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Three-Dimensional Architecture of Identified Cerebral Neurosecretory Cells in an Insect

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ABSTRACT The organization of identified neurosecretory cell groups in the larval brain of the tobacco hornworm, *Manduca sexta,* was investigated immunocytologically. Computer-assisted three-dimensional reconstruction was used to examine the architecture of the neurosecretory cell groups. The group III lateral neurosecretory cells (L-NSC III) which produce the prothoracicotropic hormone are located dorsolaterally in the protocerebrum and extend axons medially that decussate to the contralateral lobe prior to exiting the brain through the nervi corporis cardiaci I + II. The group IIa_2 medial neurosecretory cells (M-NSC IIa_2) are located anteriorly in the medial dorsal protocerebrum. The axons of these cells also exit the brain via the contralateral nervi corporis cardiaci I + II. However, their axons traverse a different pathway through the brain from that of the L-NSC III axons. Each of the cell groups possesses elaborate dendrites with terminal varicosities. The dendrites can be classified into specific fields based upon their location and projection pattern within the brain. The dendrites for these two neurosecretory cell groups overlap in specific regions of the protocerebral neuropil. After the axons of these neurosecretory cells exit the brain through the retrocerebral nerve, they innervate the corpus allatum where they arborize to form neurohemal terminals in strikingly different patterns. The L-NSC III penetrate throughout the glandular structure and the M-NSC IIa_2 terminals are restricted to the external sheath. A third group of cerebral neurosecretory cells, the ventromedial neurons (VM) which stain with the monoclonal antibody to prothoracicotropic hormone in *Manduca,* are located anteriorly in the medial region of the brain. The axons of these cells do not exit the brain to the retrocerebral complex, but rather pass through the circumesophageal connectives and ventral nerve cord. These neurons appear to be the same VM neurons that produce eclosion hormone. One dendritic field of the L-NSC III terminates in close apposition to the VM neurons. The distinct morphologies of these neurosecretory cell groups in relation to other cell groups and the distribution of neuropeptides within the neurons suggest that insect neurosecretory cells, like their vertebrate counterparts, may have multiple regulatory roles.

Neurosecretory cells (NSC) in vertebrates and invertebrates are peptidergic neurons, the products of which act on distant targets as important regulators of cellular function (Hökfelt et al., '80; Krieger, '83). In vertebrates, the physiological roles of NSC peptide products are more complex than previously thought, involving local modulatory function in addition to their classical neuroendocrine function. The site and nature of peptide release into the extracellular space is important in determining how the peptide functions, i.e., as a neurohormone, neuromodulator, or neurotransmitter. Studies of NSC morphology and distribution of NSC peptides have provided significant insight into the structural relationships of these cells with others, and thus, have been critical to understanding the multifunctional nature of peptidergic neurons. Arginine vasopressin, for example, acts not only as a neurohormone but also is released within the central nervous

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system (CNS) (Buijs, '87); here it acts as a neuromodulator (Brinton and McEwen, '89).

NSC in the CNS of insects have important neuroendocrine roles both during development and adult life (see Downer and Laufer, '83). It is not known, however, if these NSC are multifunctional. Their morphology has been investigated extensively using histological staining and prograde and retrograde nerve filling techniques. In the tobacco hornworm, *Manduca sexta,* such studies have identified the major groups of cerebral NSC and have established their general features (Borg and Bell, '77; Buys and Gibbs, '81; Carrow et al., '84; Copenhaver and Truman, '86a; Nijhout, '75). In addition, the neuroendocrine products of several of the NSC groups have been identified (Copenhaver and Truman, '86b; O'Brien et al., '88; Truman and Copenhaver, '89).

Although the neuroendocrine functions of some of the cerebral NSC groups of *Manduca* are known, local neuromodulatory effects within the brain have not been investigated. In order to provide the foundation for an analysis of neuromodulatory effects, the three-dimensional (3-D) architecture of identifiable groups of cerebral NSC was determined using antibodies to the NSC's peptide phenotype (neurohormone). This immunocytological approach is particularly appropriate because it reveals the distribution of the neuron's peptidergic product(s) and avoids the technical problems of nerve filling methods. Antibodies to several of these cerebral NSC groups are now available (O'Brien, '88; O'Brien et al., '88). Two identified groups of *Manduca* cerebral NSC were examined: 1) the lateral group III NSC (L-NSC III) which produce one molecular variant (~28 kD) of the prothoracicotropic hormone termed "big" PTTH that evokes postembryonic development (Bollenbacher and Granger, '85); and 2) the medial group IIa_2 NSC (M-NSC IIa_2) which may produce a second molecular form (~6 kD) of PTTH termed "small" PTTH (Bollenbacher et al., '84; N. Agui, K. Tomioka, and W.E. Bollenbacher, unpublished). The structural relationship between the L-NSC III and a third group of cerebral NSC, the ventromedial (VM) neurons, was also examined. The VM neurons recently have been identified as a primary source of eclosion hormone that evokes the behaviors for ecdysis and eclosion (Truman and Copenhaver, '89). Computer-assisted 3-D reconstruction was employed to examine the unique spatial arrangement of these NSC within the brain and associated retrocerebral complex. The results suggest that communication and interregulation may occur among the NSC, possibly via their peptide products.

MATERIALS AND METHODS

Animals

Larvae of the tobacco hornworm, *Manduca sexta,* were reared on an artificial diet (Bell and Joachim, '76) under nondiapausing photoperiod (LD 16:8) at 26°C and 60% relative humidity. Day 3, gate II, fifth (last) stadium larvae, staged as previously described (Vince and Gilbert, '77), were used in this study.

Antibodies

Several monoclonal antibodies (MAbs) (O'Brien '88; O'Brien et al., '88) have been generated to identified cerebral NSC in *Manduca.* The nomenclature used to describe these NSC is according to Copenhaver and Truman ('86a). One of these MAbs, designated A2H5, is specific to big PTTH produced by the L-NSC III. A second MAb, AlCll, is to an unidentified peptide produced by the M-NSC IIa_2. The A2H5 and AlCll MAbs were used as hybridoma supernatant, either undiluted for whole mount immunostaining or diluted 1:10 in phosphate buffered saline (PBS) for immunostaining of sectioned material. The VM neurons were visualized using DEAE Affi-Gel Blue purified A2H5 MAb from ascites fluid (Bruck et al., '86) at a high concentration (20.5 $\mu g\ ml^{-1}$).

Immunocytological staining of whole mount brain complexes

Brains and brains with attached bilaterally paired retrocerebral corpora cardiaca (CC) and corpora allata (CA) (Br-CC-CA) were dissected in Grace's insect tissue culture medium (Gibco, Grand Island, NY), fixed in aqueous Bouin's fixative (2 hr, room temperature), and washed overnight at 4°C in PBS (0.02 M, 0.25 M NaCl, pH 7.6). After removing the neurolemma, the fixed tissues were incubated overnight at 4°C in PBS containing 2% Triton X-100 (Sigma, St. Louis, MO). The specimens were then incubated overnight at 4°C in primary antibody (A2H5 or AlCll). Tissues were then washed in PBS (2 × 15 min at room temperature) and incubated for 2 hr at room temperature in peroxidase labelled rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove,

PA) diluted 1:50 in PBS. After washing in PBS (2 × 30 min at room temperature), specimens were incubated for 30 min in 0.05% 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.05 M Tris-HCl (pH 7.6) containing 0.01 M imidazole (Sigma). The immunoenzymatic reaction was initiated by adding 3% H_2O_2 (4–5 drops), and its progress was monitored under a dissecting microscope. The reaction was terminated by washing with PBS (2 × 30 min). Specimens were then dehydrated (ethanol series: 70%, 95%, and 100%, 20 min each), cleared with methyl salicylate, and mounted in Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistological staining of paraffin sections

Brains and Br-CC-CA were dissected and fixed as above, except that the neurolemma was not removed. They were then dehydrated in ethanol, cleared in xylene, and embedded in Paraplast Plus Tissue Embedding Medium (melting point 56°C) (Fisher). Serial sections (7 μm) were cut with a rotary microtome.

For immunostaining, the sections were deparaffinized in xylene, rinsed in 100% ethanol, and treated with H_2O_2 (0.03%) in methanol. The slides were then washed in 0.05 M Tris-HCl (pH 7.6) (2 × 5 min). The sections were incubated with primary antibody overnight at 4°C in a humid chamber, then washed in Tris-HCl buffer (3 × 5 min). This was followed by incubation in a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Jackson) (diluted 1:20 in PBS) for 1 hr at room temperature. After washing in Tris-HCl (3 × 5 min), the slides were developed in Tris-HCl containing 0.05% DAB and 0.005% H_2O_2. The sections were washed in distilled water (2 × 5 min), dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher).

Computer-assisted three-dimensional reconstructions

The architectures of NSC within the Br-CC-CA were examined using a computer program for three-dimensional reconstruction (PC3D Three-Dimensional Reconstruction Software, Jandel Scientific, Corte Madera, CA). A camera lucida drawing of the NSC somata, axons, and dendrites within the brain or retrocerebral organ was made for each serial section. Alignment of a series of tracings was done according to the visual best-fit method (Gaunt and Gaunt, '78; Young et al., '85). The tracings were digitized with a Numonics 2210 digitizing tablet. The PC3D software, running on a CompuAdd 286/12 AT microcomputer, stacked the outlines of specific NSC features from each section to produce a three-dimensional image. The reconstructions were plotted on a Hewlett-Packard HP 7475A plotter and are presented according to Tucker ('89). The left and middle members of the reconstruction should be viewed if using direct stereopsis, e.g., with a stereoscope, and the middle and right members should be viewed if using the crossed-visual axis method of stereopsis.

RESULTS

In order to describe clearly the spatial arrangements of identified cerebral NSC, the orientation of the Br-CC-CA within the larva must be defined (Fig. 1). The brain is located in the head capsule, dorsal to the esophagus and connected to the ventral nerve cord via the circumesophageal connectives. The bilaterally paired retrocerebral complexes (CC-CA) are positioned posterior to the brain lateral to the pharynx (Eaton, '88).

The unipolar L-NSC III and M-NSC IIa_2 have distinct morphologies. The L-NSC III are located laterally in the protocerebrum (Fig. 2A; O'Brien et al., '88) and the M-NSC IIa_2 are located medially (Fig. 2B). The numerous dendritic collaterals of these NSC groups appear to be restricted primarily to the axon segments proximal to their decussation. The dendrites extend into both the lateral protocerebral neuropil and the medial region of the brain. The L-NSC III and the M-NSC IIa_2 axons decussate to the contralateral brain lobe, but have distinct pathways that converge at the nervi corporis cardiaci (NCC I + II) to innervate the CC-CA. The axons of both groups pass unbranched through the CC and, via the nervus corporis allati (NCA), terminate in the CA where they arborize forming the neurohemal organ for peptide release.

Although the Br-CC-CA whole mounts revealed the basic organization of the L-NSC III and M-NSC IIa_2, the fine structural details and the spatial relationships between the neurons were not discernible. Immunohistological data and 3-D reconstructions of these NSC groups were used to provide this information.

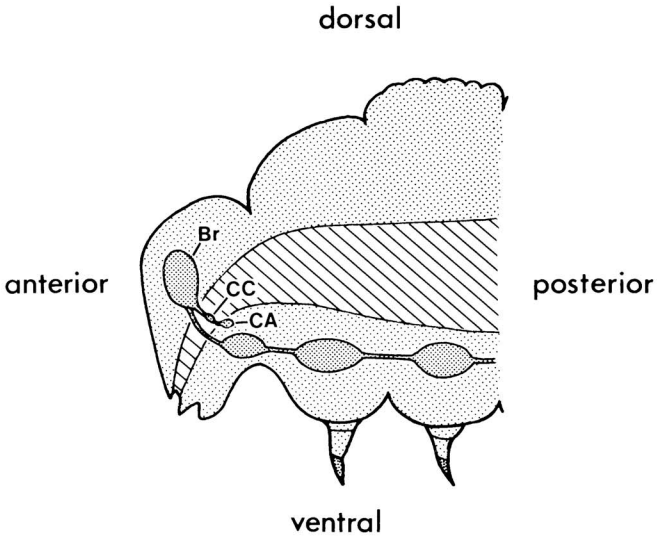

Fig. 1. Schematic diagram illustrating the position of the brain (Br) and retrocerebral organs, the corpus cardiacum (CC) and corpus allatum (CA), in the *Manduca* larva.

Architecture of the L-NSC III

Three-dimensional reconstructions of the L-NSC III (Fig. 3A) revealed the ~20 μm diameter somata (Fig. 3A, 4A) of the two bilaterally paired L-NSC III located dorsolaterally in the protocerebrum approximately midway along the anterior-posterior axis. The somata are positioned anterior and medial to the optic lobes and anterior and lateral to the corpora pedunculata. Their axons originate from the ventral side of the somata and initially project ventrally in the brain before making a sharp turn to project medially (Fig. 3A). Between this flexure and the axons' decussation to the contralateral lobe, elaborate dendritic collaterals project ventrally. These dendrites arc both anteriorly and posteriorly from the axons and branch extensively as they penetrate into the cerebral neuropil (Fig. 4B). At the decussation point, the axons fasciculate with the L-NSC III axons from the contralateral lobe to form a loop (Fig. 4C). The axons traverse medially through the brain via axon tract C (Buys and Gibbs, '81) and decussate to the contralateral lobe, anterior to the central body. After decussation, the axons remain fasciculated with the contralateral L-NSC III axons and extend dorsolaterally in the opposite brain lobe (Fig. 3A). Approximately halfway to the opposite L-NSC III somata, the axons defasciculate from the contralateral axons and project posteriorly and ventrally along axon tract A (Buys and Gibbs, '81) to exit via the NCC I + II (Fig. 3A).

The dendritic projections from the L-NSC III axons possess significant amounts of big PTTH and appear to be restricted to the ipsilateral axon segment between the sharp medial flexure and the point of decussation, as suggested by intracellular fills (Carrow et al., '84; Copenhaver and Truman, '86a). The distinctive regional branching of dendrites within the brain has led to their classification into three dendritic fields based upon location and projection pattern (Fig. 5A). The first field, designated lateral cell dendritic field 1 (L-df1), is composed of the dendrites that branch from the axons just after their medial flexure (Fig. 5A). The L-df1 dendrites extend ventrally and laterally from the axons ~70 μm in length. The dendrites possess numerous bulbous varicosities at their terminals. The second dendritic field, lateral cell dendritic field 2 (L-df2), is comprised of the dendrites that project from the axons just medial to the L-df1. The dendrites in this field are much shorter, ~20 μm, than those in L-df1, but they also possess terminal varicosities. The third field, lateral cell dendritic field 3 (L-df3), projects from the axons just proximal to decussation. This field is composed of relatively few collaterals that may be ~85 μm in length and, as in the other fields,

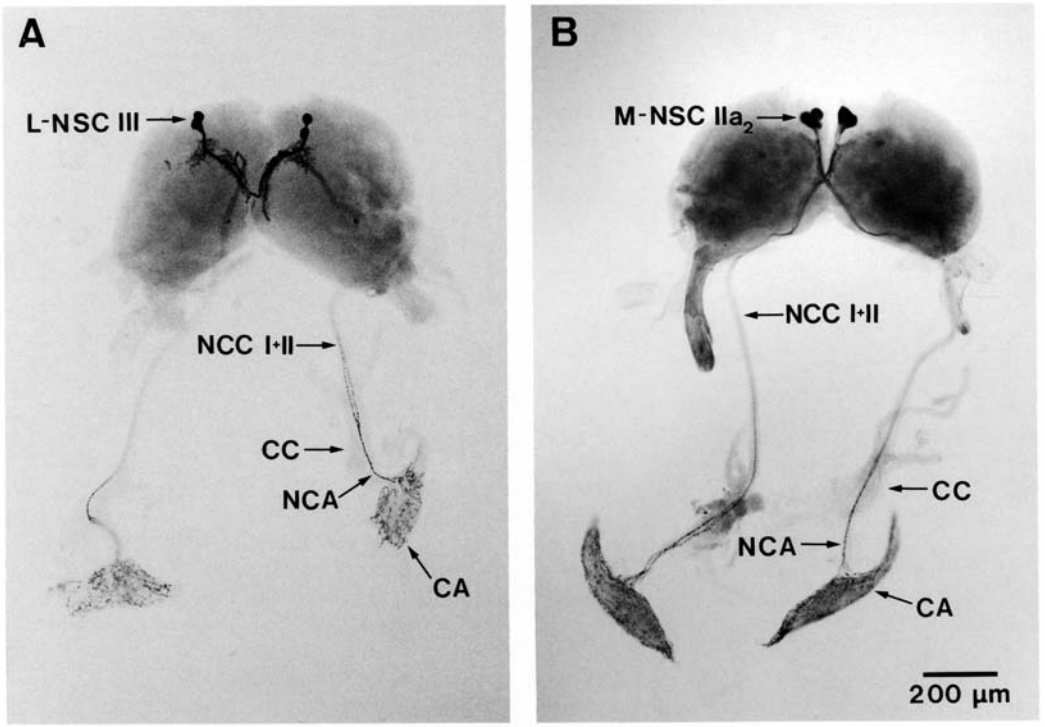

Fig. 2. Photomicrographs of immunostained NSC groups in whole mount brain-retrocerebral complexes of day 3 fifth instar *Manduca* larvae. **A:** The complete architecture of the L-NSC III immunostained with the A2H5 MAb to big PTTH. **B:** The architecture of the M-NSC IIa_2 immunostained with the A1C11 MAb specific for these neurons. CA, corpus allatum; CC, corpus cardiacum; NCA, nervus corporis allati; NCC I + II, nervi corporis cardiaci I + II.

these dendrites also possess terminal varicosities. The L-df3 dendrites extend ventrally and anteriorly from the decussation point.

The target neurons in the CNS with which the L-NSC III may interact via their dendritic fields are unknown but are likely to include diverse neuron types. The 3-D reconstructions suggest that other peptidergic neurons may be one of these neuron types. The L-df3 dendrites, for example, terminate in close proximity to a set of bilaterally paired NSC located ventromedially in the anterior portion of the brain (Fig. 5). These ~20–25 µm diameter cells, the ventromedial neurons (VM), are the primary source of eclosion hormone in *Manduca* (Truman and Copenhaver, '89). The VM neurons immunostain with affinity purified A2H5 MAb to big PTTH at a high concentration, suggesting big PTTH is coexpressed with eclosion hormone in these NSC. This possibility is supported by the absence of VM neuron immunostaining when the MAb is pre-incubated with big PTTH (Westbrook and Bollenbacher, unpublished). While the A2H5 MAb is highly specific for big PTTH of *Manduca,* proof that the VM neurons coexpress eclosion hormone and big PTTH will require in situ hybridization to localize big PTTH mRNA to these neurons. Dendritic projections from the VM neuron axons were not clearly distinguishable, but their simple organization in the larval brain has been described (Truman and Copenhaver, '89). Based upon the projection pattern of the L-df3, interaction between the L-NSC III and VM neurons is possible and could occur either at the VM neuron somata or dendrites. The L-NSC III may also interact with the M-NSC IIa_2, as discussed below.

The fasciculated L-NSC III axons exit the brain via the NCC I + II and pass unbranched through the CC to innervate the CA (Fig. 2A). In the CA, the axons, which possess abundant varicosities, penetrate and interdigitate among the glandular cells of the CA (Fig. 6A). Three-dimensional reconstruc-

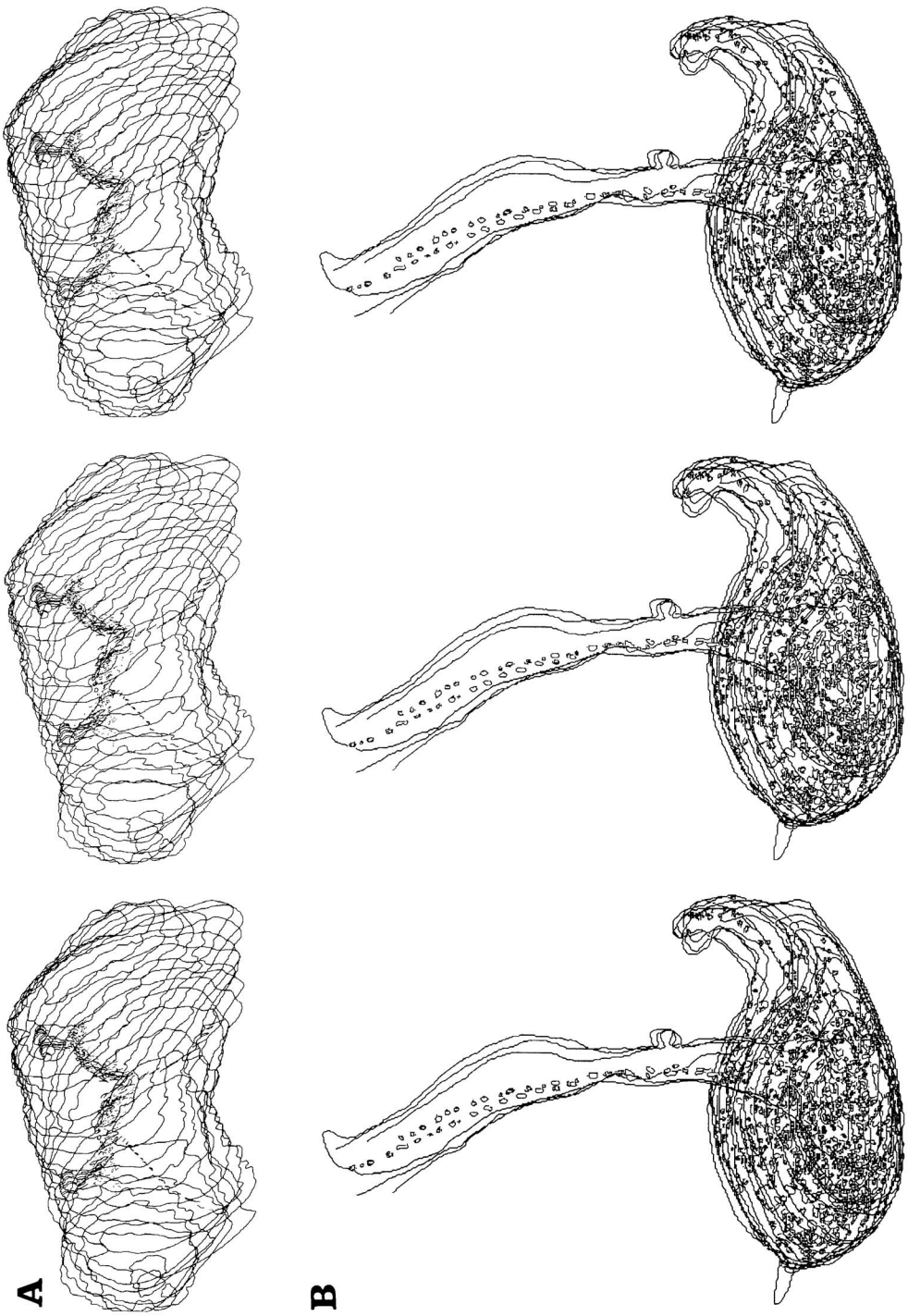

Figure 3 (See Color Section, page C2)

tions of the neurohemal terminals in the CA (Fig. 3B) revealed they are randomly distributed throughout the gland, rather than just on its periphery.

Architecture of the M-NSC IIa$_2$

The four M-NSC IIa$_2$ are located in the anterodorsal region of each protocerebral hemisphere near the brain midline (Fig. 7A, 8A), anterior to the corpora pedunculata. Their axons originate from the posterior side of the 30–35 μm diameter somata and immediately turn ventrally (Fig. 7A). They decussate to the contralateral brain lobe but, unlike the L-NSC III axons, do not fasciculate with the contralateral M-NSC IIa$_2$ axons. The decussated axons follow axon tract B (Buys and Gibbs, '81) traversing the ventral margin of the brain in a posterolateral direction to reach the NCC I + II.

The dendrites of the M-NSC IIa$_2$, like those of the L-NSC III, arborize from the axons and can be classified into distinct dendritic fields based upon their projection patterns (Fig. 9). One field, the medial cell dendritic field 1 (M-df1), extends ~110 μm laterally from the axons into the protocerebral neuropil (Fig. 8B, 9A). These dendrites appear to arborize in the same region of the neuropil as the L-NSC III L-df2 dendrites (Fig. 5A). The second field, medial cell dendritic field 2 (M-df2), are very fine, short (~20–25 μm) dendrites that project from the axons between the point where the M-df1 branch and the axon decussation point (Fig. 9A). The third M-NSC IIa$_2$ dendritic field, medial cell dendritic field 3 (M-df3), projects from the axons just ventral to the decussation point (Fig. 9B). They appear to arise from the axons on the contralateral side of the brain as suggested by intracellular fills of these neurons in adult brains (Copenhaver and Truman, '86a). The M-df2 and M-df3 arborize in the areas of the L-df2 or L-df3 of the L-NSC III, suggesting they may interact. In general, the M-NSC IIa$_2$ dendrites are much finer than those of the L-NSC III, and while they do possess some terminal varicosities, the swell-

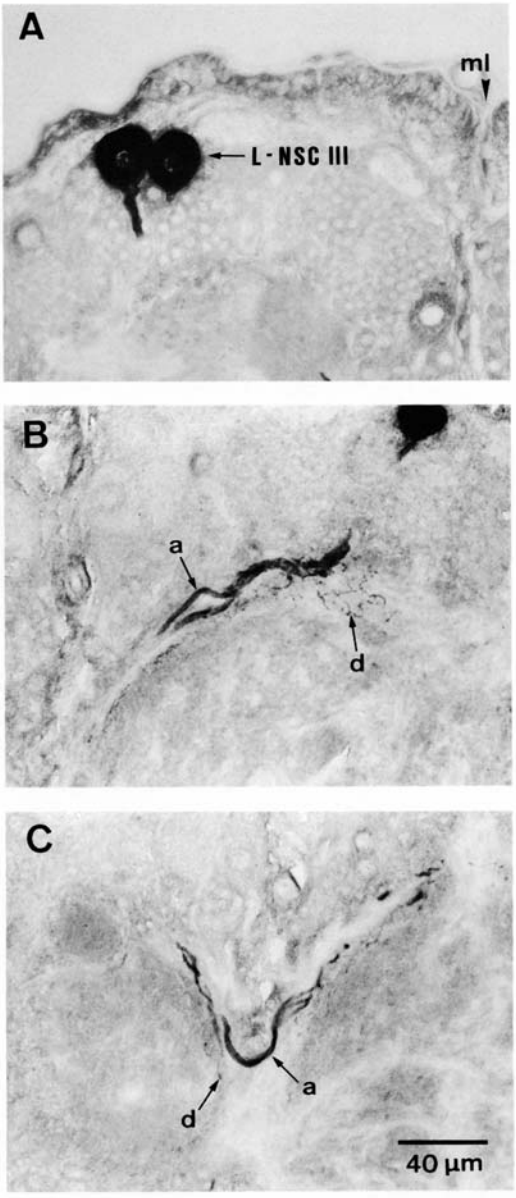

Fig. 4. Details of the L-NSC III architecture in immunohistological preparations. **A:** The two somata (~25 μm diameter) in each protocerebral hemisphere. ml indicates the midline of the brain. **B:** The axons (a) project medially through the brain, and dendritic collaterals (d) arborize from them into the protocerebral neuropil. **C:** The axons (a) decussate to the contralateral brain lobe, forming a hairpin loop. Fine dendrites (d) emerge from this region of the brain and extend ventral to the decussation.

Fig. 3. A 3-D reconstruction of the L-NSC III. **A:** The somata (purple), axons (purple), and dendrites (light blue) in the brain (outlined in green). **B:** The L-NSC III neurohemal terminals (purple) in the CA (outlined in green). A and B are not shown at the same magnification. Refer to Figure 2 for relative sizes of these structures. For instructions on viewing the 3-D reconstruction triptych, see Materials and Methods.

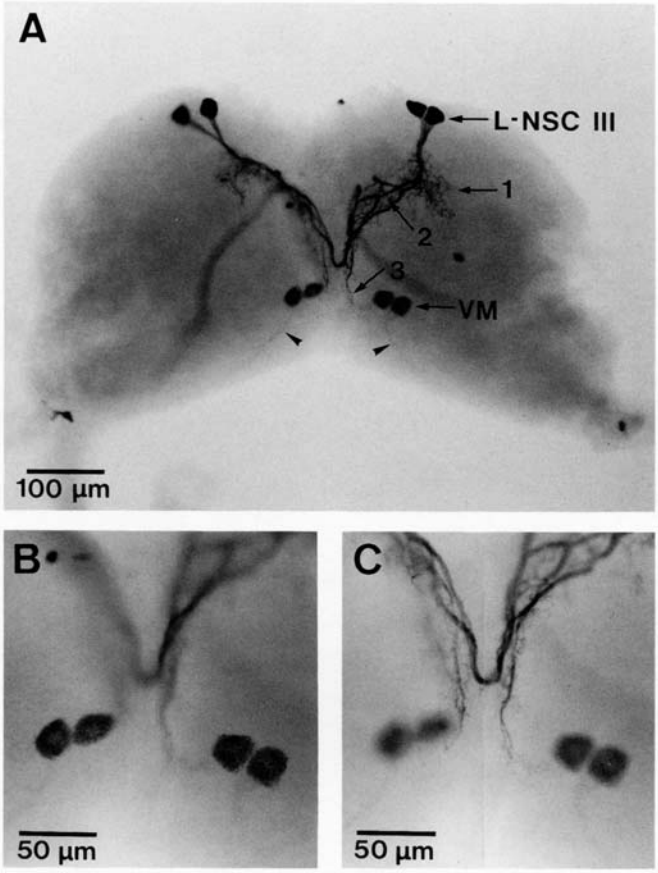

Fig. 5. Photomicrographs of the L-NSC III and ventromedial neurons (VM) in a brain whole mount immunostained with purified A2H5 MAb to big PTTH. **A:** The dendritic collaterals are classified into three groups, the L-df 1 (1), L-df 2 (2), and L-df 3 (3). **B:** Enlargement of the VM neurons which are ~20–25 µm in diameter. **C:** The L-df3 dendrites terminate in close proximity to the VM neurons.

ings are not as numerous or as large as those observed on the L-NSC III dendrites.

After traversing the contralateral lobe, the tightly fasciculated axons enter and pass through the NCC I + II to the CC and NCA (Fig. 2B). Once in the NCA, they may begin to separate from each other, and arborize extensively in the CA. Unlike the L-NSC III, arborization occurs exclusively in the periphery of the CA just beneath the basal lamina (Fig. 6A,B). This is shown clearly in the 3-D reconstruction of the neurohemal structure (Fig. 7B).

Spatial relationship of the two NSC groups

To examine more precisely the spatial relationships among these groups of cerebral NSC, alternate serial sections of brains were immunostained with the A2H5 and AlCll MAbs to generate a 3-D reconstruction of both the L-NSC III and M-NSC IIa_2 (Fig. 10). The somata of the L-NSC III and M-NSC IIa_2 are in distinctly different brain regions with the L-NSC III being posterior to the M-NSC IIa_2. The axon tracts of these two NSC groups do not overlap; the decussation of the M-NSC IIa_2 axons occurs ~7–10 µm anterior to the L-NSC III axon decussation. Unlike the L-NSC III axons that begin their posterolateral projection toward the NCC I + II while in the mid-dorsal region of the neuropil, the M-NSC IIa_2 axons begin projecting posteriorly only after they have traversed to the ventral margin of the brain. Despite their distinct pathways, the axons of both of these NSC groups converge at the ventral margin

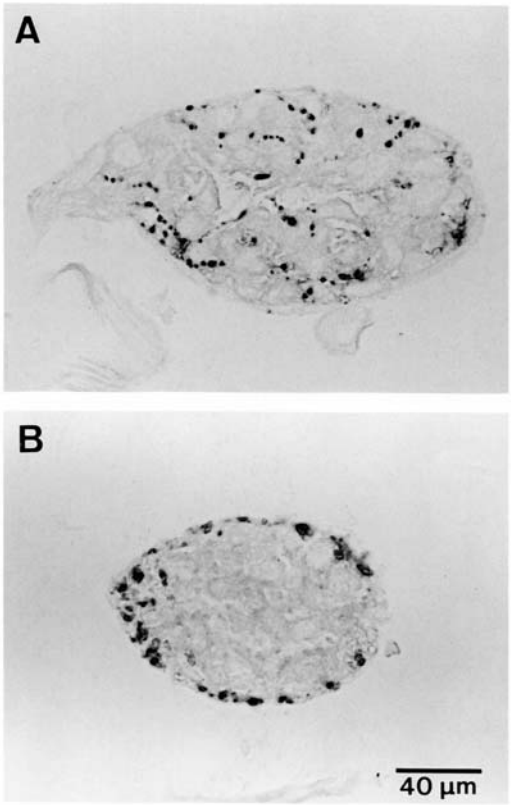

Fig. 6. Photomicrographs of immunohistological sections of the CA illustrating the unique termination patterns of the L-NSC III (**A**) and M-NSC IIa_2 (**B**) axons.

of the brain at the origin of the NCC I + II. The dendritic fields overlap in the brain, particularly the L-df2 and M-df1 in the lateral protocerebral neuropil. Although the locations of the fine M-df2 and M-df3 could not be clearly resolved in alternate sections, they do occupy the same medial protocerebral area(s) as the L-df2 and L-df3.

DISCUSSION

The basic morphologies of the cerebral NSC groups of *Manduca* have been determined in previous studies that employed nerve filling techniques (Buys and Gibbs, '81; Carrow et al., '84; Copenhaver and Truman, '86a; Nijhout, '75). The availability of antibodies to the peptides produced by specific NSC groups (O'Brien, '88; O'Brien et al., '88) enables the morphology of several of these NSC groups to be described in terms of the distribution of their peptide phenotype. Three-dimensional reconstruction of the L-NSC III and M-NSC IIa_2 reveals the complex structural organization of these neurons and provides insight into the possible functional roles for the peptides they express.

Immunocytological staining of the L-NSC III and M-NSC IIa_2 revealed details that were not resolved in previous studies. This improved resolution was especially evident for the cerebral dendritic collaterals and allowed their categorization into discrete fields. Previous studies either did not reveal the cerebral dendrites (Nijhout, '75), used techniques in which the dendrites of different NSC types overlapped (Buys and Gibbs, '81; Copenhaver and Truman, '86a), or examined animals at different stages in which the cerebral dendrites have rearranged during metamorphosis (Carrow et al., '84; Copenhaver and Truman, '86a).

The projection patterns of the elaborate dendrites in the brain suggest that these neurons communicate with other neurons, thus supporting the hypothesis that the neuropil permits communication among neurosecretory neurons (Taghert, '81). For example, the overlap of the L-df2 and M-df1 suggests that the L-NSC III and M-NSC IIa_2 may communicate via these dendritic fields. Such communication could coordinate the synthesis and/or release of big PTTH from the L-NSC III and small PTTH from the M-NSC IIa_2 to regulate ecdysteroid production by the prothoracic glands for molting and metamorphosis. Conversely, the L-NSC III and other cerebral NSC may not communicate directly but may share input or output sites. The L-NSC III may also communicate with the VM neurons via the L-df3 which appear to terminate near the VM neuron somata or their sparse dendrites. This interaction could be important in regulating the circadian clock controlled release of both PTTH (Bollenbacher and Granger, '85) and eclosion hormone (Truman et al., '81). Studies are currently underway investigating the relationship between the L-df3 and the VM neurons at the ultrastructural level.

The cerebral NSC of *Manduca* undoubtedly interact with non-neurosecretory neurons as well. This type of interaction probably occurs at each dendritic field to modulate the activity of the NSC. The L-df1, for example, overlap with the terminals of a pair of neurons whose somata are located in the far lateral region of the brain. These neurons express big PTTH immunoreactivity but do

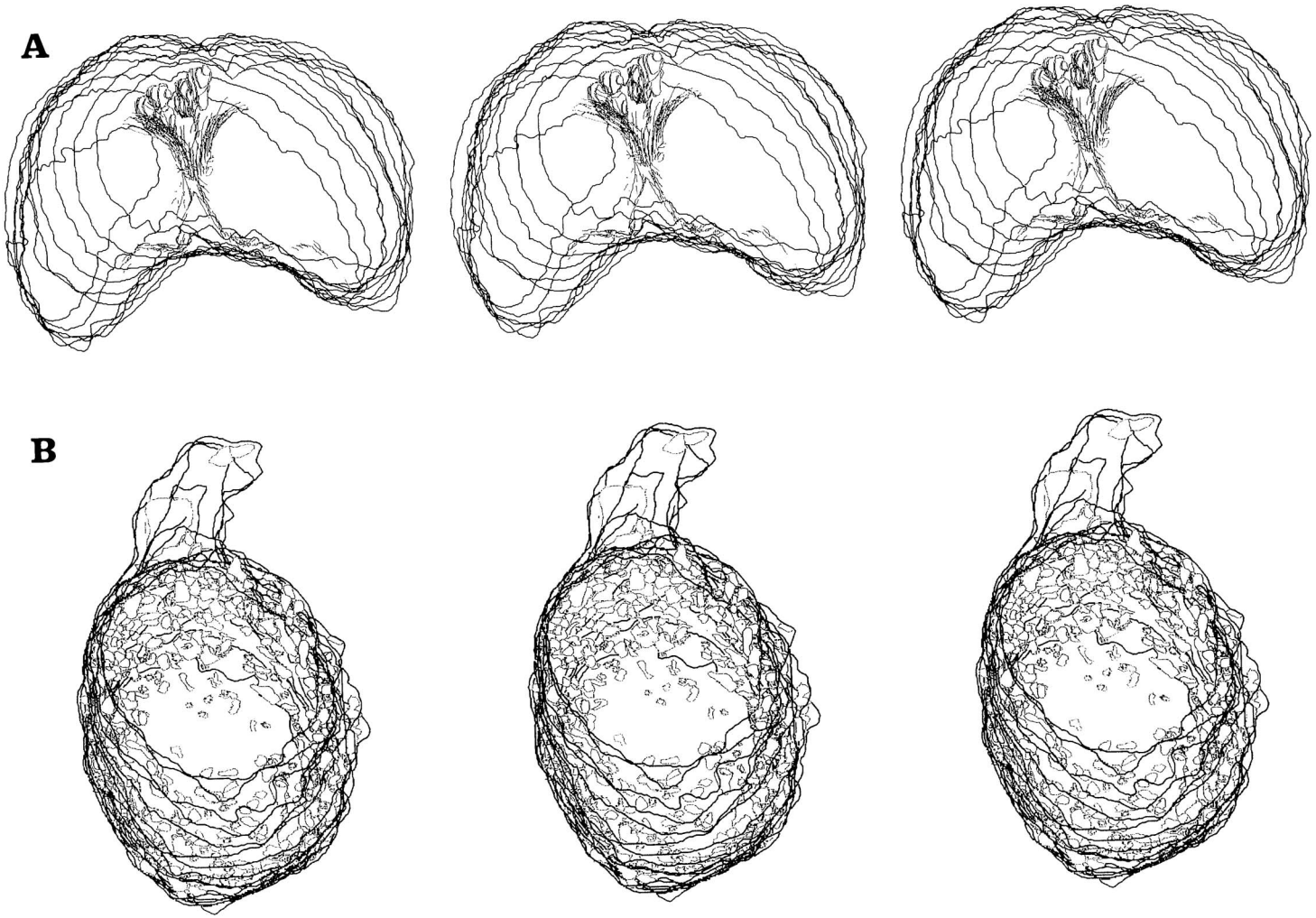

Figure 7 (See Color Section, page C3)

not possess the classical NSC structure (Westbrook and Bollenbacher, unpublished).

If communication does occur among the cerebral NSC, it could be effected by the release of the cell's peptide product(s). The terminal varicosities present on the L-NSC III and, to a lesser extent the M-NSC IIa_2, suggest that dendrites are a site of release. Dendritic release of neurotransmitters and neuropeptides into the nervous system has been documented in both vertebrate (Cheramy et al., '81; Greenfield, '85; Morris et al., '88; Pow and Morris, '89) and invertebrate systems (Schmidt and Roubos, '88). The released peptide may act as a neuromodulator of nervous and/or neuroendocrine function (Brinton and McEwen, '89; Pow and Morris, '89). The morphological relationships revealed in this study provide a foundation to begin ultrastructural and physiological investigations of potential neuropeptide-mediated interregulation of *Manduca* cerebral NSC.

The 3-D reconstructions of the retrocerebral complexes revealed distinct spatial arrangements of the axon endings for each NSC type. As shown previously, both the L-NSC III and M-NSC IIa_2 terminate at the CA (Copenhaver and Truman, '86a), but the 3-D reconstructions clearly reveal their unique termination patterns. This specialized morphology suggests that neurosecretory material is released into different compartments. Previously, it was thought that the terminals for the PTTH-producing NSC were almost exclusively limited to the periphery of the CA for PTTH release into the hemolymph (Sedlak, '81, '85). The random arborizations of the L-NSC III axons throughout the organ suggests that big PTTH may be released locally in the CA as well as into the hemolymph. Thus, big PTTH may be acting as a neurohormone and as a local regulator of CA glandular function, i.e., a regulator of juvenile hormone synthesis. The latter possibility together with possible dendritic release in the brain suggests that big PTTH may be multifunctional during insect development. Unlike the L-NSC III termi-

Fig. 7. Three-dimensional reconstructions of the M-NSC IIa_2. **A:** The somata (red), axons (red), and dendrites (purple) in the brain (outlined in green). **B:** The M-NSC IIa_2 neurohemal terminals (red) in the CA (outlined in green). A and B are not shown at the same magnification. Refer to Figure 2 for relative sizes of these structures.

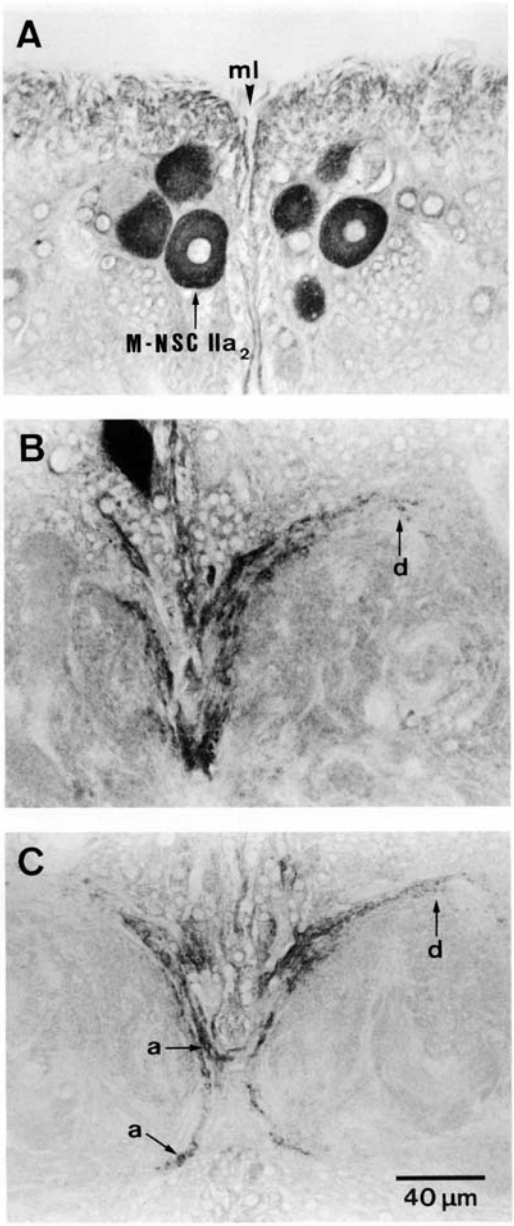

Fig. 8. Details of the M-NSC IIa_2 architecture in immunohistological preparations. **A:** Four somata (~30 µm diameter) are located in each protocerebral hemisphere. The brain midline is denoted by ml. **B:** Dendrites (d) extend laterally in the protocerebrum up to ~100 µm from the axons. **C:** The axons (a) decussate to the contralateral lobe.

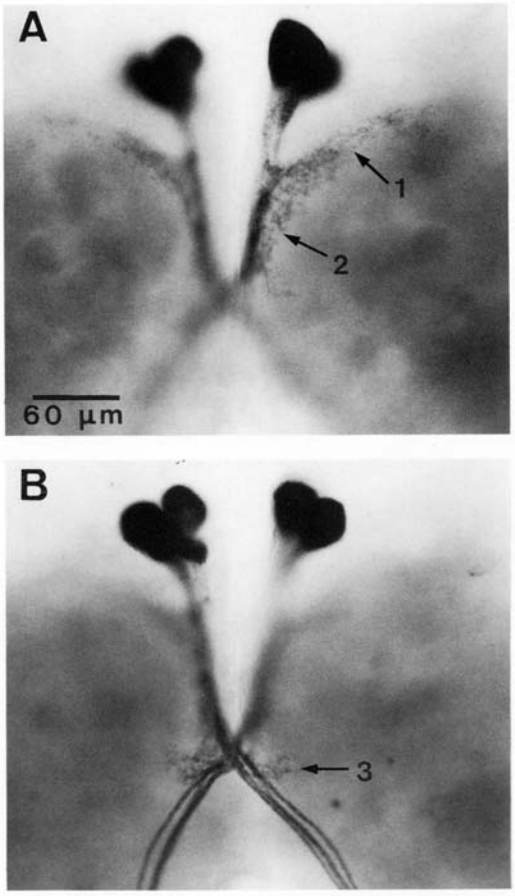

Fig. 9. Photomicrographs illustrating the M-NSC IIa_2 dendritic fields in a brain whole mount. **A:** Dendritic fields M-df1 (1) and M-df2 (2). **B:** The contralateral dendritic field M-df3 (3) at a different optical plane.

nals, the M-NSC IIa_2 axons arborize exclusively on the periphery of the CA just beneath the basal lamina. This organization suggests that neuropeptide is released into the hemolymph for neuroendocrine activity. Interestingly, the large medial NSC in the commercial silkmoth, *Bombyx mori,* which produce bombyxins (an insulin-like family of peptides), also terminate at the periphery of the CA (Mizoguchi et al., '87), suggesting that the large medial NSC of *Manduca* and *Bombyx* may be homologous. Together, the stereotypic neurohemal organ morphologies for these two groups of cerebral NSC implies different functional roles for their peptide products.

An unexpected finding from this study concerns the expression of multiple peptide phenotypes by a particular NSC group. The VM neurons which express big PTTH immunoreactivity were first identified using histological stains (Group V, Nijhout, '75) and are now known to be the principal source of eclosion hormone in *Manduca* (Truman and Copenhaver, '89). Unlike the L-NSC III and M-NSC IIa_2, these neurons do not project to the larval retrocerebral complex but pass through the circumesophageal connectives and ventral nerve cord to terminate in the proctodeal nerve at the hindgut. Big PTTH immunoreactivity is also present in the proctodeal nerve (Westbrook and Bollenbacher, unpublished) suggesting that it, in addition to eclosion hormone, may be released from this site. Although molecular probes currently are not available to determine conclusively that the immunoreactivity in the VM neurons is big PTTH, the A2H5 MAb is highly specific, and this supports possible coexpression of peptide phenotypes by the VM neurons. This MAb recognizes only the big PTTH from *Manduca,* appears to be directed at or near the active site of the peptide (O'Brien et al., '88), and recognizes big PTTH only in its native dimeric form (D.P. Muehleisen, E.J. Katahira, R.S. Gray, and W.E. Bollenbacher, unpublished).

The ability of neurons to express more than one phenotypic product has been recognized for many neuron types (Bondy et al., '89; Lundberg and Hökfelt, '83). In *Manduca,* for example, identified NSC in the abdominal ganglia express the peptides bursicon and cardioacceleratory peptide 2 (Tublitz and Sylwester, '90). The apparent expression of big PTTH by the VM neurons and the L-NSC III raises the possibility that big PTTH is an important regulatory molecule in the VM neurons. The big PTTH expressed in the VM neurons could possibly act either as a neurohormone or neuromodulator depending upon where it is released from these neurons. The expression of a peptide by different neurons, e.g., big PTTH in the L-NSC III and VM neurons, could impart a functional diversity to the neuropeptide by permitting both long- and short-term effects on different targets depending upon the titer and site of release.

In summary, this study of the 3-D architecture of identified cerebral NSC of *Manduca* has provided evidence for a highly interactive peptidergic neuron system whose functions may be dictated by the compartments into which their neuronal product(s) are released.

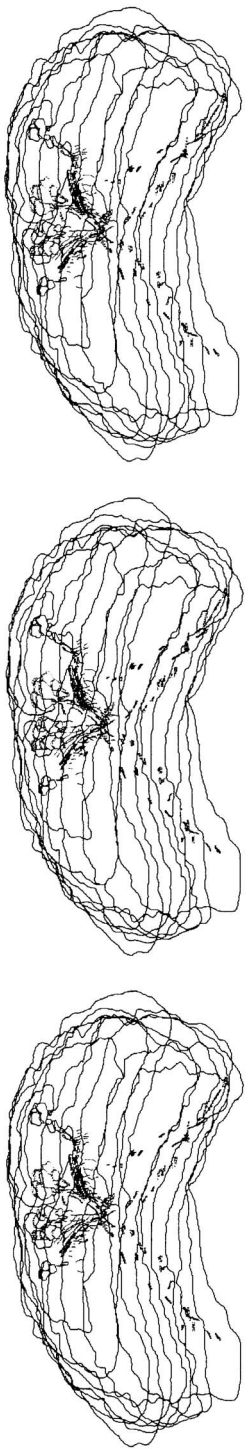

Figure 10 (See Color Section, page C4)

Fig. 10. Three-dimensional reconstruction of the L-NSC III (purple) and M-NSC IIa_2 (red) from alternate serial sections of a larval brain (outlined in green) stained with the A2H5 and A1C11 MAbs. The M-NSC IIa_2 are positioned medially and anteriorly to the L-NSC III and dendritic fields of the 2 NSC groups overlap in the protocerebral neuropil and medial region of the brain.

Future investigations will examine these neurons at the ultrastructural level to define communication in terms of the sites of neuropeptide release in order to understand the developmental and homeostatic processes directed by neuropeptide action.

ACKNOWLEDGMENTS

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COLOR SECTION

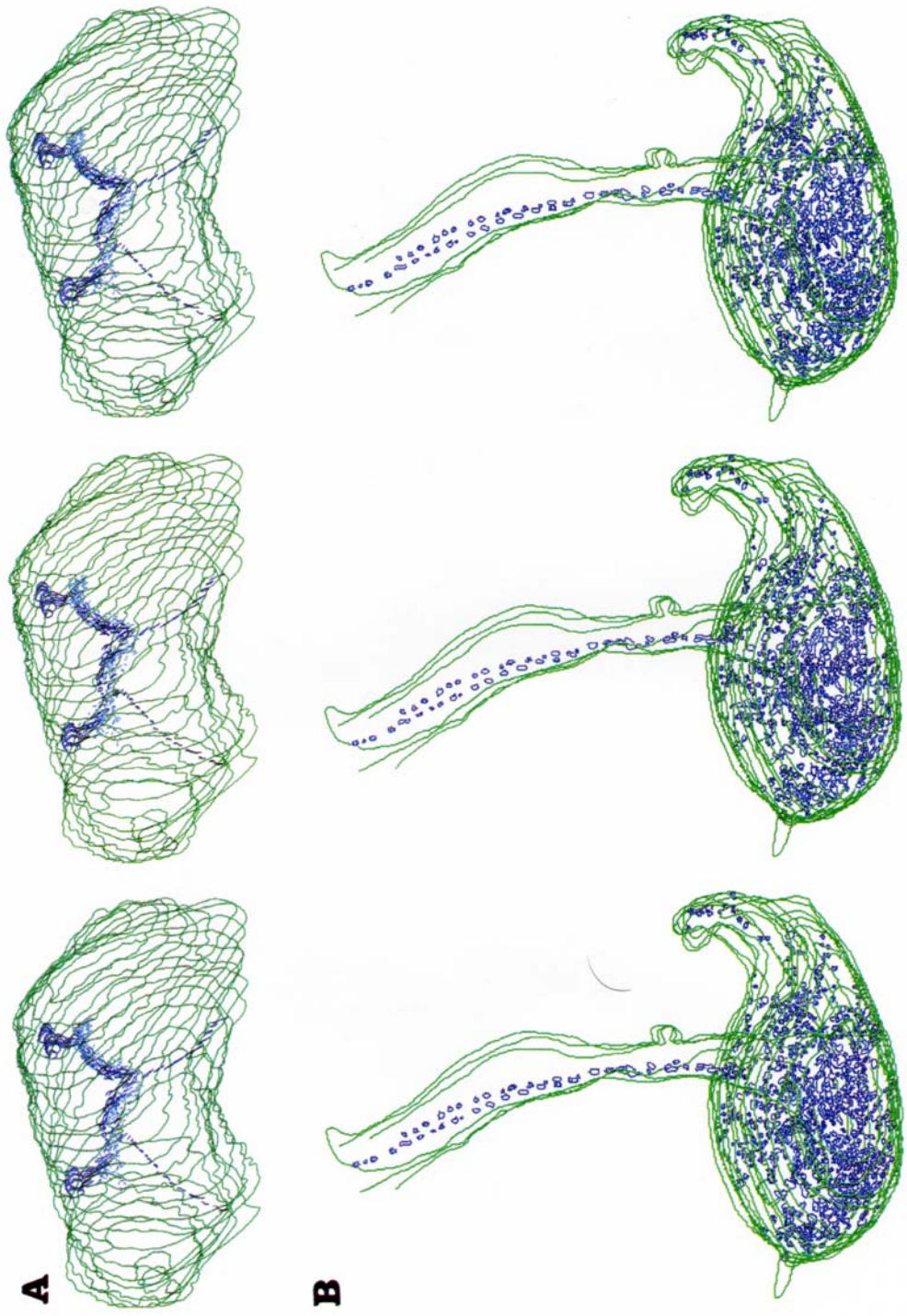

Fig. 3. (See page 166) A 3-D reconstruction of the L-NSC III. A: The somata (purple), axon (purple), and dendrites (light blue), in the brain (outlined in green). B: The L-NSC III neurohemal terminals (purple) in the CA (outlined in green). A and B are not shown at the same magnification. Refer to Figure 2 (page 165) for relative sizes of these structures. For instructions on viewing the 3-D reconstruction triptych, see Materials and Methods (page 162).

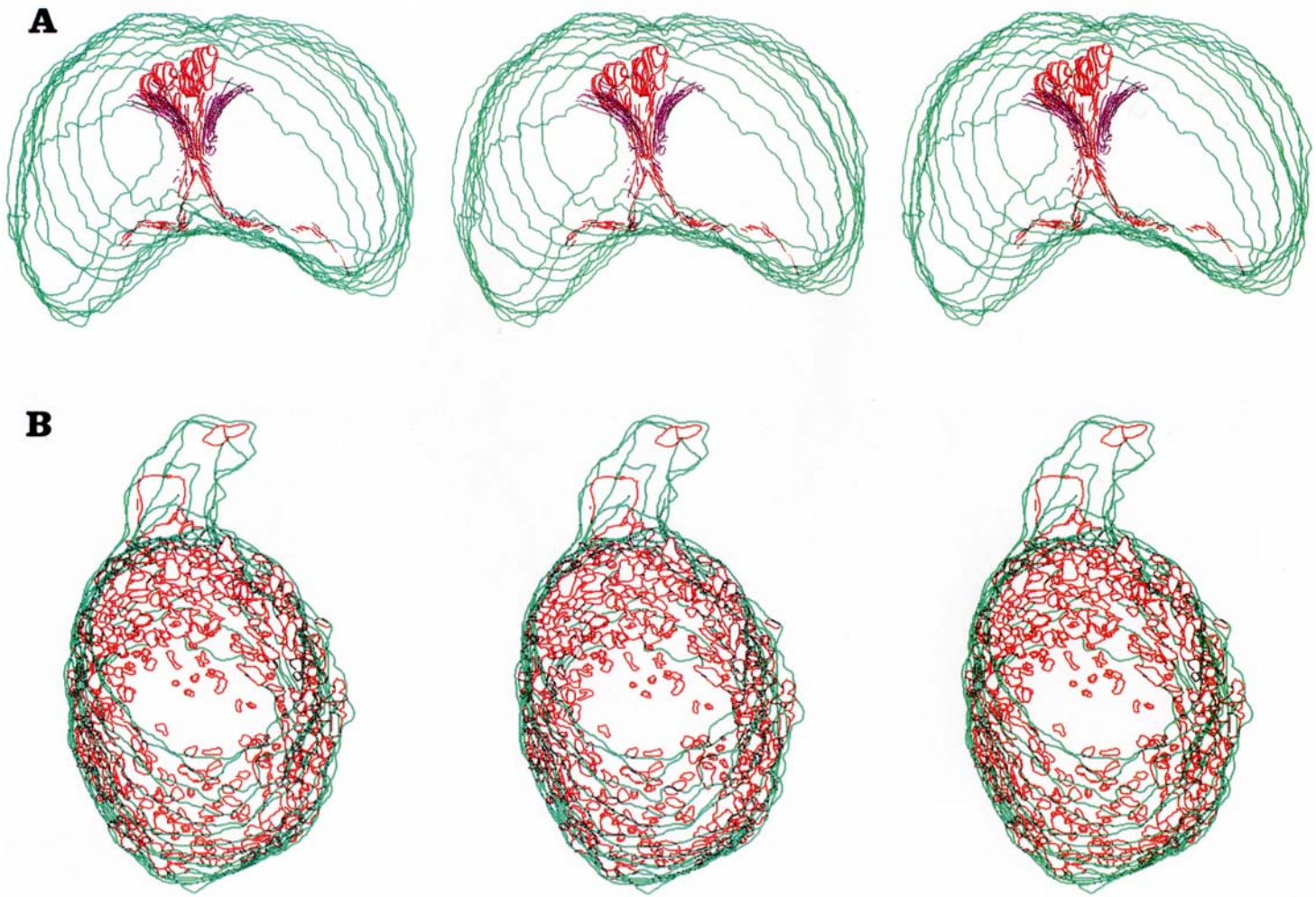

Fig. 7. (See page 170) Three-dimensional reconstructions of the M-NSC IIa_2. A: The somata (red), axons (red), and dendrites (purple) in the brain (outlined in green). B: The M-NSC IIa_2 neurohemal terminals (red) in the CA (outlined in green). A and B are not shown at the same magnification. Refer to Figure 2 (page 165) for relative sizes of these structures.

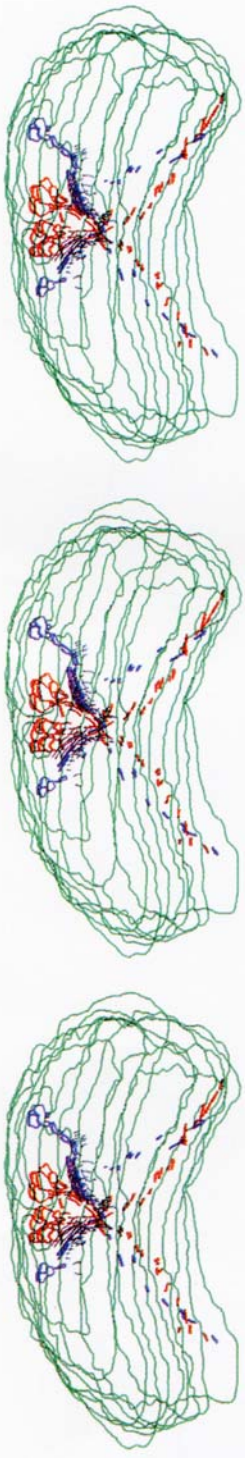

Fig. 10. (See page 173) Three-dimensional reconstruction of the L-NSC III (purple) and M-NSC IIa_2 (red) from alternative serial sections of a larval brain (outlined in greed) stained with the A2H5 and A1C11 MAbs. The M-NSC IIa_2 are positioned medially and anteriorly to the L-NSC III and dendritic fields of the 2 NSC groups overlap in the protocerebral neuropil and medial region of the brain.