

## Structure of Neurosecretory Cells with Immunoreactive Diapause Hormone and Pheromone Biosynthesis Activating Neuropeptide in the Silkworm, *Bombyx mori*

TOSHIO ICHIKAWA\*, KOH HASEGAWA<sup>1\*\*</sup>, ISAMU SHIMIZU<sup>1</sup>,  
KENJI KATSUNO<sup>2</sup>, HIROSHI KATAOKA<sup>2</sup> and AKINORI SUZUKI<sup>2</sup>

Department of Biology, Faculty of Science, Kyushu University, Fukuoka  
812-81, <sup>1</sup>Center for Ecological Research, Kyoto University, Kyoto  
606, and <sup>2</sup>Department of Applied Biochemical Chemistry,  
Faculty of Agriculture, The University of Tokyo,  
Tokyo 113, Japan

**ABSTRACT**—Immunocytochemistry of diapause hormone (DH) or pheromone biosynthesis activating neuropeptide (PBAN) revealed three clusters of neurosecretory cells present on the ventral midline of the subesophageal ganglion (SG) of the silkworm: there were 4 cells in the anterior, 6 cells in the medial, and 2 cells in the posterior cluster. Intracellular injection of Lucifer Yellow into an anterior or a medial cell revealed that the cell has a dendritic arborization at the anterior region of the SG and that it projects an axon to the corpus cardiacum (CC) via a branch of the maxillary nerve and an associated nerve of the CC (NCC-V). A dye-filled posterior cell in the larva projects bilaterally-symmetric dendritic branches to the anterior half of the SG that is to expand laterally to fuse the brain after pupation. The axon of the cell, passing through the brain, enters the NCC 3 to spread varicose terminal branches in the CC and associated nerves of the CC.

### INTRODUCTION

Neurosecretory cells in the subesophageal ganglion (SG) of lepidopteran insects secrete several distinct neurohormones. In the silkworm *Bombyx mori*, at least two peptide hormones originate from the SG. One is diapause hormone (DH) that acts on the developing ovary to induce diapause eggs [5–7, 9], and the other is pheromone biosynthesis activating neuropeptide (PBAN) that stimulates the production of sex pheromone in the pheromone glands of virgin females [1]. DH and PBAN are 24 and 33–34 amino acid peptides, respectively, with a common C-terminal sequence, FXPRLamide [15, 18, 19].

The cloning and characterization of cDNA for PBAN or DH of *Bombyx mori* showed that a single gene encoded a common precursor polypeptide, from which DH, PBAN and three other FXPRLamide peptides were released through post-translational processing [16, 30]. cDNA with a similar organization of multiple neuropeptides, including PBAN, has been identified in another moth, *Helicoverpa zea* [21]. The gene for DH-PBAN precursor was expressed in three groups of neurosecretory cells clustered in the ventral midline of the SG [31]. In *Helicoverpa zea*, three similar clusters of neurosecretory cells in the SG were visualized by immunocytochemistry of PBAN [17]. Because neuropeptides with a

FXPRLamide motif have a myotropic activity [20] and induce integumental pigmentation [23, 24], the three sets of neurosecretory cells may be involved in processes other than diapause induction and pheromone production, and they may functionally differentiate to secrete specific (sets of) neuropeptides at different stages of postembryonic development of the insect. Characterization of anatomical profiles of the cells, including their terminal structures, has to be done, when attempting to elucidate functions and functional differentiation of the cells.

In the present study, we immunocytochemically identified three clusters of neurosecretory cells, using anti-DH or -PBAN antisera, and we injected Lucifer Yellow into the cells to visualize complete structures, including neurohaemal terminals in the corpora cardiaca. Functional differentiation of DH/PBAN immunoreactive cells, identified by surgical ablation of particular clusters of cells is reported elsewhere [14].

### MATERIALS AND METHODS

#### Animal

The female silkworms, *Bombyx mori* L., we used were F1 hybrids of the bivoltine races 'Kinshu' and 'Showa'. The eggs were incubated at 26°C under illumination. The larvae were reared on a commercially available artificial diet (Yakult Co., Japan) at 26±1°C with a photoperiod of 16L:8D. Pupae and adults were maintained under the same conditions.

#### Lucifer Yellow filling

The head of a 5th instar larva was isolated and mounted in an experimental chamber using beeswax. The chamber was filled with physiological saline designed to mimic extracellular conditions within

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\* To whom all correspondence should be addressed.

\*\* Present address: Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., LTD., 1689 Kashima 3-chome, Yodogawaku, Osaka 532, Japan.

the brain [25]. The SG was exposed by removing surrounding tissues. A pair of insect pins was placed under the SG of a larva to provide a supporting platform for the larval brain. The head of a pupa or a pharate adult was isolated and mounted, ventral or frontal side up, in the chamber. The SG was exposed by removing part of the cuticle over the ganglion. After incubation for 10–20 s in saline containing 1% pronase (Kaken Seiyaku Co. Japan), the sheath of the SG was partially removed, using a fine tungsten needle.

Under visual guidance, a soma of a neurosecretory or non-neurosecretory cell located around the ventral midline of the SG was penetrated with a bevelled glass microelectrode filled with 10% Lucifer Yellow CH (Sigma). An indifferent electrode was placed in the chamber. The microelectrode was connected to a preamplifier (MEZ-8201, Nihon Kohden), which allowed simultaneous recording and current injection *via* a bridge circuit. An action potential was induced from the soma by passing a depolarizing current pulse (3–4 nA, 3 ms in duration). Intracellular action potentials and signals for injected currents were displayed and stored on a memory oscilloscope (VC-10, Nihon Kohden).

Lucifer Yellow (LY) was iontophoretically injected by passing a hyperpolarizing current (3 nA) for 5–10 min. Subsequently the preparation was maintained at room temperature (19–23°C) for 1 hr. The tissues containing the dye were dissected out together with parts of associated structures (e.g. aorta and oesophagus) and fixed with 4% formaldehyde in 0.15 M sodium phosphate buffer (pH 7.4) for 30 min. The tissues were then washed for 30 min in the buffer, dehydrated, cleared with methyl salicylate and gently compressed under the weight of a coverslip or by a finger. The preparations were viewed and photographed immediately at various focal planes, using a fluorescence photomicroscope (Nikon). The structure of LY-filled cells was reconstructed from photographic films, using a photographic enlarging projector.

#### *Antisera production*

Two peptides corresponding to the amino-terminal 12-amino acid sequence of DH and the amino-terminal 15-amino acid sequence of PBAN, both added a cysteine at the carboxyl terminus, were synthesized at The University of Tokyo. These peptides were conjugated with bovine serum albumin (BSA). Six female BALB/c mice were immunized at 2–3 week intervals by giving an intraperitoneal injection of DH(1–12)-Cys-BSA (20 µg/mouse) in Freund's complete adjuvant. Three days after each injection, the mice were bled from the tail vein and the blood was centrifuged. Antibody detection in each mouse serum was carried out by dot-immunobinding assay using the synthetic DH(1–12)-Cys. After injecting the antigen five times, the antibody activity of all mice was detected at 6,000-fold dilution of the antiserum. An antibody for PBAN(1–15)-Cys-BSA was developed by immunizing a rabbit. After injecting 0.4 mg of antigen four times at 2 week intervals, the antibody was detected at 5,000-fold dilution of the antiserum.

#### *Immunocytochemistry*

Larval SGs were isolated and the ventral sheath of the ganglions was partially removed. The tissues were fixed in Bouin's solution for 2 hr at room temperature, then were rinsed for 15 hr in phosphate-buffered saline containing 0.2% Triton X-100 (PBT, pH 7.4) and incubated at 4°C for 3 days in rabbit anti-PBAN antiserum diluted 1:250 in PBT containing 0.25% BSA. These tissues were then washed in PBT at room temperature and soaked for 2 hr in rhodamine-labeled goat anti-rabbit IgG antiserum diluted 1:40 in PBT. Next, they were washed in phosphate-buffered saline (PBS),

dehydrated and cleared with methyl salicylate. Immunoreactivity of some LY-filled cells was examined in whole mount preparations stained using the same immunocytochemical method described above. The primary and secondary antisera were sometimes replaced with mouse anti-DH and rhodamine-labeled rabbit anti-mouse IgG antisera, respectively.

DH/PBAN immunoreactive cells of a pupa and an adult moth were usually examined in sectioned preparations because of relatively strong autofluorescence of the SG after pupation. The isolated brain-SG complex was placed in the fixative, dehydrated, soaked in xylene and embedded in paraffin. Serial sections were deparaffinized, rehydrated, washed in PBT and preincubated with PBT containing 10% normal goat serum for 1 hr. These sections were then incubated in anti-PBAN (or anti-DH) antiserum overnight at 4°C. The primary antiserum was diluted 1:500 in PBT. The sections were then rinsed in PBS and exposed to a rhodamine-labeled goat anti-rabbit (or anti-mouse) IgG antiserum diluted 1:30 for 1 hr. After another rinse in PBS, the sections were mounted with glycerol. Sections were sometimes double-labeled using two primary antisera (rabbit anti-PBAN and mouse anti-DH antisera) and two secondary antisera labeled with fluorescein or rhodamine.

For controls concerning the specificity of the immunocytochemical procedure, sections were incubated with the primary antisera inactivated by adding synthetic PBAN-I or DH (1 µg/ml). Immunostaining was almost totally abolished, though in some preparations the somata were faintly stained.

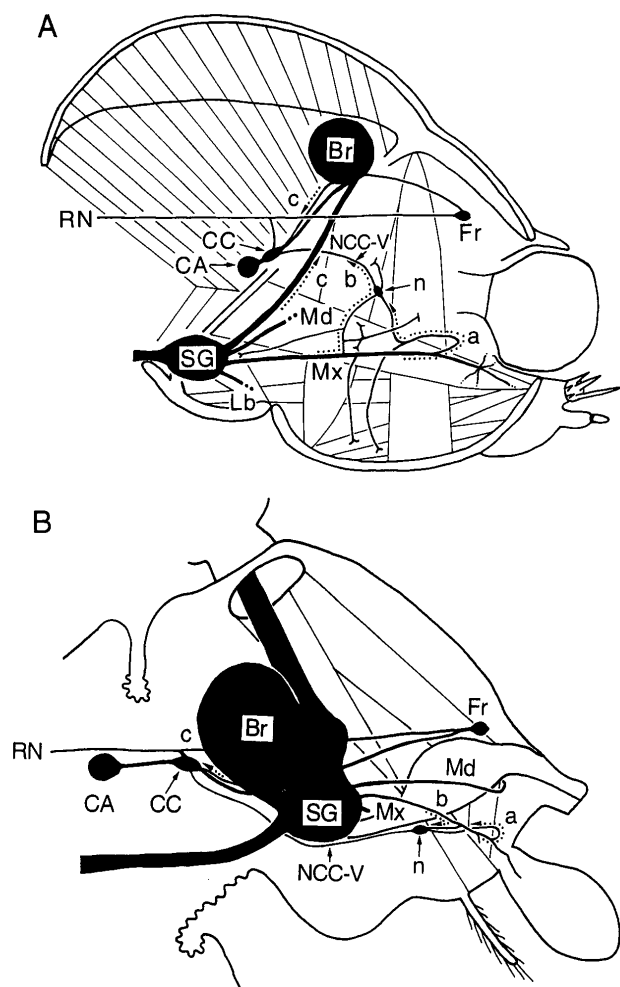
## RESULTS

### *Gross anatomy of brain-SG-retrocerebral neurohaemal organs*

Anatomical profile of the brain and SG of the larva appears markedly different from that of the adult as there are gross metamorphic changes in the CNS during adult development. Larval SG is linked to the brain by a pair of long connectives, the circumoesophageal connective, while adult SG is completely fused with the tritocerebral lobe of the brain (Fig. 1). However, all of the major nerve branches associated with the retrocerebral neurohaemal organs, the corpus cardiacum (CC) and the corpus allatum (CA), remain through metamorphosis, despite extensive restructuring of central and peripheral nervous system in the head. The CC-CA complex is connected to the brain by two nerves, nervus corporis cardiaci (NCC) 1+2 and NCC 3 and is connected to the SG by NCC-ventralis (NCC-V) and two branches of the maxillary nerve (a and b in Fig. 1). Many thin branches of the maxillary nerve innervating larval muscles in the maxilla appear to be greatly diminished or entirely lost at pupation as the result of changes in target muscles during development. There is a small ellipsoidal node at the point where the two branches of the maxillary nerve join together into the NCC-V. A thin nerve (NCC-RN) extending medially from the CC joins the recurrent nerve.

### *Localization of DH/PBAN immunoreactive cells*

Twelve neurosecretory cells in the SG showed potent immunoreactivity to the rabbit anti-PBAN antiserum. In larva, the somata are localized at three distinct regions, as clusters on the ventral midline of the SG (Fig. 2A). The



anterior, medial and posterior clusters are comprised of four, six and two somata, respectively. All neurosecretory cells at larval stage were strongly immunoreactive. At an early pupal stage, SG fuses to the brain and the anterior portion of SG extends laterally to connect to the ventral edge of the brain. The anterior cells move laterally to separate into two clusters as a result of metamorphic changes of the SG (Fig. 2B). The anterior and medial cells at pupal and adult stages were also strongly immunoreactive, whereas immunoreactivity of the posterior cells at the late pupal and adult stages were usually weak (Fig. 2B).

Immunocytochemical visualization of the DH/PBAN immunoreactive cells revealed that the anterior and medial cells project an axon into the maxillary nerve while the posterior cells extend an axon to the circumoesophageal connective. Visualization of the entire length of axonal tracts of the cells was not feasible as immunoreactivity of the axons usually disappeared in the maxillary nerve or the connective. Immunoreactive axons often re-appeared in a branch of the maxillary nerve before reaching the node (cf. Fig. 3E).

FIG. 1. Semi-schematic diagrams of the brain-suboesophageal ganglion and connections to retrocerebral neurohaemal organs in the larval (A) and adult silkworm moth (B). Br, brain; CA, corpus allatum; CC, corpus cardiacum; Fr, frontal ganglion; Lb, labial nerve; Md, mandibular nerve; Mx, maxillary nerve; NCC-V, nevus corporis cardiaci ventralis; RN, recurrent nerve; n, node; SG, suboesophageal ganglion. a-c, axonal pathways of DH/PBAN immunoreactive cells.

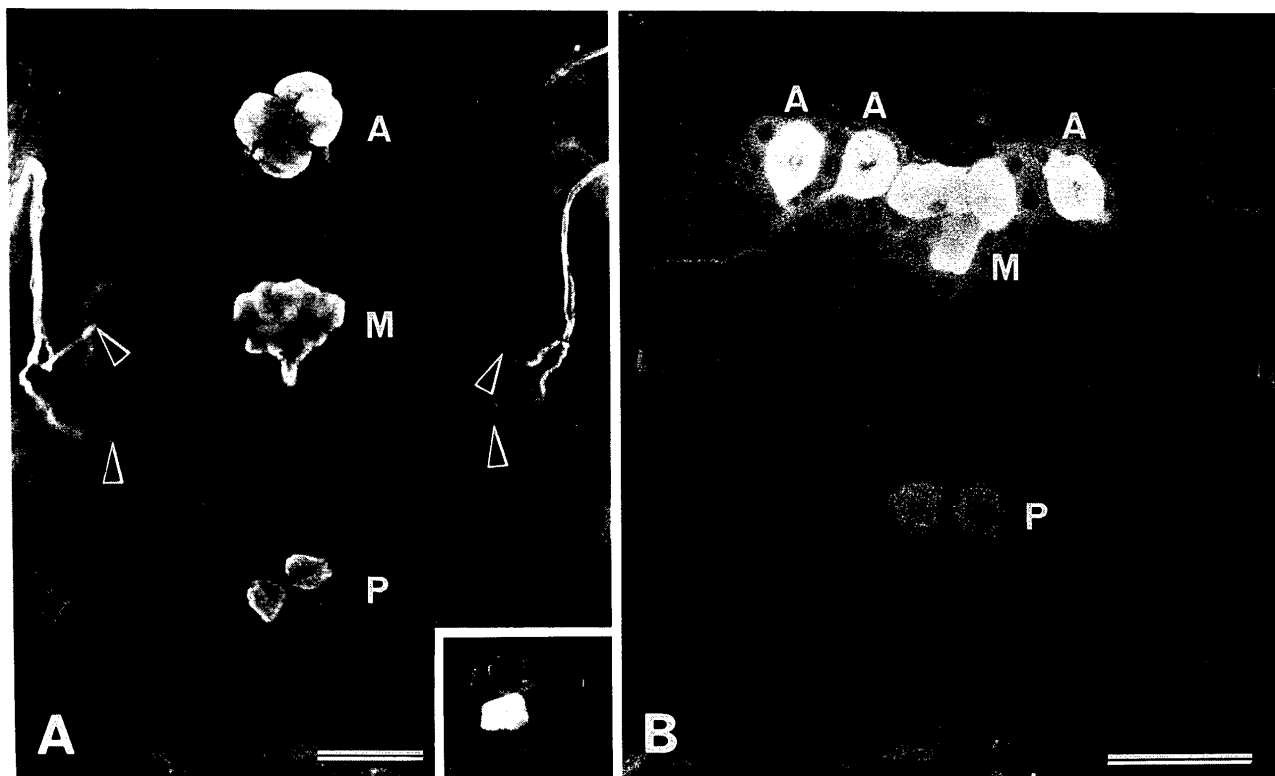


FIG. 2. Ventral views of PBAN immunoreactive cells' somata in the larval (A) and adult SG (B). A, anterior cell (cluster); M, medial cell cluster; P, posterior cell cluster. Bundles of immunoreactive axonal processes (arrowheads) are seen, though they are out of focus in this picture. Inset illustrates a medial cell that has been intracellularly injected with Lucifer Yellow. Scale bars, 100  $\mu$ m.

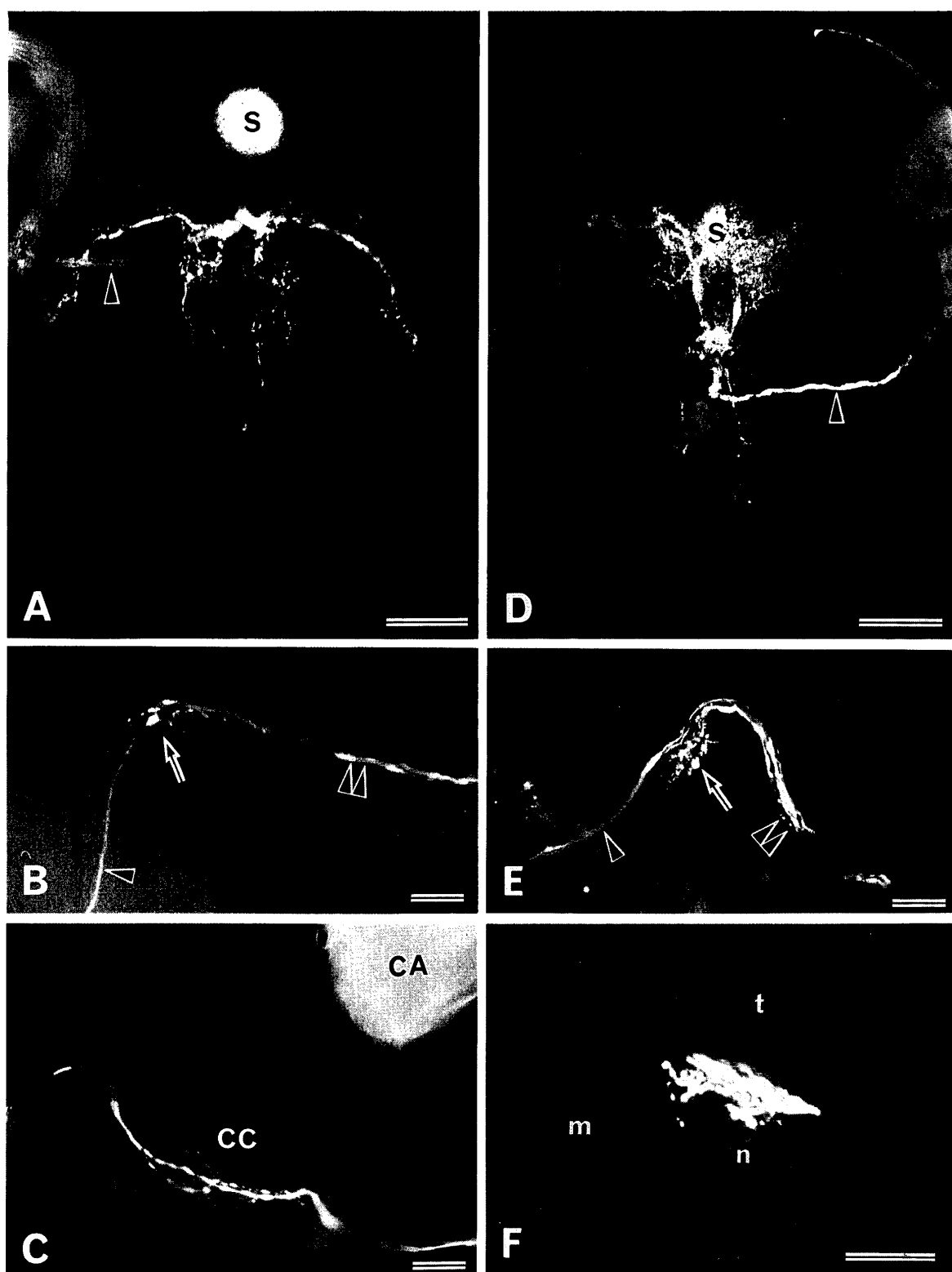


FIG. 3. Photomicrographs of an anterior and a medial cell in the larval SG visualized by injection of Lucifer Yellow (A-D) or PBAN immunocytochemistry (E, F). A. Ventral view of an anterior cell. S, a soma (out of focus); arrowhead, an axon. B. Axonal profile of the anterior cell. The axon (single arrowhead) is smooth in appearance before reaching the node (arrow), but has varicose appearance in the node and the NCC-V (double arrowheads). C. Varicose axonal processes in the corpus cardiacum (CC). CA, corpus allatum. D. Ventral view of a medial cell. S, a soma; arrowhead, an axon. E. PBAN immunoreactive axonal processes. They (single arrowhead) appear smooth before invading the node (arrow), but give rise to varicose branches in the node and the NCC-V (double arrowheads). F. Varicose branches in the node (n). m, a muscle; t, trachea. Scale bars, 100  $\mu$ m (A, D), 50  $\mu$ m (B, C, E, F).

Although we used mostly the anti-PBAN antiserum, similar results were obtained when the anti-DH antiserum was used as a primary antiserum. Double labeling of cells using anti-DH and anti-PBAN antisera revealed that the same three clusters of neurosecretory cells were immunoreactive with the two antisera (data not shown).

#### Structure of DH/PBAN immunoreactive cells

Somata of neurosecretory cells in the SG could often be distinguished by their opalescence. We impaled many opalescent somata at the three regions where DH/PBAN immunoreactive cells were expected to be present. Before injecting LY, action potentials of an impaled cell were recorded because a neurosecretory cell was discernible from a non-neurosecretory cell by large and prolonged action potentials [12, 25]. All those neurosecretory cells present at medial and posterior regions were DH/PBAN immunoreactive cells (inset of Fig. 2A), but cells at the anterior region were sometimes different neurosecretory cells with no immunoreactivity to the antisera and an axon projecting to the mandibular nerve.

*Anterior and medial cells:* Injection of LY into a soma of an

anterior or a medial cell of the larva revealed that the primary neurite originating from the soma extended for some distance posteriorly or dorsally and bifurcated into dendritic processes. The dendritic processes of the anterior and medial cells are shaped " $\pi$ " (Fig. 3A) or T (Fig. 4A) and X (Figs. 3D, 4B), respectively. An axon originating from one of the dendritic processes extends laterally and turns anteriorly to enter the maxillary nerve contralateral to cell's soma. All seven anterior cells examined in this study had an axon that ran in the maxillary nerve toward the mandible and turned posteriorly to reach the ellipsoidal node (route a in Fig. 1A). Six out of eleven medial cells examined had such a long axon and the others had a relatively short axon that entered a shortcut branch on the halfway (route b in Fig. 1A) to reach the node. Axons of both anterior and medial cells had a few varicose branches in the node where two cells of unknown origin were present (Fig. 3B,F). Axons leaving the node passed through the NCC-V and reached the CC (Figs. 3C and 4A, B). They usually invaded into a nerve (NCC-RN) and had a varicose appearance not only in the CC but also in the full length of the NCC-V (Fig. 3B). Such a structural specialization is often present in immunocytochemical preparations (Fig. 3E).

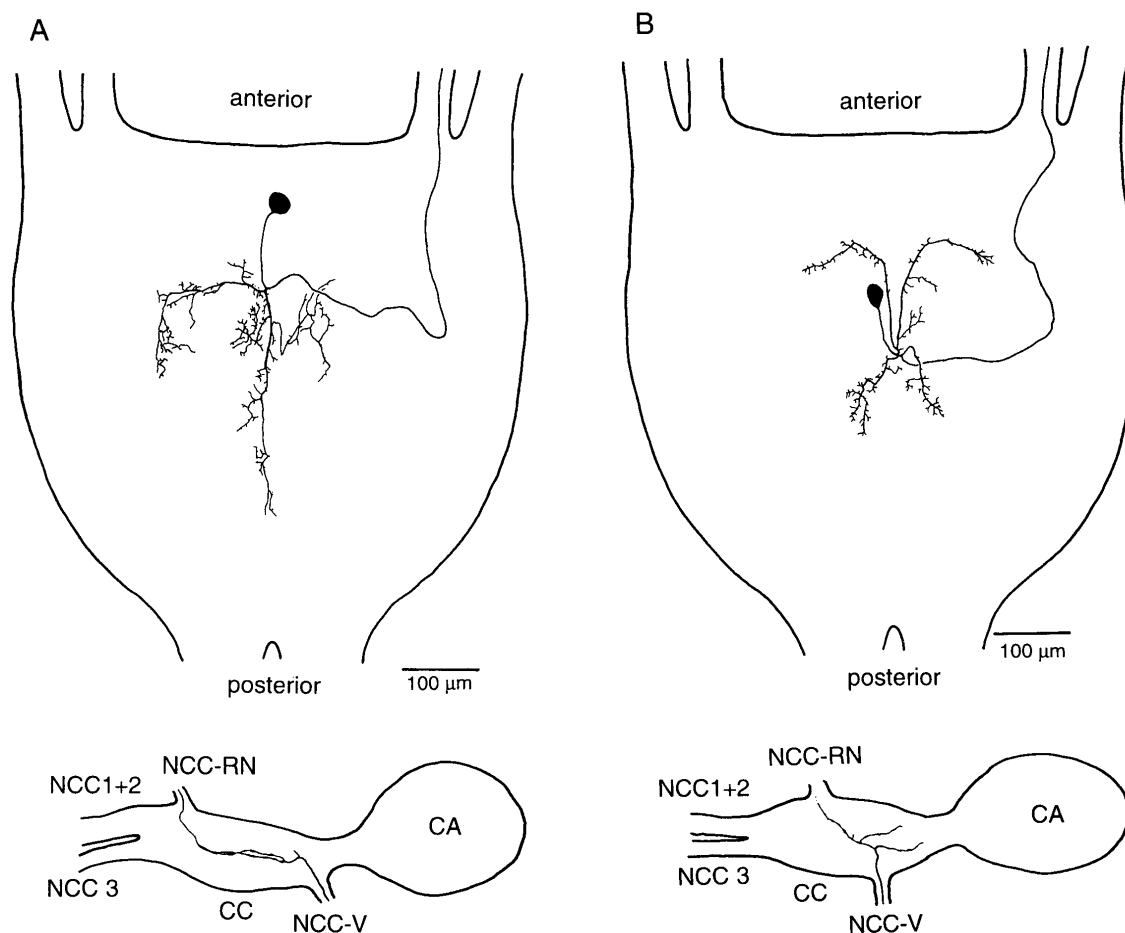


FIG. 4. Drawings of an anterior (A) and a medial cell (B) in the larval SG. CA, corpus allatum; CC, corpus cardiacum; NCC 1+2, fused nerve of nervus corporis cardiaci 1 and 2; NCC 3, nervus corporis cardiaci 3; NCC-V, nervus corporis cardiaci ventralis; NCC-RN, nervus corporis cardiaci recurrens that joins the recurrent nerve.

Although the anatomical appearance of neurosecretory cells at a late pupal or an adult stage somewhat differs from that at the larval stage because of the gross anatomical change of SG during adult development, branching patterns of primary dendrites of the cells are basically similar to those of larval cells (Fig. 5). Axons of pupal or adult cells, like the larval cells, extended toward the node and NCC-V via two branches of the maxillary nerve (route a and b in Fig. 1B). Varicose branches of those axons could be observed in the node, but no axon could be traced to its terminal in the CC, using the dye-filling method because of elongation of NCC-V

during development.

**Posterior cells:** Fig. 6A shows a pair of posterior cells visualized by LY injection. Position of both somata, dendritic branches and axonal pathways are almost symmetric around the midline of the ganglion. LY injection into a single cell reveals that each cell has a symmetric dendritic arbor and dendritic branches of the two cells overlap (Fig. 7A). The primary neurite extending from the soma runs toward the dorsal surface for a short distance, then turns anteriorly to bifurcate into dendritic processes. Two dendritic processes run side by side along the midline of the SG for some distance and each process turns laterally and then posteriorly to give off many fine branches. An axon originating from the primary neurite or one of the dendritic processes extends laterally and turns anteriorly to enter the circumoesophageal connective contralateral to the cell's soma. The axon, after running up the connective (route c in Fig. 1A) and passing through the lateral part of the brain, enters the nerves corpus cardiaci 3 (NCC 3) to project to the CC, an area where the terminal branches form a complex network of numerous varicosities. A few terminal branches invaded the associated nerves of the CC, NCC-RN and NCC-V (Figs. 6B, 7B) but did not invade the corpus allatum (CA).

Figure 6C shows a ventral view of a posterior cell of a pupa at 4 days after pupation, a time when the brain-SG complex takes on an adult form. The bilateral dendritic branches expand to cover the anterior edge of the neuropile around the oesophageal foramen. The axon first projects postero-dorsally and finally leaves the brain posteriorly after a few turns in the brain-SG complex. The anatomical profile of the cells in the brain-SG complex at the adult stage is almost identical to that in the complex at the mid-pupal stage (Figs. 6D and 8A,B). Their axons are characterized by dense fasciculation in the CC and many fine, parallel branches in the recurrent nerve under the aorta (Figs. 6E and 8C). A few branches invading the allatal nerve (NCA) reach the proximal portion of the corpora allata (CA) (Fig. 8C).

## DISCUSSION

DH and PBAN of *Bombyx mori* are generated along with three other structurally-related peptides from a common precursor polypeptide that is translated from a single species of mRNA [16, 30]. *In situ* hybridization with cDNA encoding the precursor polypeptide revealed twelve neurosecretory cells that localize somata as clusters on the ventral side of three different neuromeres of SG: mandibular, maxillary, and labial neuromeres [31]. Since the number, size and position of neurosecretory cells we visualized immunocytochemically are identical to those of the cells revealed by cDNA hybridization, it is apparent that the anterior, medial and posterior DH/PBAN immunoreactive cells correspond to the neurosecretory cells in the mandibular, maxillary and labial neuromeres, respectively. It remains to be determined if all the neurosecretory cells produce functional

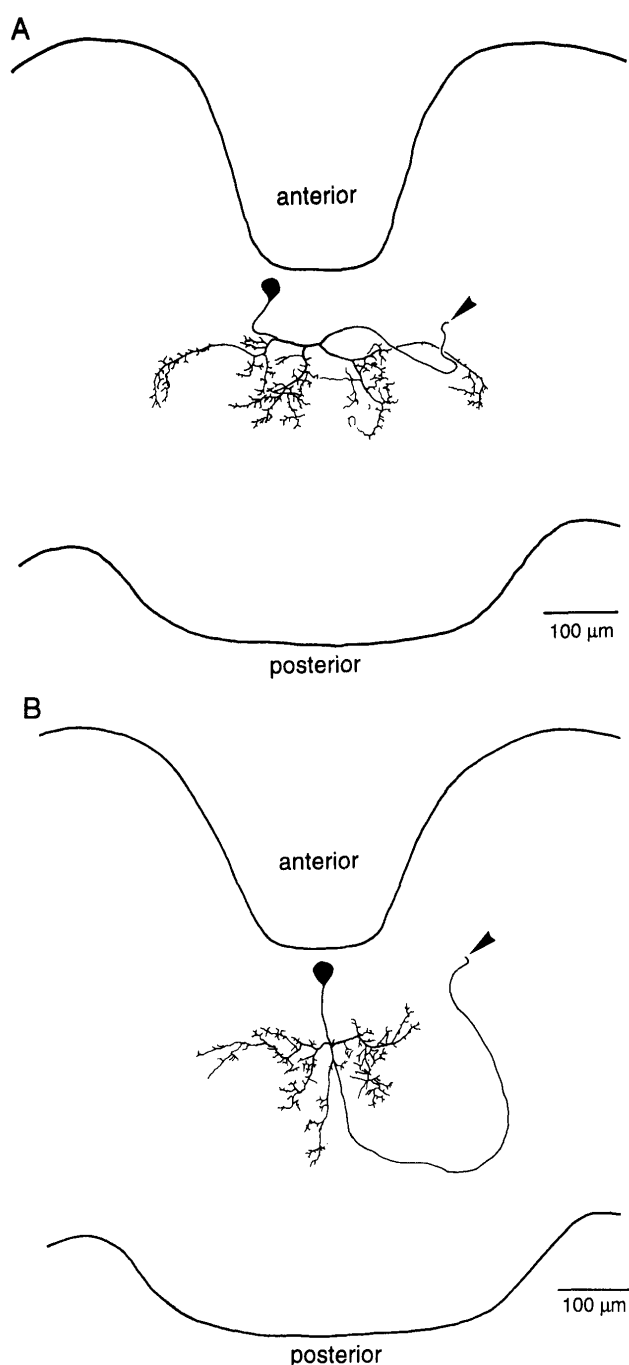


FIG. 5. Dorsal views of an anterior (A) and a medial cell (B) in adult females. Axons exit the SG into the maxillary nerve at the points indicated by arrowheads.

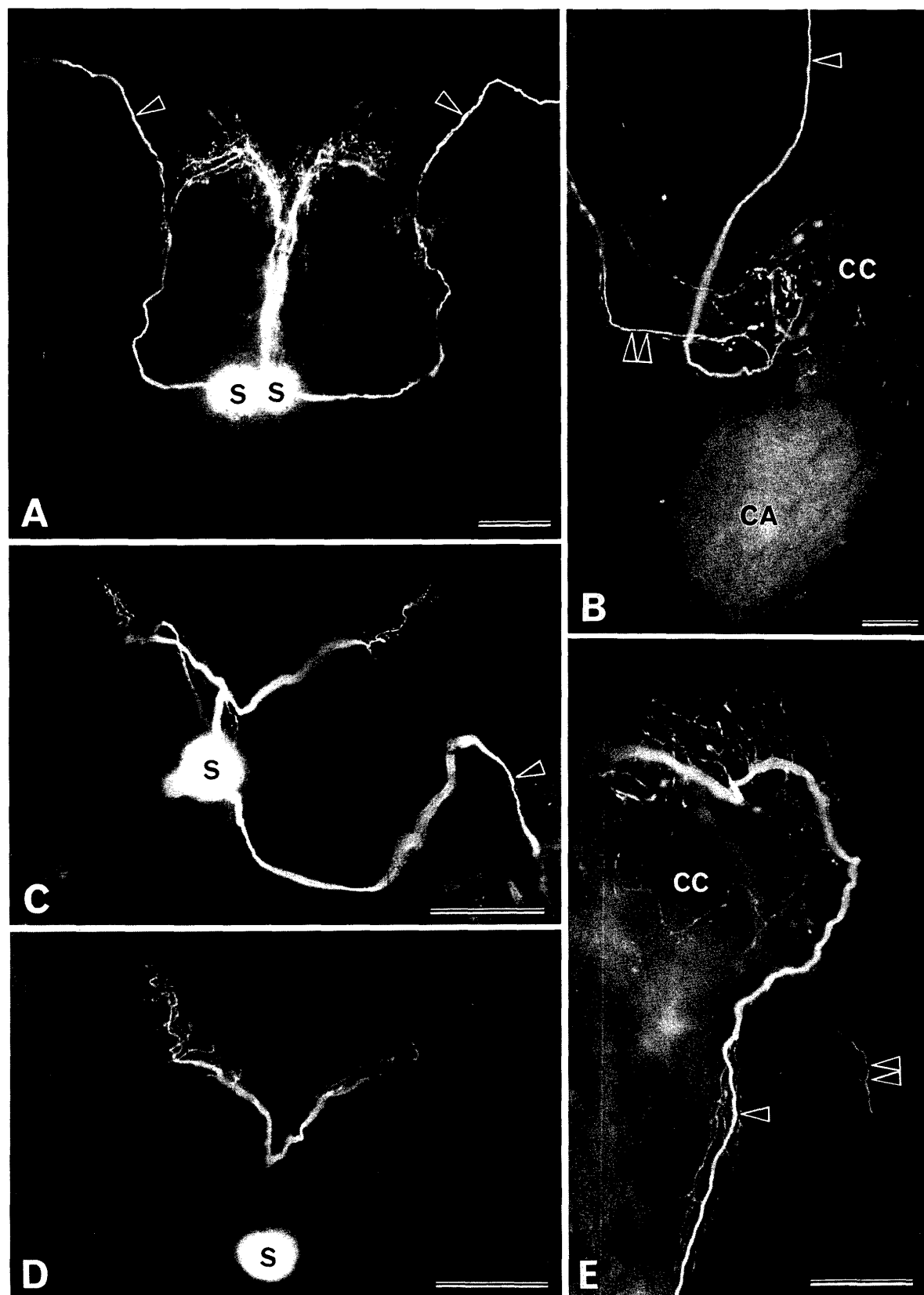


FIG. 6. Photographs of posterior cells in the SG of a 5th instar larva (A,B), a 4-day old pupa (C) and an adult moth (D,E). A. Ventral view of larval SG. S, a soma (out of focus); arrowhead, an axon. B. Dorsal view of larval neurohaemal organs, the corpus cardiacum (CC) and the corpus allatum (CA). arrowhead, a smooth axon in the NCC 3; double arrowheads, varicose axonal branches projecting to the recurrent nerve. C. Ventral view of pupal SG. S, a soma (out of focus); arrowhead, an axon. D. Frontal view of adult SG. S, a soma. E. Ventral view of adult corpus cardiacum (CC). arrowhead, an axonal process invading the recurrent nerve; double arrowheads, an axonal branch in the allatal nerve. Scale bars, 100  $\mu$ m (A, C, D) or 50  $\mu$ m (B, E).

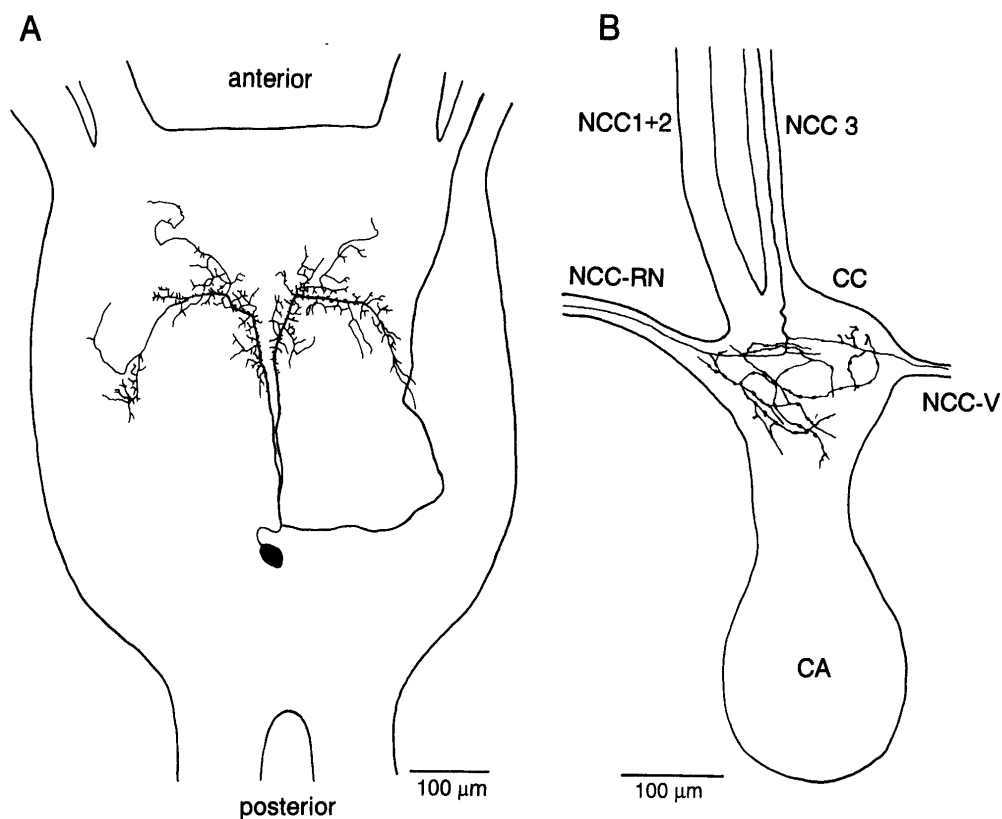


FIG. 7. A. Ventral view of a posterior cell in a 5th instar larva. B. Dorsal view of an axon terminal of the cell. Abbreviations are the same as for Fig. 4.

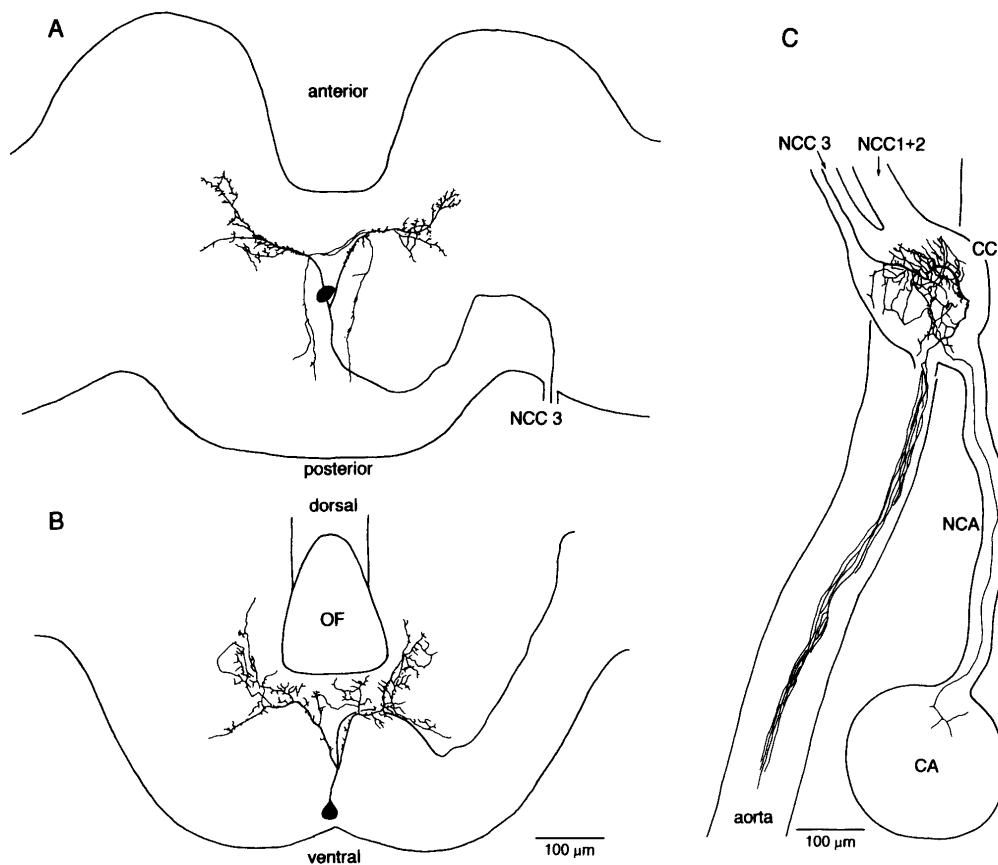


FIG. 8. Ventral (A) and frontal view (B) of a posterior cell and its axon terminal (C) of an adult female. OF, oesophageal foramen; CC, corpus cardiacum; CA, corpus allatum; NCC 1+2, nervi corporis cardiaca 1 and 2; NCC 3, nervus corporis cardiaci 3; NCA, nervus corporis allati.



(mature) DH and/or PBAN and secrete these into the haemolymph at appropriate stages of postembryonic development. Specific morphological profiles of the three sets of cells revealed by injection of LY suggests that they may be under the control of self neural mechanisms to play specific physiological functions. Some evidence for such a functional specialization has been obtained from a surgical experiment showing that potent DH activity is responsible for the posterior cells while potent pheromonotropic activity is linked to the medial cells [14]. However, functional specialization of cells does not simply mean that they produce a specific neuropeptide, because five neuropeptides generated from the precursor usually have significant DH and pheromonotropic activities [30].

Since a group of FXPRLamide peptides, including DH and PBAN, is widely distributed among insects and can regulate many physiological processes [22–24], DH/PBAN immunoreactive cells reported here may be involved in regulation of other processes besides induction of embryonic diapause and pheromone production. The neurosecretory cells are strongly immunoreactive at the larval stage (Fig. 2A) as well as at the pupal and adult stages of the female when DH and PBAN function, respectively (Fig. 2B). In a preliminary experiment, similar DH/PBAN immunoreactive cells were also found in males at the larval, pupal and adult stages. These distributions of DH/PBAN immunoreactivity suggest a multiplicity of functions.

Many FXPRLamide peptides have a myotropic activity [11, 20, 26, 27]. The anterior and medial cells have numerous varicosities (putative neurohaemal structure) in the NCC-V which pass closely between musculatures of the mandible and the maxilla (Fig. 3B, E), though they showed no significant sign of direct innervation into particular muscles of the mouthparts. Thus, neurohormones released from the nerve may act locally on the musculatures. FXPRLamide peptides have an effect on cuticular melanization and epidermal reddish brown pigmentation of some species of armyworms [23, 24]. Because PBAN-I of *Bombyx mori* can produce strong melanization of cuticle of *Pseudaleia separata*, the neuropeptide and other structurally-related neuropeptides from *Bombyx mori* may be involved in regulating integumental pigmentation of the silkworm. The anterior and medial cells have a few varicose branches in the small node (Fig. 3B, E, F), thereby suggesting that two cells present in the node could be possible targets of their modulatory actions. Although origin and functional nature of the cells are unknown, these may be a kind of peripheral (neurosecretory) neuron localized in the course of peripheral nerves [3, 4, 8, 10, 29]. Some peripheral neurons appear to function as a monitor for changes in the internal environment such as concentrations of amino acids and sugars in the haemolymph [28].

Fukuda and Takeuchi [6] found a pair of neurosecretory cells with an azocarminophilic material at the particular area in the ventral side of presumptive labial neuromere of SG and these were regarded as being putative diapausing factor

producing cells, because the amount of the neurosecretory material apparently differed between a diapausing-egg producer and a non-diapausing producer [7]. It is apparent that these neurosecretory cells correspond to posterior DH/PBAN immunoreactive cells, because in the present study we detected no other putative neurosecretory cell with a prolonged action potential and located around the posterior cells. Axons extending from the posterior cells pass the brain and reach the CC (Figs. 7, 8). If the cells produce DH in the somata and transport it to the CC along such an axonal pathway, the pathway would provide an anatomical basis for the distribution of DH activity obtained from the brain and the CC [32, 33].

PBAN immunocytochemistry revealed three clusters of neurosecretory cells in the same regions of the SG of *Helicoverpa zea*, though the number of cells comprising each cluster differs from that of *Bombyx mori* reported here [17]. However, the medial (maxillary) cluster contains two pair of cells that project an axon into the ventral nerve cord (VNC) and which terminate in the terminal abdominal ganglion (TAG). This axonal pathway seems to be involved in the transportation of PBAN and/or PBAN-like peptides, because an intact VNC is needed for normal pheromone production [34]. The peptides transported to the TAG appear to be released into the ganglion and stimulate aminergic neurons innervating pheromone glands to produce sex pheromone [2, 34]. In *Bombyx mori*, there was no cell with such an axon descending the VNC, thereby suggesting that pheromonotropic peptides produced in the somata may be transported to the CC via branches of the maxillary nerve and the NCC-V, then are released into the haemolymph to act directly on pheromone glands.

DH/PBAN immunoreactive cells send their axons to the CC through specific axonal pathways. The anterior and medial cells' axons in particular project into the peripheral nerve that runs under the cuticle of the pupal and adult head (Fig. 1B). Secretion of pheromonotropic peptides is assigned to the medial and anterior cells [14], and seems to be completely inhibited at a critical period of time after the initiation of copulation [13]. These characteristic features of the neurosecretory system make this system an interesting model suitable for electrophysiological analyses of secretory activities of neurosecretory cells at a particular stage of development and control mechanisms of secretion in insects.

## ACKNOWLEDGMENTS

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