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ARCHITECTURE OF CEREBRAL NEUROSECRETORY CELL SYSTEMS IN THE SILKWORM *BOMBYX MORI*

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Summary

Anatomical and physiological characteristics of putative neurosecretory cells (NSCs) in the medial and lateral areas of the larval brain of *Bombyx mori*, identifiable by the opalescent appearance of their somata, were examined by means of intracellular recording and staining. Intracellular injection of Lucifer Yellow revealed that the medial cell group consisted of at least six subgroups of cells distinguishable by the geometry of their dendritic branches. Five subgroups of cells project axons to the contralateral corpus allatum (CA) or to the corpus cardiacum (CC). The remaining subgroup sends an axon to the ipsilateral ventral nerve cord. Three subgroups of cells were identified in the lateral group, projecting axons to the ipsilateral CC, to the CA or to the contralateral CA. Large and prolonged action potentials, similar to those recorded in some neurosecretory systems, were recorded from these medial and lateral cells. However, two pairs of medial cells containing paraldehyde–fuchsin-positive (neurosecretory) material and with axons extending to the contralateral nerve cord had action potentials of a short duration, more typical of non-NSCs such as tritocerebral cells innervating the stomodeal dilator muscles *via* the CC.

Introduction

Insect nervous systems have many specific cells that can be visualized by the characteristic chromophilic materials present in their cytoplasm. Most such 'neurosecretory cells' (NSCs) make a specific terminal structure in specialized neurohaemal regions in order to release their products into the haemolymph (Rowell, 1976; Raabe, 1983). Many neurohormones identified in insect nervous systems seem to be derived from these NSCs (Holman *et al.* 1990).

Morphological and physiological characterization of individual NSCs as well as identification of their hormonal products are vital when attempting to elucidate related neuroendocrine functions. Retrograde cobalt fills combined with silver intensification have revealed the dendritic fields and axonal pathways of NSCs in various species of insects (Pipa, 1978; Koontz and Edwards, 1980; Buys and Gibbs, 1981; Copenhaver and Truman, 1986*b*). Intracellular injection of a marker dye

Key words: insect, neurosecretory cell, dye injection, action potential, *Bombyx mori*.

from a micropipette into a single cell can reveal its complete structure: this is important for identifying a particular cell (Copenhaver and Truman, 1986*a*), for classifying a cell (Carrow *et al.* 1984; Copenhaver and Truman, 1986*b*) and for the possible identification of homologous cells in different species. In addition, intracellular micropipettes can pick up electrical activity that provides clues about the neurosecretory nature of the impaled cell, since the time course of action potentials differs between NSCs and non-NSCs in both vertebrates and invertebrates (Yagi and Iwasaki, 1977; Miyazaki, 1980; Carrow *et al.* 1984).

The silkworm, *Bombyx mori*, is one insect in which (neuro)endocrine mechanisms have been extensively studied. The brain has many types of putative NSCs distinguishable by the size and position of their somata, by the characteristic appearance of their cytoplasmic chromophilic inclusions (Panov and Kind, 1963; Bassurmanova and Panov, 1967) and by retrograde cobalt fills (Morohoshi *et al.* 1977). Recently, three types of NSCs have been identified immunohistochemically (Mizoguchi *et al.* 1987, 1990; Kono *et al.* 1990). In the present study, the central and terminal structures of all putative NSCs in the silkworm brain were individually examined using intracellular injections of the fluorescent dye Lucifer Yellow after recording electrical potentials.

Materials and methods

The fifth-instar larvae of *Bombyx mori* L. used throughout this study were F1 hybrids of the races 'Kinshu' and 'Showa'. The larvae were reared at 26°C on a commercially available artificial diet (Kyodo Shiryo Co., Japan).

The gross anatomical features of the cerebral neuroendocrine system and its associated targets were examined by dissecting anaesthetized animals in a physiological saline and fixing the tissues with a 70 % ethanol solution.

For electrophysiological studies, the head of a larva was usually isolated and mounted in an experimental chamber using beeswax. Sometimes the head of an intact larva was fixed to the chamber after plugging the mouthparts with a quick-drying glue. The brain was exposed by making a window in the front of the head. The chamber was filled with physiological saline designed to mimic extracellular conditions within the brain (Miyazaki, 1980). A pair of insect pins was placed under the brain to provide a supporting platform. After incubation for 20–30 s in saline containing 1 % pronase, the sheath of the brain was partially removed using a fine needle.

Putative NSC somata in the viable brain can be identified under the dissecting microscope by their reflective opalescence. Under visual guidance, an opalescent soma was penetrated with a bevelled glass microelectrode filled with $1\,mol\,l^{-1}$ potassium acetate or 5 % Lucifer Yellow CH (Sigma) dissolved in $0.15\,mol\,l^{-1}$ LiCl. 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. Intracellular potentials and signals for injected currents were displayed and stored on a memory

oscilloscope (VC-10, Nihon Kohden). Stored signals were replayed and recorded on a chart recorder.

Lucifer Yellow (LY) was ionophoretically injected by passing 300 ms hyperpolarizing pulses of current (10 nA) at 1 Hz for 5–10 min. Subsequently the preparation was maintained at room temperature (19–23 °C) for 1–2 h (isolated head) or at 4 °C for 1 day (whole animal). 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 mol l^{-1} sodium phosphate buffer (pH 7.4) for 1 h. 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 with a fluorescence photomicroscope (Nikon). The structure of LY-filled cells was reconstructed from photographic films, using a photographic enlarging projector.

Some LY-filled cells were examined for paraldehyde–fuchsin-positive materials. After observing the fluorescence, the brains were rehydrated and refixed in aqueous Bouin's solution for 24 h in the dark. The tissues were then stained with

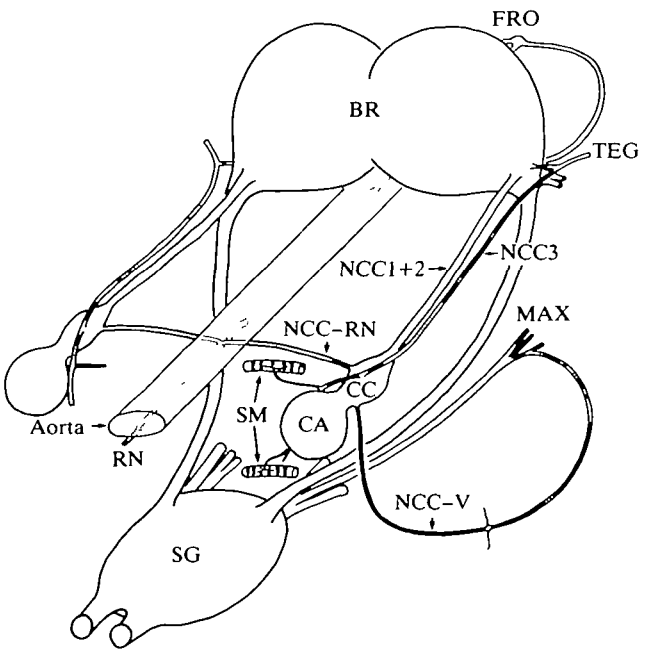

Fig. 1. Schematic diagram of the cerebral neurosecretory system in a fifth-instar larva of *Bombyx mori*. Only the right-hand side of the bilaterally paired retrocerebral complex and associated nerves is completely illustrated. BR, brain; CA, corpus allatum; CC, corpus cardiacum; FRO, frontal ganglion; MAX, maxillary nerve; NCC1+2, nervi corporis cardiaca 1 and 2; NCC3, nervus corporis cardiaci 3; NCC–RN, nervus corporis cardiaci–nervus recurrens; NCC–V, nervus corporis cardiaci ventralis; RN, recurrent nerve; SG, suboesophageal ganglion; SM, stomodeal dilator muscle fibres; TEG, tegumentary nerve.

paraldehyde–fuchsin according to Dogra and Tandan (1964) for whole-mount preparations.

Results

Gross anatomy of the cerebral neuroendocrine system and distribution of putative neurosecretory cells

The morphological organization of the retrocerebral complex and its associated structures in *Bombyx* larvae is illustrated in Fig. 1. It is similar to that of *Manduca sexta* larvae (Nijhout, 1975; Copenhaver and Truman, 1986*b*). A pair of corpora cardiaca (CC) lies posterior to the brain and each CC is connected to the posterior face of the brain by a fused nerve, the nervus corporis cardiaci 1 and 2 (NCC1+2). The CC receives contacts from the nervus corporis cardiaca 3 (NCC3), which arises from the lateral face of the brain as part of the tegumentary nerve and projects to the stomodeal dilator muscles *via* the CC. The corpus allatum (CA) lies posterior to the CC. The allatal nerve, nervus corporis allati (NCA), connecting the CC to the CA, is very short, being little more than a constriction between the two organs. Each CC gives rise to two additional nerves, medially the nervus corporis cardiaci–nervus recurrens (NCC–RN) and ventrally the nervus corporis cardiaci ventralis (NCC–V). The two NCC–RNs from the bilaterally paired CC join beneath the aorta and run for several hundred micrometres on the surface of

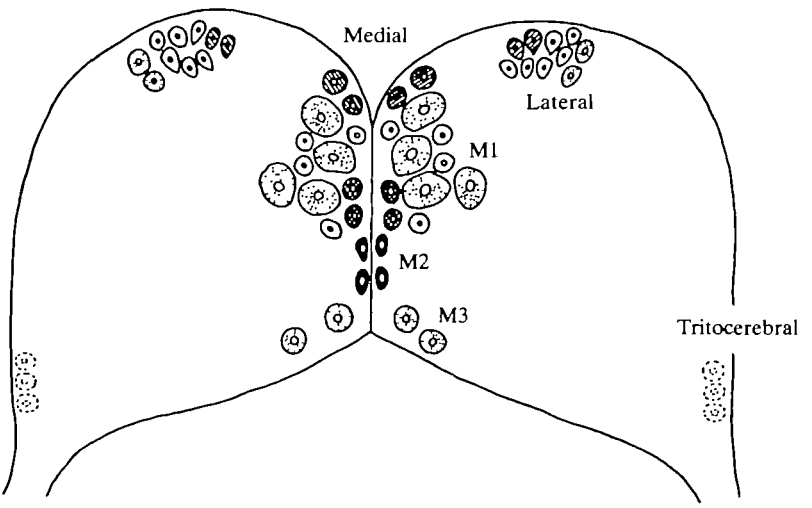

Fig. 2. Highly diagrammatic representation of the distribution of somata of putative neurosecretory cells (NSCs) distinguishable by their opalescent appearance in living brains or from their chromophilic cytoplasmic inclusions. Frontal view. M1, M2 and M3 represent the first, second and third groups of medial cells, respectively. Different shadings of M1 cells and lateral cells represent different staining properties (after Panov and Kind, 1963; Bassurmanova and Panov, 1967). Three tritocerebral cells (interrupted circles) were visualized by retrograde fills of Lucifer Yellow *via* the NCC3.

Table 1. *Summary of the characteristics of putative neurosecretory cells in the brain of* Bombyx mori

Cell group	Location of major dendrites	Axonal pathway	Terminal site	Mean action potential duration ±S.D. (ms)	*N*	Neuropeptides identified
Protocerebral cells						
Medial group						
M1 cells						
Subgroup a	Ipsi dorsal	Contra NCC1+2	CA	33±14	24	Bombyxin[1]
b	Both medial	Contra NCC1+2	CC complex	25±8	12	
c	Both dorsal	Contra NCC1+2	CC complex	24±9	7	
d	Ipsi dorsal	Contra NCC1+2	CC complex	23±8	4	
e	Contra ventral	Contra NCC1+2	CC complex	26±10	9	
M2 cells	Both medial	Contra VNC	?	2.5±0.3	11	
M3 cells	Both medial	Ipsi VNC	PN[4]	24±6	4	Eclosion hormone[2]
Lateral group						
Subgroup a	Ipsi dorsal	Contra NCC1+2	CA	26±9	8	PTTH[3]
b	Ipsi medial+ventral	Ipsi NCC1+2	CA	22±10	10	
c	Ipsi medial+ventral	Ipsi NCC1+2	CC complex	26±8	11	
Tritocerebral cells	Ipsi tritocerebrum	Ipsi NCC3	Stomodeal muscles	1.2±0.2	8	

Ipsi, ipsilateral (hemisphere); Contra, contralateral (hemisphere); Both, both hemispheres; CA, corpus allatum; CC complex, corpus cardiacum and associated nerves of the CC; NCC1+2, nervi corporis cardiaci 1 and 2; NCC3, nervus corporis cardiaci 3; VNC, ventral nerve cord.

[1] Mizoguchi *et al.* (1987); [2] Kono *et al.* (1990); [3] PTTH, prothoracicotropic hormone, Mizoguchi *et al.* (1990); [4] PN, proctodeal nerve (T. Ichikawa, unpublished immunohistochemical observation).

the recurrent nerve (RN) before merging into it. The NCC–V runs through the underlying hypopharyngeal musculature and joins a branch of the maxillary nerve of the suboesophageal ganglion (SG). The SG is connected to three thoracic ganglia and eight abdominal ganglia.

The putative NSCs in the brain occur in two clusters, medial and lateral (Fig. 2). Most of the NSCs in the viable brain were clearly visible because of the way they diffracted light. Three distinct groups of cells were usually recognizable in the medial cluster, on the basis of the size, shape and position of their somata; the three groups are here termed M1, M2 and M3 cells following the nomenclature of Panov and Kind (1963). The relative locations of the somata within the M1 group varied considerably from animal to animal. However, four cells with large somata (40–50 μm in diameter) were readily distinguishable from other cells with small somata (20–30 μm in diameter) in each hemisphere of the brain. Two pairs of M2 cells were usually distinguishable by their ellipsoidal somata located close to the midline of the brain. One or two M3 cells with relatively large and round somata (30–35 μm in diameter) were sometimes faintly discernible in the anteroventral area of both brain hemispheres.

The lateral cluster was always made up of several cells. No individual cell in this cluster was accurately identifiable on the basis of the appearance of its soma in the viable brain, though histological studies revealed three types of cell in this cluster (Panov and Kind, 1963; see Fig. 2).

Retrograde LY filling *via* the NCC3 in a preliminary experiment revealed three cells with somata in the lateral surface of the tritocerebrum (Fig. 2) and thick

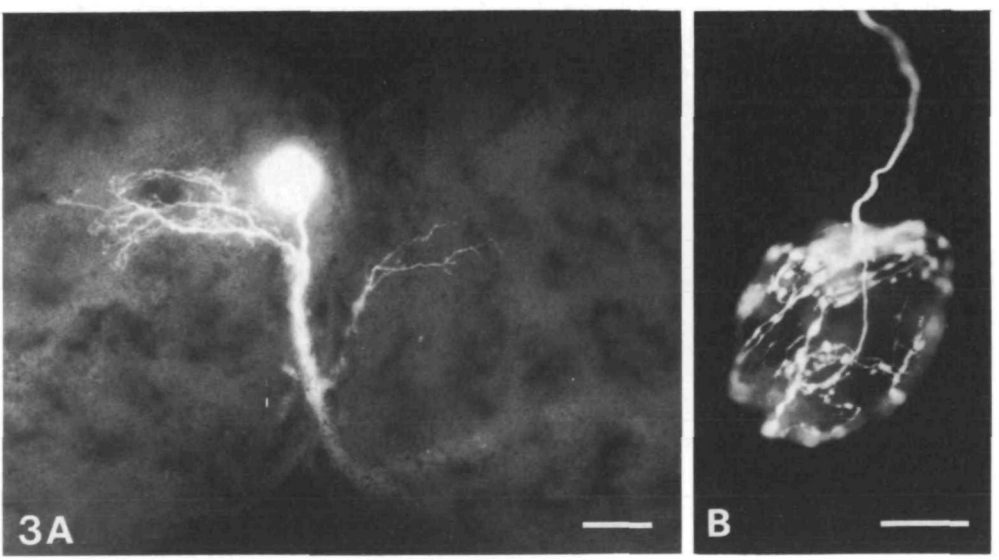

Fig. 3. Photomicrographs of a cell of group M1 with a large soma (subgroup a), as revealed by an ionophoretic injection of Lucifer Yellow. (A) Frontal view of the central structure in the brain. (B) Terminal ramification and varicosities on the dorsal surface of the corpus allatum. Scale bars, 50 μm.

dendrites in a ventral region of the tritocerebral neuropile. The fill was carried out because these tritocerebral cells had been visualized previously by retrograde cobalt fills *via* the NCC3 and were considered to be members of an NSC group (Morohoshi *et al.* 1977). It was very difficult to identify the tritocerebral cells as they lacked the opalescent appearance of NSCs and they did not stain with paraldehyde–fuchsin.

Structures of putative neurosecretory cells

One hundred and eight cells in 102 animals were filled with Lucifer Yellow. The distribution and overall characteristic profiles of the cells are summarized in Table 1. Examples of the dye-filled cells are shown in Figs 3–5. Fig. 3 shows central and terminal arborizations of an M1 cell with a large soma. A single neurite extending from the soma runs ventrally for a short distance and bifurcates into an

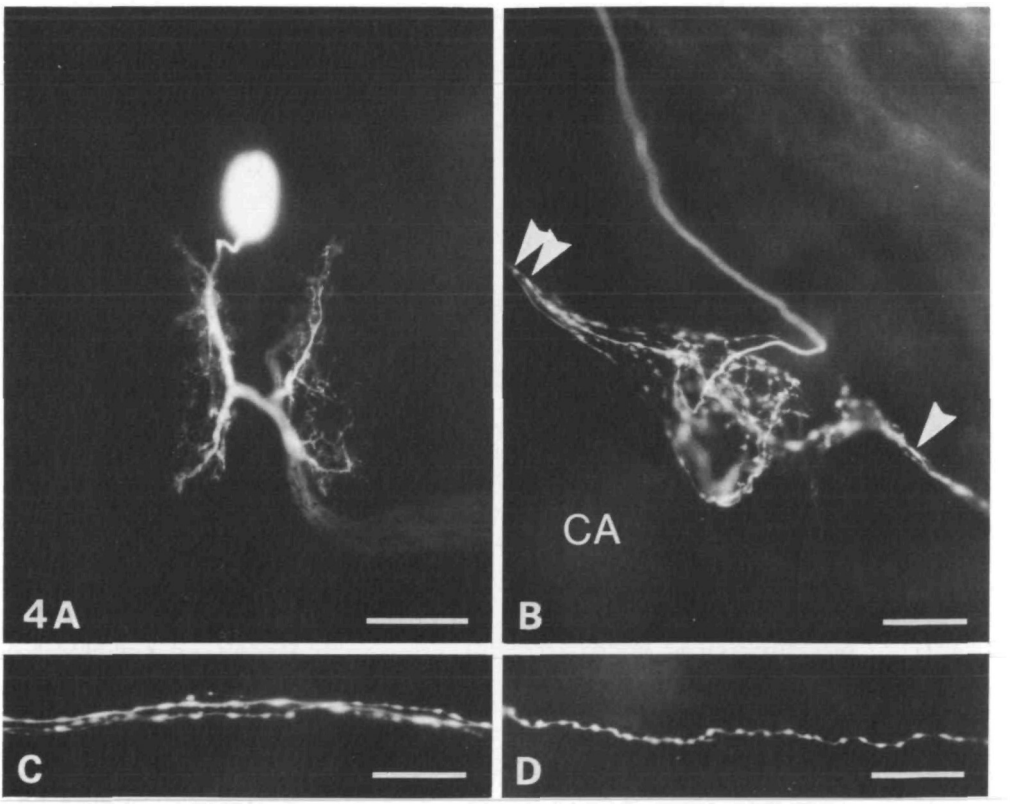

Fig. 4. Photomicrographs of various portions of a cell of group M1 with a small soma (subgroup b). (A) Frontal view of the dendritic arborization in the medial part of the brain. (B) Terminal arborization in the corpus cardiacum. A few varicose processes extend to the nervus corporis cardiaci–nervus recurrens (single arrowhead) and the nervus corporis cardiaci ventralis (NCC–V) (double arrowhead). CA, corpus allatum. (C) Varicose processes running along the recurrent nerve. (D) Varicose processes invading the NCC–V. Scale bars, 50 μm.

axonal process and a large dendritic process that extends branches to the dorsal region of the ipsilateral protocerebrum. The axonal process produces a few thin branches before and after crossing the midline of the brain. A contralateral branch extends to the dorsal protocerebrum. The axon runs along the ventral surface of the contralateral protocerebral neuropile, enters the NCC1+2 and passes through the CC without branching to terminate in the CA. The terminal neurohaemal arborization was confined to the superficial region of the CA (Fig. 3B). Fig. 4 shows an M1 cell with a small soma. The cell has a relatively narrow dendritic field in the medial region of the protocerebrum in both hemispheres of the brain. The axon of the cell projects to the contralateral CC *via* NCC1+2, where the terminal branches form a complex network of numerous varicosities (Fig. 4B). A few terminal branches leave the CC towards the recurrent nerve *via* the NCC–RN (Fig. 4C) and the NCC–V (Fig. 4D). The terminal branches do not invade the CA. Fig. 5 shows a lateral cell that projects an axon to the contralateral CA. The neurite originating from the soma travels ventromedially towards the midline and crosses into the contralateral hemisphere of the brain. The neurite is densely fasciculated. The axon, after taking a characteristic pathway in the contralateral hemisphere (for details, see Fig. 10), leaves the brain and terminates in the CA without branching in the CC (Fig. 5B). The axon usually bifurcates in the NCA or CA and spreads varicose terminal processes on the surface of the CA. The terminal of the lateral cell was easily distinguishable from that of the medial cell shown in Fig. 3 by its relatively thin and sparse terminal branches.

Medial cells

M1 cells. This group of cells could be divided into at least five subgroups

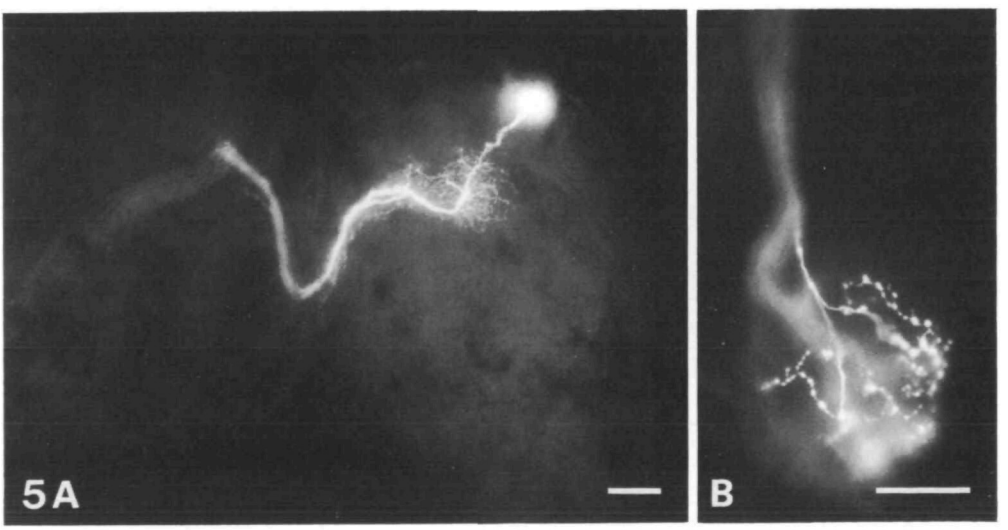

Fig. 5. Micrographs of a cell of the a subgroup of the lateral group of cells. (A) Central structure in the brain. (B) Terminal branches on the dorsal surface of the contralateral CA. Scale bars, 50 μm.

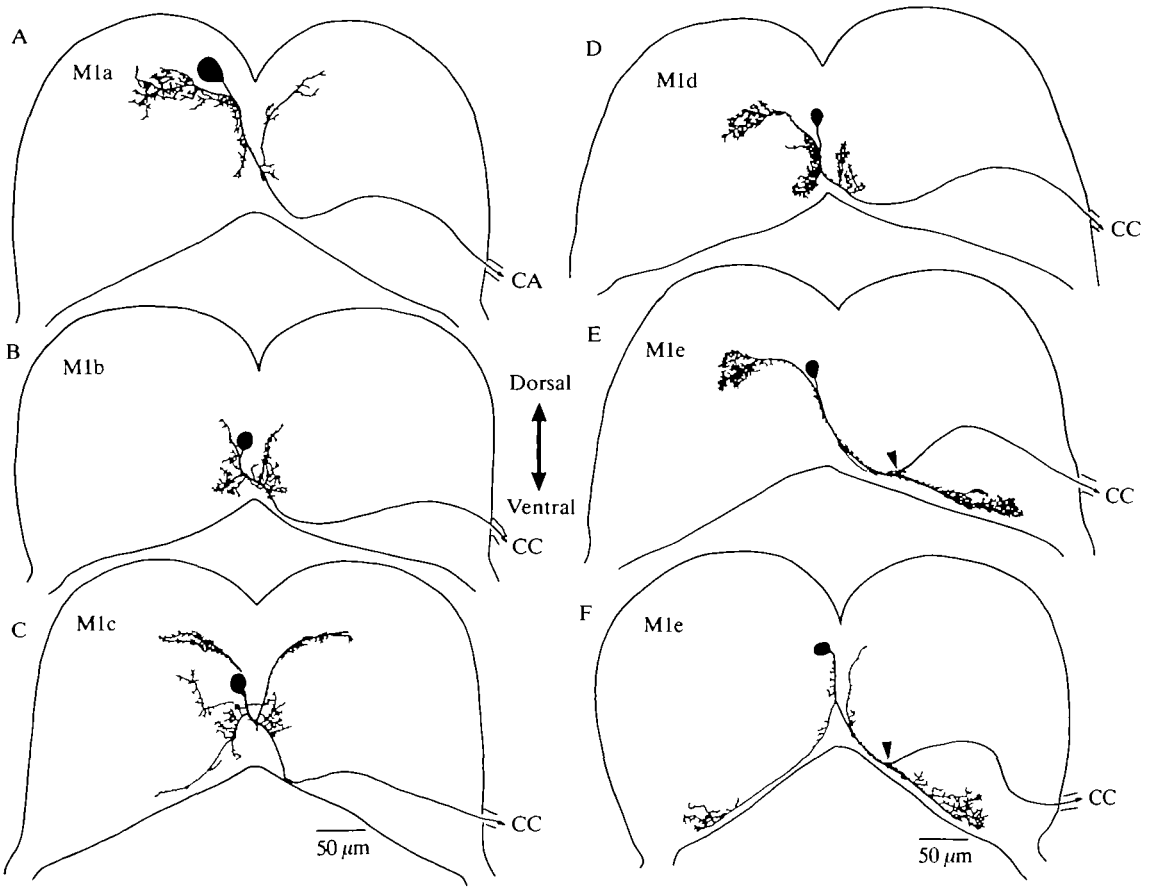

Fig. 6. Five subgroups of M1 cells. (A) The cell of subgroup M1a is characterized by a large soma and a large ipsilateral dendrite spreading in the dorsal protocerebrum. (B) The M1b cell has a narrow dendritic field in the medial region of the brain. (C) The M1c cell has two major dendritic branches extending to the dorsal superficial region of the protocerebrum in both hemispheres. (D) The M1d cell sends a major dendrite into the same protocerebral region as the M1a cell, but has a small soma. (E,F) The two cells of subgroup M1e are characterized by thickening of the axons (arrowheads) and by the ventral collaterals originating from the axonal thickening. They show considerable variability in the distribution of additional (thin) processes. CA, corpus allatum; CC, corpus cardiacum.

according to the pattern of the dendritic branches (Fig. 6). The cells of subgroup M1a are characterized by thick ipsilateral and thin contralateral dendrites extending to the dorsal protocerebrum as well by large somata (Fig. 6A). Another cell of the same subgroup is illustrated in Fig. 3. M1a cells always terminated in the CA without branching in the CC. The branching pattern of their dendrites, including other short processes in the medial area of the brain in different animals, showed little variability, suggesting that the M1a cells may represent a functionally

homogeneous group. The structural profile of the M1a cells is identical to that of bombyxin-producing cells (Mizoguchi *et al.* 1987).

M1b cells usually have a dendritic field confined to the medial region of the protocerebrum (Fig. 6B, see also Fig. 4). A few cells extended a slender process to the ventral area of the ipsilateral protocerebrum. The axon terminals always formed a complex network of branches and varicosities in the CC and there were a few branches extending out of the CC to the NCC–RN and NCC–V (Fig. 4B–D). The branches extending to the NCC–RN usually terminated after running for several hundred micrometres on the surface of the RN, although in a few cells these branches terminated before reaching the RN or after running the entire length of the contralateral NCC–RN. Some varicose processes were also found in the NCA, but they did not invade the CA. These terminal profiles of M1b cells in the CC complex (the CC and accessory nerves of the CC) were common to the other three subgroups of cells with small somata, as described below.

The M1c cells gave rise to dorsal dendrites in both hemispheres, whereas the M1d cells lacked the contralateral dendrite (Fig. 6C,D). Cells of both subgroups showed variability in the length of the additional minor branches.

Cells of subgroup M1e are characterized by the large ventral dendrite that arises from a thickening of the neurite in the contralateral hemisphere (Fig. 6E,F). The axon also originates from the thickening and projects to the CC complex. The cells of this subgroup showed considerable variations in additional dendritic processes. Most cells had a thin process extending dorsally (and then turning laterally) in the contralateral hemisphere (Fig. 6F). Some cells extended a (slender) ipsilateral process to the ventral (Fig. 6F) or dorsal area of the protocerebrum (Fig. 6E). This relatively large variability in distribution of the minor processes may suggest a further division of this subgroup of cells, although in this report I put them into the same group.

M2 cells. These cells spread dendritic branches in the anteromedial region of the ipsi- and contralateral hemispheres of the brain (Fig. 7A). The axons run along the ventral surface of the contralateral protocerebral neuropile, send out several fine processes to the tritocerebrum, and travel down the circumoesophageal connective to the suboesophageal ganglion (SG) where they have a few short processes (inset to Fig. 7A). Although the axons were observed to run down the ventral nerve cord connecting the three thoracic ganglia, they could not be traced to their terminals because the dye was only faintly fluorescent in the axons and was partially obscured by the autofluorescence of the thoracic and abdominal ganglia.

Electrical stimulation of the somata of the M2 cells induced a short-duration action potential apparently differing from the typical NSC's action potential (see Fig. 12). This result could have been obtained because the electrode was introduced into a non-neurosecretory cell located in the close vicinity of the M2 cell rather than in the M2 cell itself. However, the impaled cell was shown to be the M2 cell following staining with paraldehyde–fuchsin after LY injection (Fig. 8). The location, shape and staining properties of the cell soma were all typical of M2 cells.

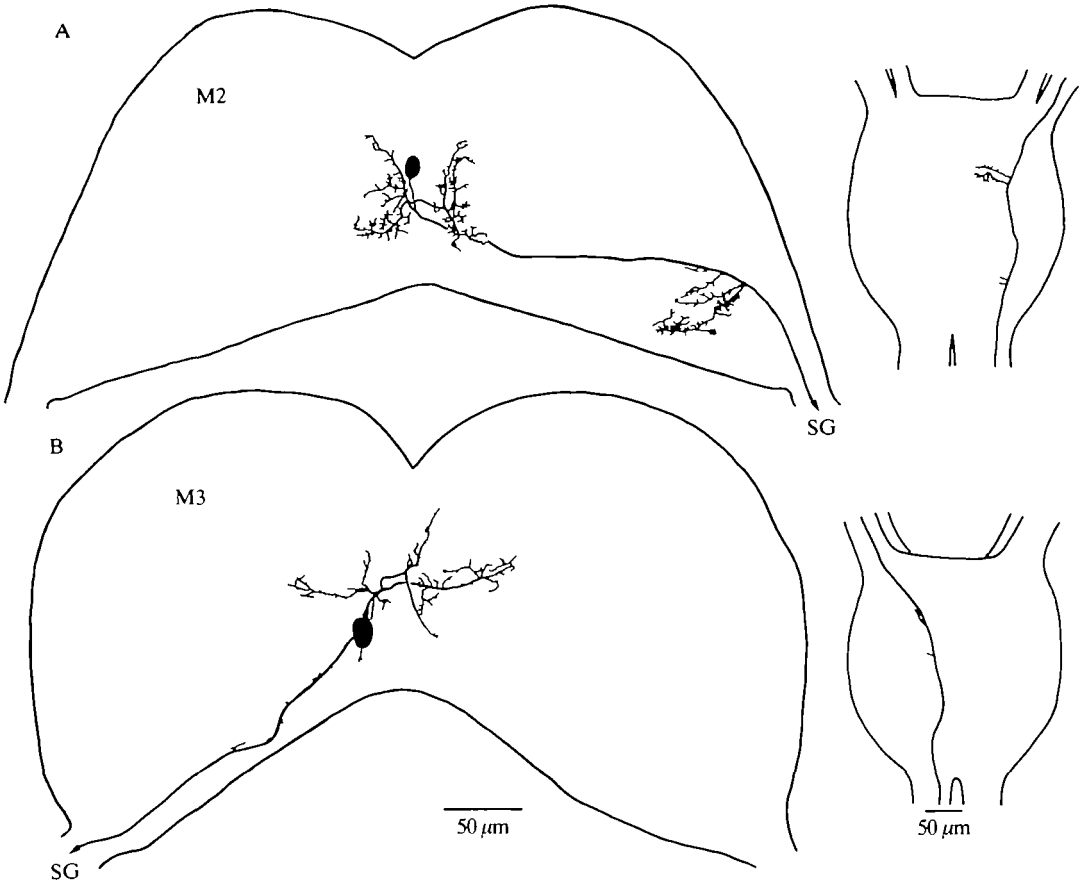

Fig. 7. Structures of two cells projecting axons to the ventral nerve cord. (A) The M2 cell sends an axon through the contralateral circumoesophageal connective to the suboesophageal ganglion (SG). (B) The M3 cell sends its axon through the ipsilateral connective. Insets illustrate axons passing through the SG.

M3 cells. This group of cells also has dendritic fields in the anteromedial region of the brain (Fig. 7B). However, the axons do not cross the midline of the brain, but traverse the ventral surface of the ipsilateral protocerebral neuropile and enter the SG where they give off few branches (inset to Fig. 7B). Although the axons could be traced only to the SG and to the adjacent ganglion using the dye-filling method described in this study, immunohistochemical techniques, using a monoclonal antibody against the eclosion hormone, have revealed that the axons of the M3 cells extend the full length of the ventral nerve cord and project into the proctodeal nerve (T. Ichikawa, in preparation).

Lateral cells

Cells located in the lateral cluster were divided into three subgroups, La–Lc, according to their central and terminal structures.

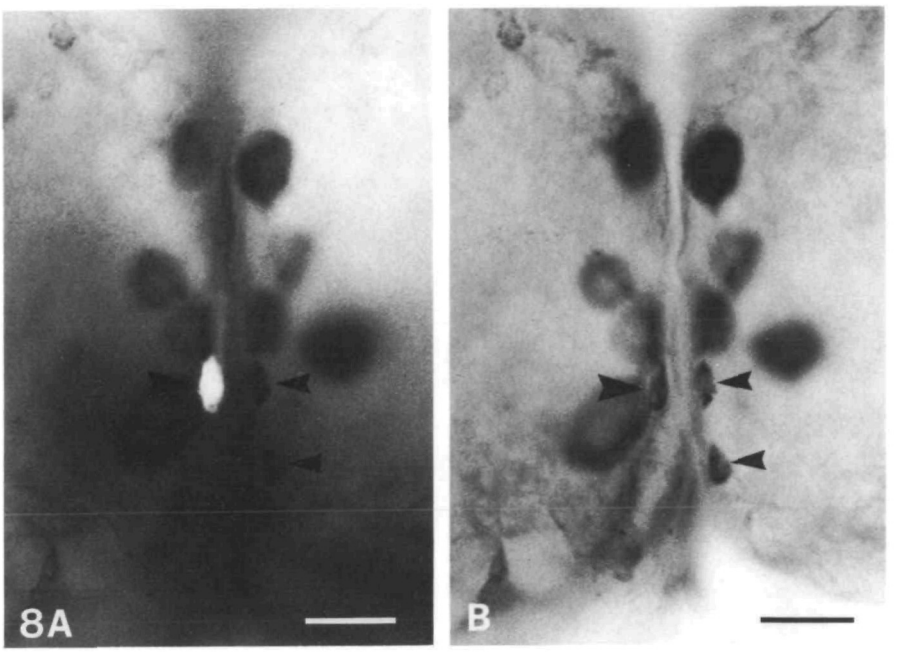

Fig. 8. (A) Fluorescent and (B) bright-field photomicrographs of the medial part of the same brain showing that the Lucifer-Yellow-filled cell is the paraldehyde–fuchsin-positive M2 cell (large arrowheads). Small arrowheads indicate other M2 cells not stained with Lucifer Yellow. Scale bars, 50 μm.

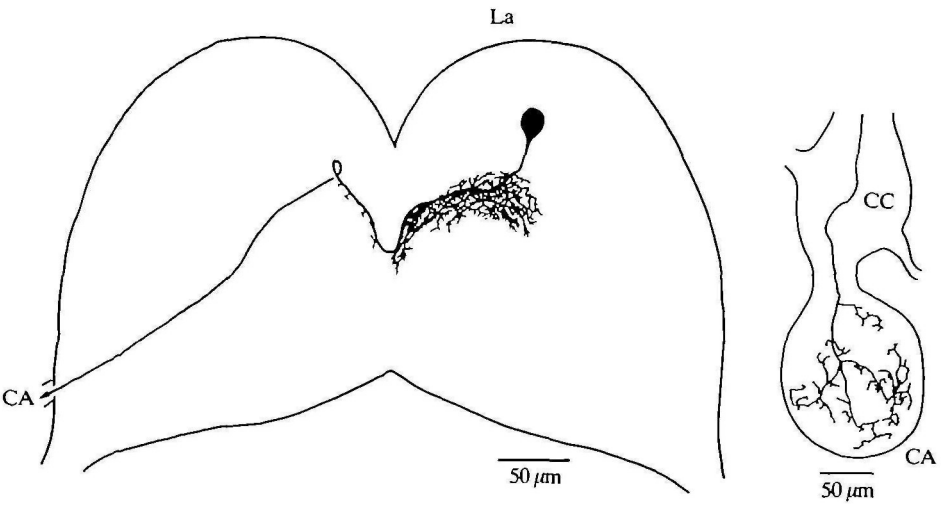

Fig. 9. Frontal view of the central structure of a lateral cell in subgroup La. Another cell of the same group is illustrated in Fig. 5. The inset shows the axon passing through the corpus cardiacum (CC) and forming terminal branches on the dorsal surface of the corpus allatum (CA).

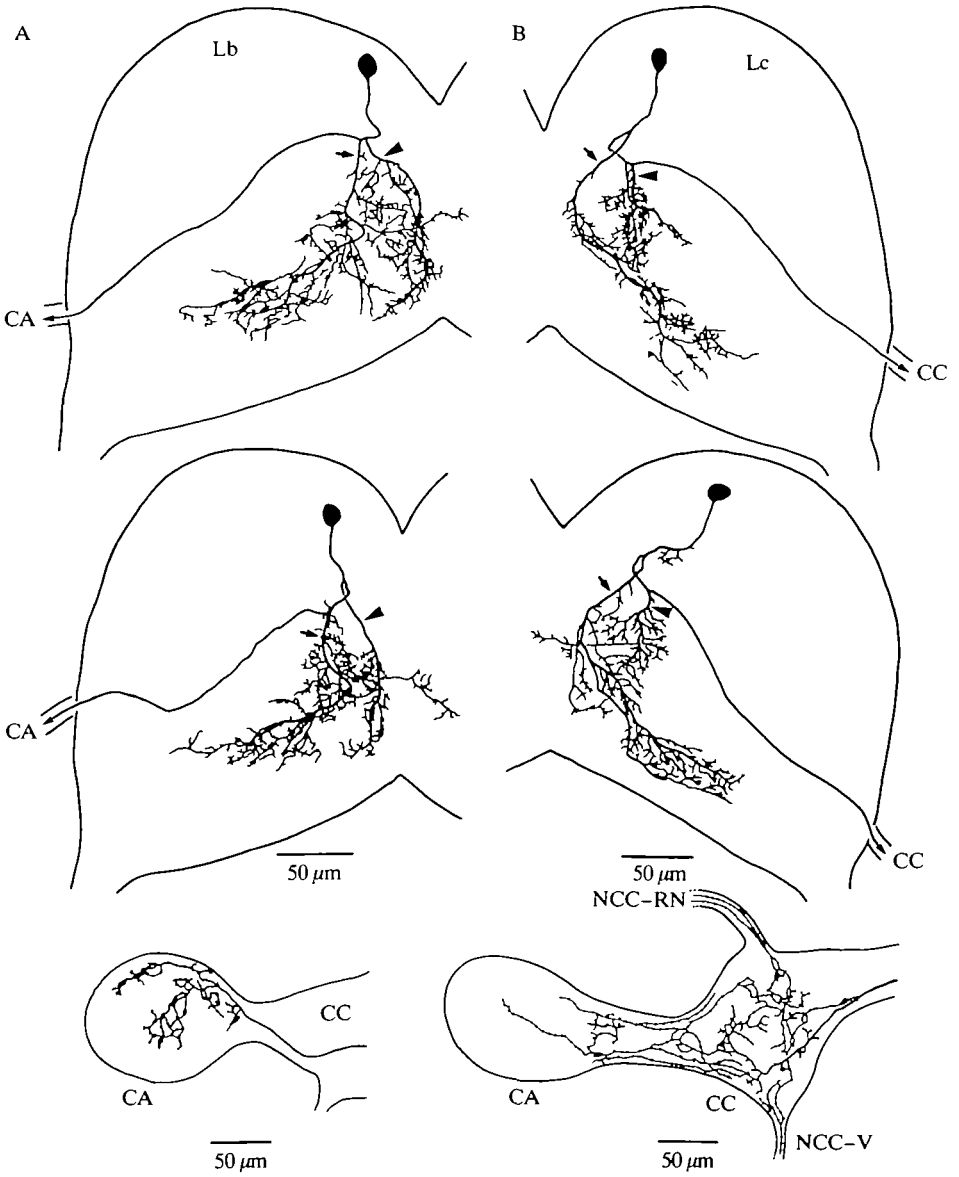

Fig. 10. Comparison of the dendritic and terminal geometry of lateral cells of subgroups Lb (A) and Lc (B). Both groups of cells have medial (arrowheads) and ventral (arrows) collaterals. The medial collaterals of the Lb cells originate from the neurite leading to the ventral collaterals, whereas those of the Lc cells originate from the axon. The Lb cells terminate in the CA, whereas the Lc cells terminate in the CC (terminal ramifications on the dorsal surface of the neurohaemal organ are shown at the bottom of each column). NCC–RN, nervus corporis cardiaci–nervus recurrens; NCC–V, nervus corporis ventralis.

Fig. 9 shows the central pattern of branching of a cell from the first subgroup. The neurite arising from the soma first runs ventromedially towards the midline of the brain and then dorsolaterally for some distance after crossing the midline, finally turning ventrally to enter the NCC1+2. The densely fasciculated neurite, the axonal pathway and the terminal profile in the CA are identical to those of the prothoracicotropic hormone (PTTH)-producing cells identified immunohistochemically (Mizoguchi *et al.* 1990).

Fig. 10 shows four cells belonging to the second (Lb) and third subgroups (Lc) of the lateral cells. They send two large dendritic collaterals into the medial and ventral regions of the protocerebrum and axons to the ipsilateral NCC1+2. The Lb cells can be differentiated from the Lc cells on the basis of their terminal structures; the former have terminal branches in the CA (Fig. 10A, bottom), whereas the latter, like the M1b cells (Fig. 4), send terminal branches to the CC complex (Fig. 10B, bottom), though some Lc cells have a few terminal processes running onto the surface of the CA (Fig. 10B). Although the dendritic arborizations of the two subgroups of cells appear complicated and similar, the two can be distinguished on the basis of the branching point of the medial dendrites (arrowheads in Fig. 10). The dendrites of Lc cells originate from the axons, whereas those of Lb cells arise from neurites connecting the somata and the ventral dendritic branches (arrows in Fig. 10).

Tritocerebral cells

The dye-filled electrode was advanced into the ventral region of the neuropile, where the tritocerebral cells extended thick dendrites, until it encountered a cell to fill with LY. About 40% of the filled cells sent axons into the NCC3 and had a dense dendritic arborization in the neuropile (Fig. 11). The axons running down the NCC3 passed the CC without branching and extended several long terminal branches among the stomodeal dilator muscles (data not shown).

Electrical action potentials of putative neurosecretory cells

The duration of action potentials recorded from *B. mori* NSCs has been reported to be significantly longer than that of non-NSCs (Miyazaki, 1980). Action potentials were recorded from impaled cells, before injecting the dye. They were induced by current injection because many cells showed no spontaneous activity. All impaled cells in the medial and lateral clusters, except the M2 cells, showed large, overshooting and prolonged action potentials (Table 1). Fig. 12A shows such action potentials obtained by injecting current pulses (1 nA, 300 ms in duration) into a cell of subgroup M1a. The cell produced three action potentials with a duration of 20–30 ms at half-amplitude. Fig. 12B shows a typical action potential with a short duration recorded from the soma of an M2 cell. The duration of the action potential of M2 cells, 2.5 ms, was about one-tenth of that of other medial and lateral cells (see Table 1). It also differed significantly from the duration of action potentials (1 ms) recorded from a neurite (dendrite) of the

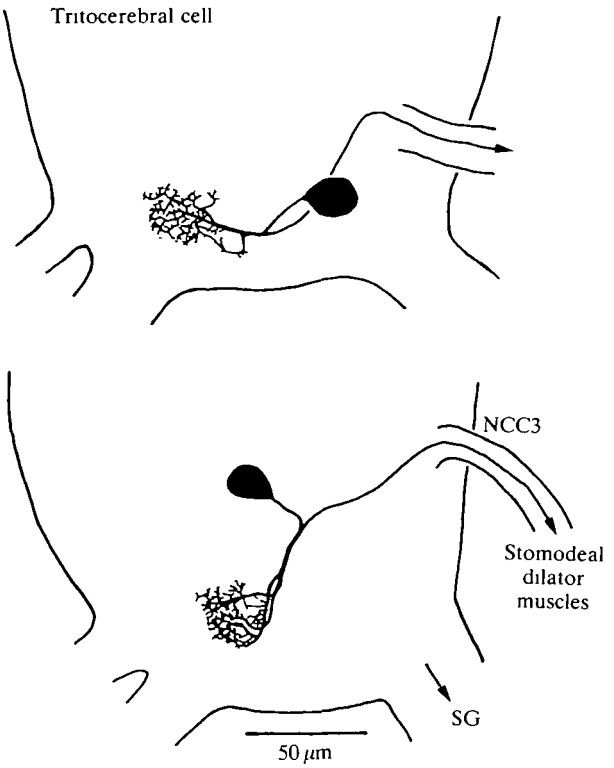

Fig. 11. Lateral aspect of tritocerebral cells projecting axons to the stomodeal dilator muscles *via* the nervus corporis cardiaca 3 (NCC3).

tritocerebral cells (Fig. 12C), which innervate the stomodeal dilator muscles (Fig. 11).

Discussion

Histological studies using specific 'neurosecretory' stains have shown that in the brain of *B. mori* the medial (group M1) cell cluster contained at least four and the lateral cell cluster at least three types of cells distinguishable by the size and position of their somata and by the staining properties of their cytoplasmic inclusions (Panov and Kind, 1963; Bassurmanova and Panov, 1967). The five M1 and three lateral cells identified in the present study (Figs 6, 9, 10) illustrate the multiplicity of morphological cell types present in the insect brain. These morphologically and histologically different cells may regulate different physiological and behavioural processes by producing different neurosecretory signals in the form of neurohormones. Various peptide hormones have been extracted from insect nervous systems and their amino acid sequences have been analyzed (Holman *et al.* 1990). In *B. mori*, three species of cerebral neuropeptides have

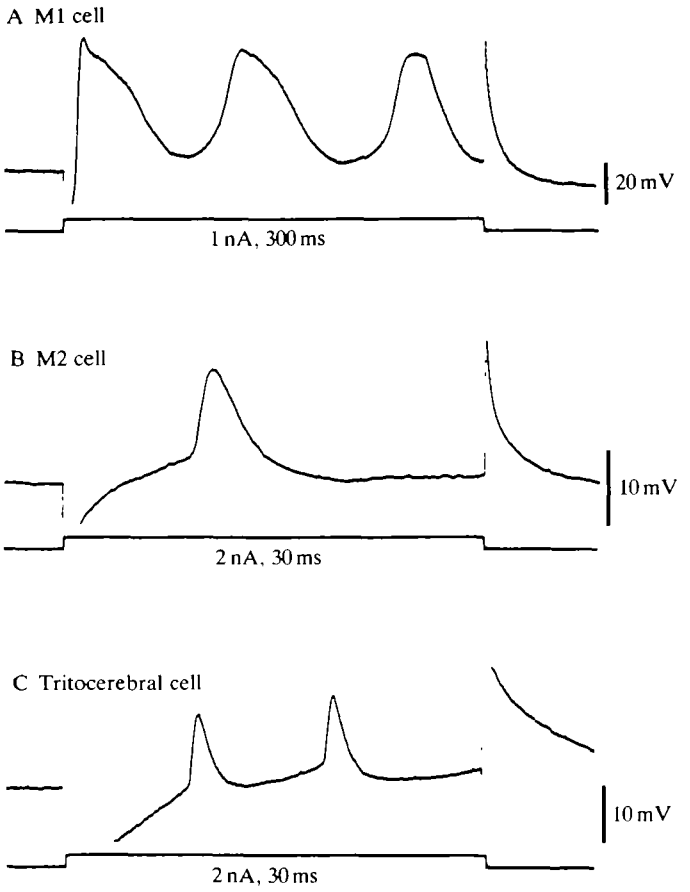

Fig. 12. Action potentials induced by the injection of electrical currents into putative neurosecretory cells. (A) Response of a cell in group M1; (B) response of a cell in group M2 and (C) response of a tritocerebral cell. Upward deflections of the current traces indicate the beginning of current injections and downward deflections signal the end of current injection. The amplitude and duration of the current are shown beneath each trace. Resting membrane potentials were 40 mV (A), 28 mV (B) and 27 mV (C).

been characterized and immunohistochemical studies using monoclonal antibodies have revealed the localization (and structural characteristics) of the cells responsible for the production of peptides. Bombyxin is an insulin-like peptide that activates the prothoracic glands of the saturniid moth *Samia cynthia ricini* (though inactive on *Bombyx mori*) and is produced by four pairs of medial cells with large somata (Mizoguchi *et al.* 1987). The bombyxin-producing cells were positively identified as M1a cells in the present study (Figs 3 and 6A) because of their uniquely large somata and from the distribution of their axon terminals in the CA. Prothoracicotropic hormone (PTTH) is a peptide that activates the prothoracic

glands of *B. mori* and is synthesized by two pairs of lateral cells that project to the contralateral CA (Mizoguchi *et al.* 1990). The immunohistochemical profiles of their characteristic axonal pathway and axon terminals in the CA clearly demonstrate that the PTTH-producing cells correspond to the La cells (Figs 5 and 9). Eclosion hormone (EH) is a peptide that triggers pupal–adult ecdysis (Fugo *et al.* 1984). Although immunohistochemistry using the anti-EH antibody did not reveal their dendritic structure and axonal pathway, the ventromedial localization of the cells reactive to the antibody suggests that they correspond to the M3 cells (Kono *et al.* 1990). In an immunohistochemical experiment, I observed that the M3 cells filled with Lucifer Yellow were immunoreactive to the anti-EH antibody produced by Kono *et al.* (1990). In addition, the experiment revealed that these cells had axonal pathways leading to the proctodeal nerve (T. Ichikawa, in preparation).

The axonal pathway of the M3 cells is interesting because it is thought that the EH synthesized by the medial cells may be transported to the CC–CA complex to be liberated from there into the haemolymph. This is suggested by the strong EH activity in extracts from the medial part of the brain and CC–CA complex and in the haemolymph (Fugo and Iwata, 1983; Fugo *et al.* 1984; Sakakibara and Fugo, 1990). However, the M3 cells do not send any processes to the CC–CA complex (Fig. 7B). In *Manduca sexta* there are two sources of EH. The EH that triggers the larval–larval and larval–pupal ecdyses is produced by two pairs of ventromedial cells (Truman and Copenhaver, 1989), whereas the EH responsible for the pupal–adult ecdysis is produced by five pairs of lateral cells that project to the ipsilateral CC–CA complex (Copenhaver and Truman, 1986*a*). The same situation may occur in *B. mori* because (1) the axonal pathways of the M3 cells of *B. mori* and of the ventromedial EH cells of *M. sexta* are the same, suggesting that they are homologous, and (2) an extract from the lateral part of the *B. mori* brain showed weak, but significant, EH activity (Fugo and Iwata, 1983). If this proposal proves to be correct, then the Lb cells are the most likely candidates for the lateral source of EH, because these cells send axons to the ipsilateral CA (Fig. 10). Confirmatory evidence could probably be acquired by assaying bioactive material released from the cells by intracellular stimulation, an approach used with success in *M. sexta* (Copenhaver and Truman, 1986*a*).

Intensive intracellular studies on the structure of single NSCs in *Manduca sexta* revealed at least three morphological classes of monopolar cells in both the medial (corresponding to the M1 group according to the terminology used in the present study) and the lateral clusters in pupal and adult brain, though an additional class of NSCs appeared in the medial cluster during adult development (Carrow *et al.* 1984; Copenhaver and Truman, 1986*b*). Intracellular fills in the present study on the larval brain of *Bombyx mori* revealed five morphological classes of NSCs in the medial (M1) and three in the lateral clusters. Despite the species difference and the different stages of development examined, the close structural similarities between some cells of *M. sexta* and *B. mori* has facilitated identification of homologous cells in the two species. In *B. mori* the NSCs projecting to the

retrocerebral complex have terminal branches in either the CC or the CA, but not both (Figs 3–5). In *M. sexta*, a similar restriction of the terminal branches has been observed in the pupal cells (Carrow *et al.* 1984). In contrast, many cells in the adult brain share two retrocerebral organs for their terminals, and some cells terminate on the surface of the aorta (Copenhaver and Truman, 1986*b*). Thus, it is easier to draw comparisons between the larval cells of *B. mori* and the pupal cells of *M. sexta*. The M1a cell of *B. mori* (Fig. 6A) apparently corresponds to the medial Ia cell of *M. sexta*, which is characterized by its large soma, ipsilateral major dendrites and a terminal in the CA (Figs 4A, 5A in Carrow *et al.* 1984). The profile of the remaining two classes of *M. sexta* medial cells whose dendritic fields are limited to the ipsilateral hemisphere does not fit that of any medial cells of *B. mori*, though these cells commonly terminate in the CC. In the case of lateral cells, it is obvious that the La cell of *B. mori* (Fig. 9) corresponds to the IIa cell of *M. sexta*, since both have a densely fasciculated neurite in the ipsilateral protocerebrum, a characteristic axonal pathway and terminate in the contralateral CA (Fig. 4C in Carrow *et al.* 1984). Immunohistochemical studies have shown that the IIa and La cells both produce the same hormone, PTTH (O'Brien *et al.* 1988; Mizoguchi *et al.* 1990). The Lb and Lc cells (Fig. 10) may also be related to the IIb(CA) and IIb(CC) cells that terminate in the ipsilateral CA and CC, respectively (Fig. 7 in Carrow *et al.* 1984).

Axon terminals in the CC always contained a few processes extending to the NCC–RN (and RN) and the NCC–V (Fig. 4). These processes have numerous varicosities along their entire length and do not leave from nerves to reach specific target tissues. Axon terminals in the CA spread their varicose processes on the surface of the CA, but few occur inside the CA (Figs 3, 5). These findings suggest that the retrocerebral complex, including the peripheral nerves of the CC, serves as a release site for the neurosecretory products of the cerebral NSCs into the haemolymph. There was no consistent picture to suggest a direct delivery of the secretory product to a specific target tissue such as the CA, though a few lateral cells did extend a small number of varicose processes to the CA (Fig. 10B). Such a direct delivery mechanism has been proposed in the cerebral NSC system of *M. sexta* (Carrow *et al.* 1984; Copenhaver and Truman, 1986*a*) and other insects (Raabe, 1983).

Intracellular potentials have been recorded from NSCs in several insect species (Gosbee *et al.* 1968; Wilkins and Mote, 1970; Cook and Milligan, 1972; Norman, 1973; Carrow *et al.* 1984; Copenhaver and Truman, 1986*a*,*b*), including *Bombyx mori* (Miyazaki, 1980). The NSC soma has a typical electrically excitable membrane and produces large, overshooting action potentials of relatively long duration. The prolonged action potentials recorded from the M1, M3 and lateral cells in the present study coincide with the general features of electrical activity that have been described for NSCs. In contrast, the M2 cells generate an action potential with a short duration that is practically identical to the action potentials of non-NSCs (Fig. 12). The duration of the action potential in M2 cells was twice that of the tritocerebral cells (Table 1). However, this may simply be due to

different recording sites, the soma in the case of the M2 cells and dendrites for the tritocerebral cells. Most insect neurones have electrically non-excitable somata. In these cells, the action potentials recorded in the soma can become much smaller and slower than those recorded in the axon (or the dendrite), depending on the passive cable properties of the cells. The relatively small and slow action potentials of the M2 cells suggest that they may be attenuated potentials that passively invade the electrically non-excitable soma of the cells (Fig. 12B).

The M2 cells contain some paraldehyde–fuchsin (PF)-positive material (Fig. 8). Chromophilic cytoplasmic inclusions, however, do not always indicate a neurosecretory function for cells, since certain pigments and cytoplasmic organelles can also react to PF and to other 'neurosecretory stains' (Rowell, 1976; Panov, 1980; Raabe, 1983). The origin of the PF-positive material and the siting of the cell terminals remain to be examined. The electrophysiological and histological characteristics of the M2 cells in *B. mori* are interesting, because the cells of group M2 (and of group M3) appear to be present not only in Lepidoptera (Panov and Kind, 1963) but also in other holometabolous insects (Panov, 1979). The cells in *Chironomus plumosus* appear to terminate in the last abdominal ganglion (Panov, 1979).

The tritocerebral cells project axons *via* the NCC3 to the stomodeal dilator muscles, with no sign of any interaction in the CC (Fig. 11). The action potential produced by the tritocerebral cells was characteristic of an ordinary non-neurosecretory neurone (Fig. 12C). There is no histological evidence that they possess chromophilic neurosecretory material. Copenhaver and Truman (1986*b*) demonstrated that electrical stimulation of the NCC3 in *M. sexta* induced contractions in the various muscle groups. All these observations taken together suggest that *B. mori* tritocerebral cells are motoneurones.

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