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

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ABSTRACT—A gene encoding a precursor polyprotein of diapause hormone (DH) and four related peptides is expressed by three groups of neurosecretory cells in the subesophageal ganglion (SOG) of *Bombyx mori*. Long-term chronic recordings of firing activities were made from a common axonal tract (the maxillary nerve) of two groups of cells localized in the mandibular and maxillary neuromeres of SOG during pupal-adult development. Mandibular and maxillary cells usually produced a cluster of action potentials at an interval of 30–60 min during pupal-adult development and there was no significant difference in the firing activity profile between diapause-egg and non-diapause-egg producers. We suggest that rather than DH secretion, pupal mandibular and maxillary cells are involved in the secretion of DH-related neuropeptides. DH secretion seems to be assigned to the third group of cells (labial cells); hence, there may be different posttranslational processing of the precursor polyprotein in different neurosecretory cell groups.

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

INTRODUCTION

Mechanisms of embryonic diapause have been extensively studied in the silkworm *Bombyx mori*, since Hasegawa (1951) and Fukuda (1951) independently reported neurohormonal control of the diapause. The hormonal factor, diapause hormone (DH) which is produced in neurosecretory cells in the subesophageal ganglion (SOG), transported to neurohaemal sites and released into the haemolymph (Ichikawa *et al.*, 1995; Shimizu *et al.*, 1997), acts on the developing oocytes to prepare the physiological conditions required for arrest of early embryogenesis (Yamashita, 1996). DH and four additional neuropeptides, including pheromone biosynthesis-activating neuropeptide (PBAN), are generated from a common precursor polyprotein that is encoded by a single gene (Kawano *et al.*, 1992; Sato *et al.*, 1993). Six pairs of neurosecretory cells expressing the gene are localized in a cluster at three distinct regions along the ventral midline of SOG: two pairs of cells at the mandibular neuromere, three pairs at the maxillary neuromere and a pair at the labial neuromere (Sato *et al.*, 1994). It has remained undetermined if neurosecretory cells functionally differentiate, though it was addressed in a physiological study (Ichikawa *et al.*, 1996) and in a theoretical

analysis of polyprotein sequence (Veenstra, 2000).

In a series of experiments, we made long-term recordings from neurosecretory cells expressing the DH/PBAN gene in female pupae of *Bombyx mori* that had experienced different environmental regimens of diapause induction. Evidence for functional differentiation of cells has now been obtained. Our first paper covered firing activity of pupal labial cells that significantly depend on environmental condi-

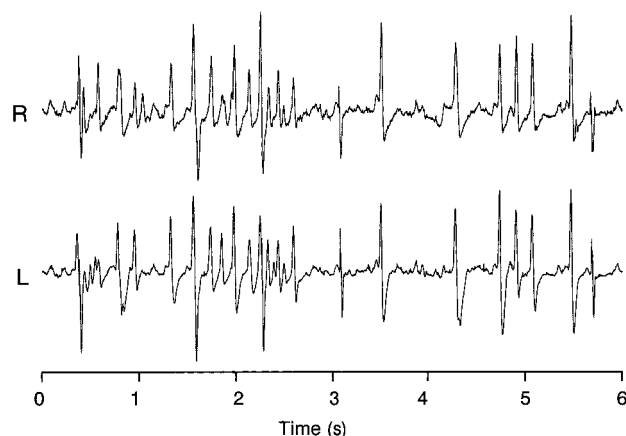


Fig. 1. Trains of compound action potentials of synchronously active mandibular and maxillary cells recorded from two groups of cells with a soma in the right and left half of subesophageal ganglion on day 5 of the pupal period. Scale bars: 0.2 mV.

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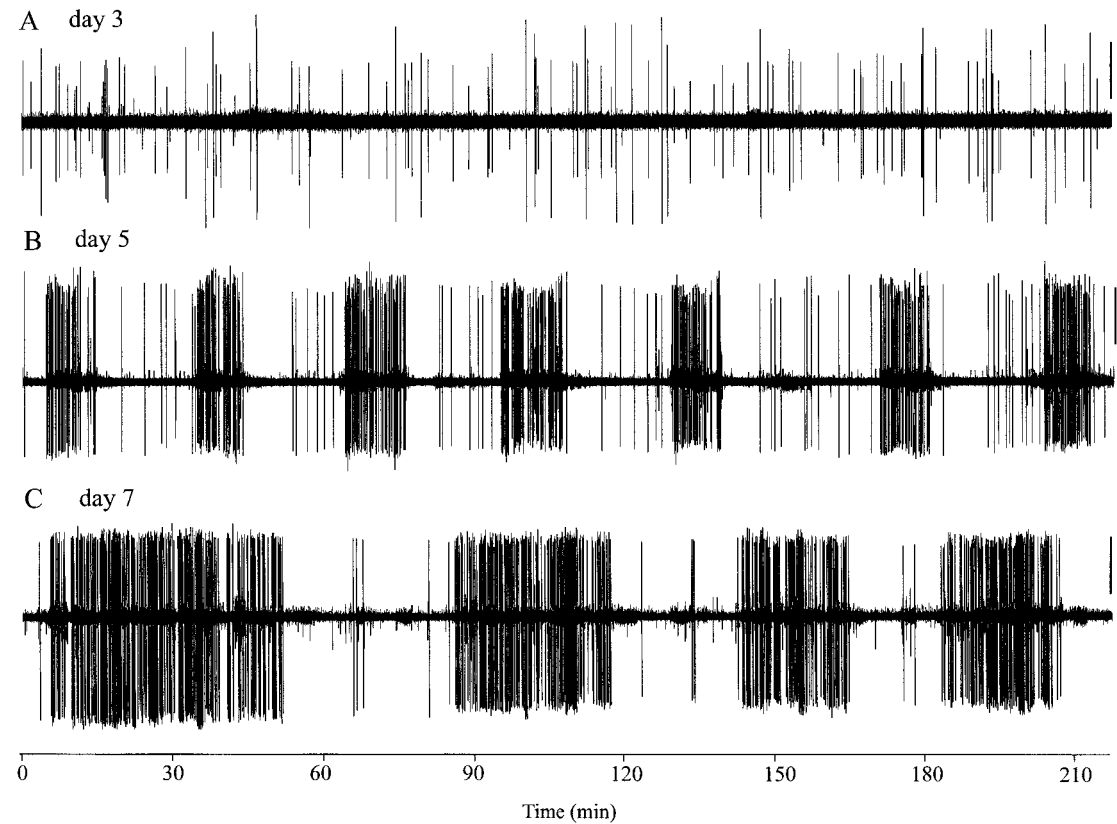


Fig. 2. Typical firing patterns of mandibular and maxillary cells at three different developmental stages of diapause egg producers. Scale bars: 0.2 mV.

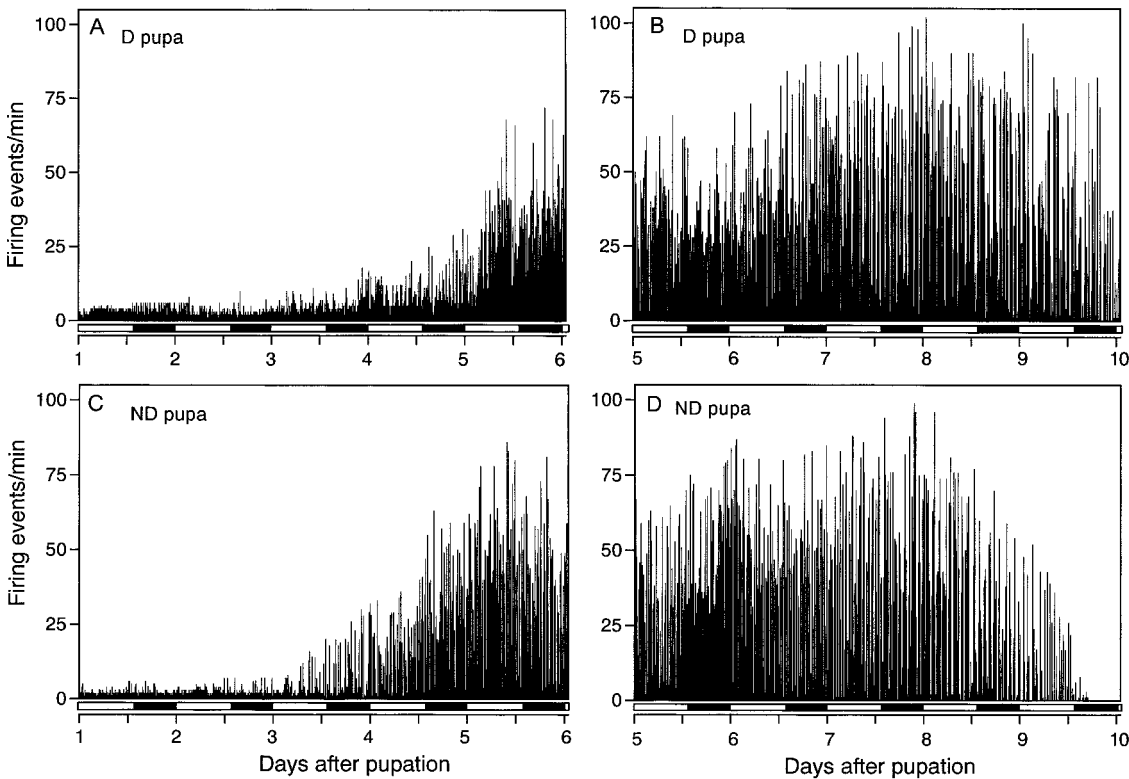


Fig. 3. Examples of long-term firing activities of mandibular and maxillary cells in diapause- (D pupa) and non-diapause-egg producers (ND pupa) during the early (A and C) and late pupal stages (B and D). Note that firing activity is expressed by the number of synchronous firing events per min. Photophase and scotophase are shown by white and black bars at the bottom, respectively.

tions during embryonic development; hence the cells are likely related to secretion of DH (Ichikawa, 2003). This present report covers firing activity patterns of pupal mandibular and maxillary cells, and as they are independent of environmental conditions, we suggest that they are not related to DH secretion.

MATERIALS AND METHODS

A bivoltine race of *Bombyx mori* (Kinshu×Showa) was used. To obtain pupae destined to produce diapause eggs or non-diapause eggs, eggs were incubated under conditions of continuous illumination at 27°C or in the dark at 16°C, 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.

The dorsal area of the thorax of a female pupa on day 0 (at 5–7 hr after ecdysis) or on day 5 was fixed with paraffin to a platform. Part of the cuticle over the SOG was removed to expose the maxillary nerve, the axonal pathway of Md and Mx cells (Ichikawa *et al.*, 1995). The maxillary nerve was cut and the proximal stump of a nerve was introduced into a suction electrode filled with a physiological saline. Other methods for recordings and signal processing were the same as those described elsewhere (Ichikawa, 2003).

RESULTS

Electrical action potentials of mandibular (Md) and maxillary (Mx) cells were recorded extracellularly from a proximal stump of the maxillary nerve rather than the NCC-V, because identification of the later at an early pupal stage was most difficult. Fig. 1 shows electrical potentials recorded simultaneously from a bilateral pair of maxillary nerves. Waveforms of electrical potentials were characterized by compound action potentials with different amplitudes and a synchrony of the compound action potentials recorded from the pair of nerves. Such characteristic waveforms of action potentials were quite similar to those of Md and Mx cells identified in adult females (Ichikawa, 1998). The synchrony of compound action potentials is due to synchronous firings of different number of active cells in the Md-Mx cell population (Ichikawa, 2002). Thus, it is evident that the compound action potentials recorded from the maxillary nerve originate from active cells in the Md-Mx cell population at the pupal stage.

Fig. 2 shows typical firing patterns of the Md-Mx cell population in diapause-egg producers (D pupae) at early, middle and late pupal stages. The firing rate during the early one third of the pupal period was relatively low (20–70 events of synchronous firing per hr) (Fig. 2A). As firing rates began to increase at a middle pupal stage, rhythmic nature of firing activity became evident (Fig. 2B, C). An active phase of firing rhythm was formed by a cluster of many trains of action potentials with a different temporal structure rather than a simple burst of action potentials with a constant firing rate. Duration of single clusters of action potentials and interval of neighboring clusters gradually increased and became maximal at a late pupal stage. Mean duration and interval (±SD) at the late pupal period were 28.5±9.7

and 60.8±12.6 min (n=10), respectively. Similar firing activity patterns were obtained from non-diapause-egg producers (ND pupae) (data not shown).

Long-term, chronic recordings of firing activity of Md and Mx cells were obtained from 20 D-pupae and 16 ND-pupae. Fig. 3 shows four examples of firing activity profiles of the cells during early and the late pupal periods of diapause-egg and non-diapause-egg producers. There is no significant difference in firing activity profiles between the two types of pupae: the firing activity is maintained at a low level for the first 3 or 4 days of the pupal period, then gradually increased toward a maximum on day 7 or 8, and finally declined to near zero at the end of pupal period. No significant diel change in firing activity was observed. Although there was a considerable variation in the absolute firing activity of the cells among individual pupae, similar patterns of daily change in firing activity were usually observed, as shown in Fig. 4, in which total numbers of synchronous firing events per day were plotted. There was no significant difference in the firing activity profile between D and ND pupae. Fig. 5 shows averaged firing activities of cells in D and ND pupae. Firing activity on day 7 reached the maximum (±SEM) of 10660±1330 events/day (D pupae) or 8780±1260 events/day (ND pupae), and the difference was statistically

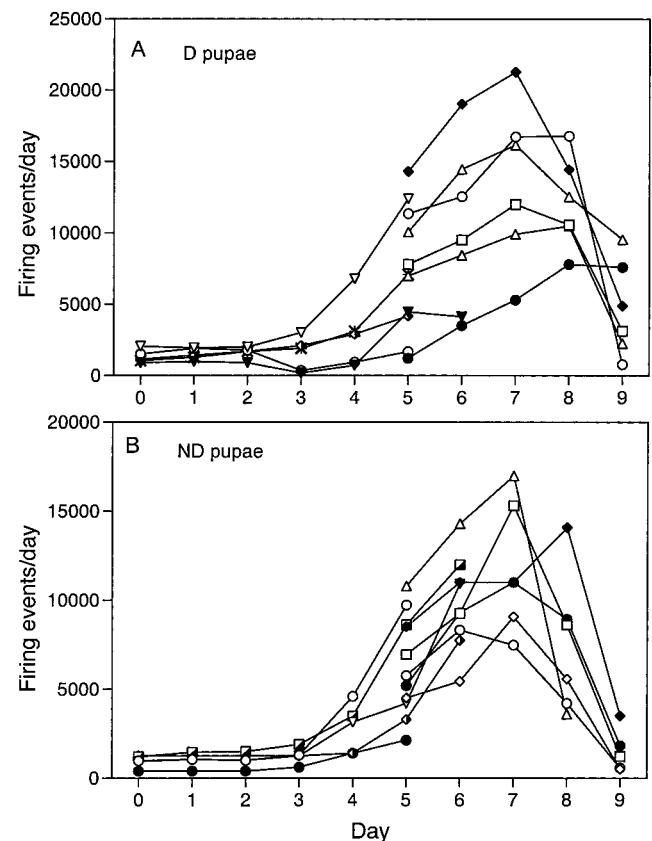


Fig. 4. Variation in daily changes in firing activities of mandibular and maxillary cells in diapause-egg (A) and non-diapause-egg (B) producers during the pupal period. Different symbols indicate different pupae.

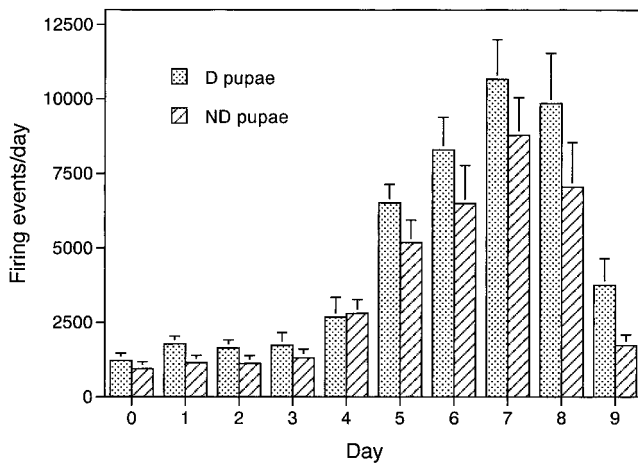


Fig. 5. Averaged daily firing activities of mandibular and maxillary cells in diapause- and non-diapause-egg producers during the pupal period. Each bar in the histogram shows the mean and S.E.M ($n=8-10$).

insignificant (Mann-Whitney *U*-test).

DISCUSSION

Because the branch of maxillary nerve, the NCC-V, is an unique axonal tract of mandibular (Md) and maxillary (Mx) cells (Ichikawa *et al.*, 1995), the branch is an ideal site to identify electrical signals from the cells (Ichikawa, 1998). In the present study, firing activity was recorded from the maxillary nerve because of the technical difficulty. Although the origin of action potentials recorded from the nerve should be carefully identified, the characteristic waveform and temporal pattern of compound action potentials greatly facilitated identification of the origin, as demonstrated in Fig. 1. In a preliminary experiment, similar characteristic action potentials could be recorded from NCC-V of a few pupae for a short period of time. The characteristic features of action potentials of a population of Md-Mx cells were first observed at the adult stage (Ichikawa, 1998), and action potentials of different amplitudes are due to synchronous firings of different numbers of active cells in the population (Ichikawa *et al.*, 1999; Ichikawa, 2002).

The DH/PBAN gene is expressed in three clusters of neurosecretory cells: mandibular, maxillary and labial (Lb) cells (Sato *et al.*, 1994). A surgical ablation experiment suggested functional differentiation of the neurosecretory cells: hormonal induction of diapause eggs in a D pupa was responsible for Lb cells, while activation of sex pheromone production in a female moth was largely responsible for Mx cells (and Md cells) (Ichikawa *et al.*, 1996). This notion was supported by later physiological studies on the firing activity of the cells (Ichikawa, 1998, 2003). Yet, it remained unknown if the Md and Mx cells were inactivated during the pupal period. The present study revealed firing activity profile of pupal neurosecretory cells: firing activity was maintained at a low level for 3 or 4 days after pupation and then

gradually increased to a maximum on day 7, then rapidly declined (Figs. 3 and 4). The expression pattern of DH/PBAN gene in ND pupae showed a quite similar time course (Xu *et al.*, 1995). These findings indicate that the pupal neurosecretory cells not only synthesize neuropeptides but also actively release the secretory products from their axon terminals into the haemolymph.

An Lb cell in a D pupa is fully active during the pupal period, whereas the same cell in an ND pupa is almost inactivated during early three quarters of pupal period (Ichikawa, 2003). Several lines of evidence indicate that DH is needed to act on developing ovaries to induce diapause eggs until day 7 (Hasegawa, 1963; Shiomi *et al.*, 1994; Shimizu *et al.*, 1997). Thus, the Lb cell is the most likely candidate responsible for secretion of DH for determination of embryonic diapause. In contrast, Md and Mx cells even in ND pupae became active during the effective period of DH (Fig. 3). If electrical activity of such cells induces releasing of their products, DH itself should be excluded from the products, because if DH were released from these cells, the DH should induce diapause eggs even in the ND pupa. Although little is known of cell-specific production or non-production of respective peptides originated from precursor polyprotein in insects, cell- or tissue-specific processing mechanisms of precursor polyproteins are well understood in vertebrates (Douglass *et al.*, 1984). Veenstra (2000) proposed possible biochemical mechanisms for exclusive production of DH in Lb cells and selective inactivation of the hormone in Md and Mx cells by different proprotein convertases.

DH is generated along with four additional neuropeptides from a common precursor polyprotein (Kawano *et al.*, 1992; Sato *et al.*, 1993). Because the four neuropeptides, including pheromone biosynthesis activating neuropeptide (PBAN), have a significantly lower DH activity (Sato *et al.*, 1993), Md and Mx cells may secrete all or some of the four neuropeptides. It is evident that neurosecretory cells become active to release a single or multiple species of pheromonotropic peptides for activation of pheromone production in adult females (Ichikawa, 1998) and that a potent pheromonotropic activity is ascribable to PBAN and β -SOG neuropeptide (Sato *et al.*, 1993). It seems likely that the same species of neuropeptides with a higher pheromonotropic activity are released from Md and Mx cells during the pupal period, but identification and physiological functions of their secretory products remain to be determined.

PBAN and PBAN-like immunoreactivity are widely distributed among lepidopteran species that apparently use no sex pheromone for mate attraction, thereby suggesting pleiotropic functions of pheromonotropic neuropeptides at various stages of development, other than the regulation of pheromone biosynthesis at the adult stage (Gäde *et al.*, 1997). Our study provides physiological evidence of multiple functions of neurosecretory cells and neuropeptides in the female silkworm. The neuropeptides may play an important role in morphogenesis during late pupal-adult development.

Rhythmic firing activity of Md and Mx cells and the resultant fluctuation of neuropeptide concentration seem suitable for reducing down-regulation of hormone receptors to maintain such a biological function of neuropeptides (Ichikawa and Kamimoto, 2003).

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