine whether electrically active Lb cells actually release a DH-active neuropeptide, a D-type male pupa on day 1 was parabiotically connected to a D-type female pupa (recipient) in which SOG had been surgically removed on day 0. DH activity was expressed as 3-hydroxykynurenine (3-OHK) content in the recipient ovaries (Sonobe and Ohnishi, 1970). Fig. 6 shows the result of this parabiotic experiment. A recipient female (D♀-SOG) had a significantly smaller amount of 3-OHK than did an intact D female (D♀) or intact ND female (ND♀). The same recipient female connected with an intact D male (+D♂), like that with a normal D female (+D♀), had a large amount of 3-OHK in ovaries. In contrast, an intact ND male (+ND♂) and a D male lacking SOG (+D♂-SOG), as a donor, had no effect on the 3-OHK content of a recipient female ovaries. Thus, it is evident that a D-type male pupa with electrically active Lb cells releases a hormone with a strong DH activity into the

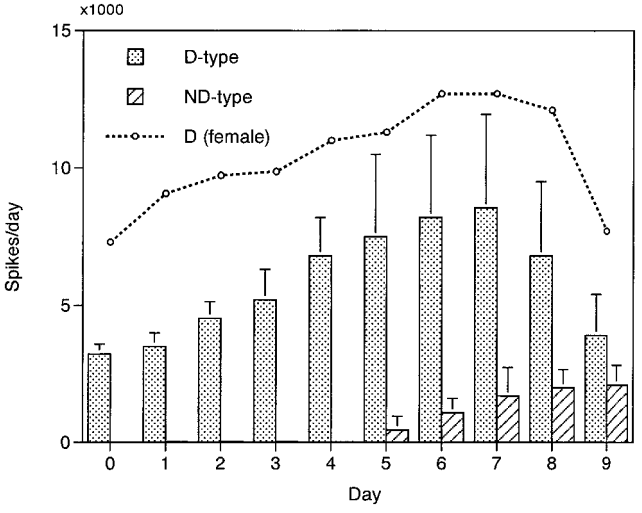

Fig. 5. Averaged daily firing activities of labial cells in D- and ND-type males. Each bar in the histogram shows the mean and S.E.M. (N=8-10). Dotted line indicates averaged firing activity of labial cells in D-type female pupae (Ichikawa, 2003).

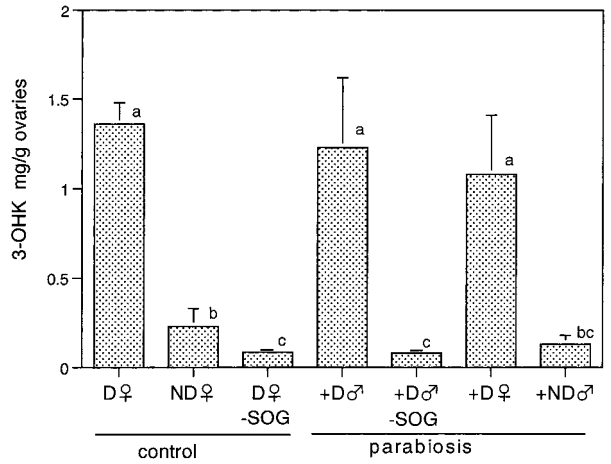

Fig. 6. DH activity of male pupae. DH activity was expressed as an amount of 3-hydroxykynurenine (3-OHK) in the ovaries of a recipient female connected parabiotically to a test pupa (donor). Control animals are an intact D-type female (D♀), intact ND female (ND♀), and D female lacking SOG (D♀-SOG)(recipient). Donors are a normal D male (+D♂), D male lacking SOG (+D♂-SOG), normal D female (+D♀), and normal ND male (+ND♂). Different letters (a-c) indicate statistically different groups ($P \leqq 0.05$, Mann-Whitney U-test).

haemolymph.

DISCUSSION

There are three known groups of neurosecretory cells expressing DH/PBAN gene (Sato *et al*., 1994), hence they are possible candidates of DH secreting cells. A previous study indicated that Lb cells are active in a diapause-egg producer but almost inactive in non diapause-egg producer (Ichikawa, 2003). The present study revealed that the same hold true for D-type and ND-type males (Fig.4). Two other groups of cells, mandibular (Md) and maxillary (Mx) cells, are active in both diapause- and non-diapause-egg producers throughout the pupal period, thereby suggesting that they have no relation to DH secretion (Ichikawa and Kamimoto, 2003). A preliminary study indicated that the same holds true for male pupae (T. Ichikawa, unpublished observation). It is evident that male pupae with active Lb cells have a DH-active substance in the haemolymph (Fig. 6). These observations strongly suggest that Lb cells in both sexes are exclusively responsible for secretion of DH and support the previous notion of functional differentiation of the neurosecretory cells (Ichikawa *et al*., 1996). Average firing activity of a male Lb cell was about 60% of the activity of female one (Fig. 5). However, a smaller amount of DH secretion due to such a lower electrical activity of a male cell may be compensated for by a smaller volume of haemolymph of a male pupa, if an electrical event would trigger secretion of the same amount of hormone from a neurosecretory cell in both sexes. Average body weight of a male pupa (1.01±0.12g (±SD), n=20) used in the present study was about 66% of that of a female (1.53±0.14g, n=20), and blood volume was estimated to be about 25% of the body weight in both sexes (Horie *et al*., 1971). These lines of evidence suggest that there is no sexual dimorphism in the physiological and biochemical natures of three groups of neurosecretory cells.

Sonobe *et al*. (1977) showed a sexual dimorphism in daily change in the amount of DH-active substances in SOG: DH content in D-type female pupae begins to decrease just after pupation, while that in D-type male pupae progressively increases during the first two-thirds of pupal-adult development and then decreases rapidly to an initial, low level of pharate pupae. Because the time course of change in DH content in the latter was similar to that in ND-type male or female, it was expected that putative DH producing cells in D males, like ND ones, were inactivated. However, firing activity profiles of Lb cells and hormonal activity in D males showed a close similarity to profiles in D females rather than in ND females (Figs. 5 and 6). Since different races of *Bombyx mori* were used in the biochemical and physiological studies, it remains unknown if there is marked biochemical and/or physiological difference between the races.

A male of bivoltine race, Daizo, of *Bombyx mori* shows a seasonal dimorphism in color patters of wings: autumn-morph with a dark-colored pattern and summer-morph with a pale pattern. These seasonal morphs of male moths, like diapause induction in female moths, are determined by environmental regimens during embryonic and larval stages: long days and high temperatures facilitate wing formation of autumn morph while short days and low temperatures induce wings of summer morph (Tsurumaki *et al*., 1999). An experimental morphological-study demonstrated neuroendocrine regulation of seasonal morph development: removal of the brain or injection of crude extracts of pupal SOGs into a young pupa of day 0 significantly facilitated change in the destiny of seasonal morph from summer type to autumn, while the same operations done on day 1 or later had no effect on the destiny; furthermore, injection of DH into day-0 pupa led to the same change in the destiny but PBAN was ineffective (Yamanaka *et al*., 2000). These findings indicate that DH originating from SOG acts on wing disks of a male pupa to form wings of an autumn morph. This manner of DH action on male wing disks appears to be basically similar to that on the developing ovaries for diapause induction, but the sensitive period differs between two tissues: the late limit of a sensitive period of wing disks is day 1 (Yamanaka *et al*., 2000), while the limit for the ovaries is day 7 (Shimizu *et al*., 1997). Electrically active Lb cells (Fig. 2) and presence of DH in the haemolymph in D-type male pupae (Fig. 6) strongly support the involvement of DH in autumn-morph wing formation. The ineffectiveness of PBAN seems reasonable, because Md and Mx cells in both types of pupae should actively release at least PBAN, as noted with female neurosecretory cells (Ichikawa and Kamimoto, 2003); hence, if PBAN were effective for wing disks, both types of males would have autumn-morph wings. The novel function

of DH in male pupae may be an example of pleiotropic characteristics of DH/PBAN family neuropeptides (Gäde, 1996).

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