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Localization of *Manduca sexta* Allatotropin Neuropeptides in Developing Ventral Nerve Cord of the Silk Moth *Bombyx mori*

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This study has been carried out to determine localization of *Manduca sexta* allatotropin (Mas-AT) neuropeptide in developing ventral nerve cord of the silk moth *Bombyx mori* with polyclonal antisera against Mas-AT. Suboesophageal ganglion (SOG) of the second to fifth instar larvae and 3-day-old pupae showed two to ten Mas-AT-immunoreactive (Mas-AT-IR) cell bodies. There were two to three pairs of labeled cell bodies in each thoracic ganglion (TG) from third instar larvae to adults, with the exception of TG from prepupae. One pair of labeled cell bodies was localized in each abdominal ganglion (AG) 1 to 6 from third instar larvae to 3-day-old pupae, whereas in 5-day-old pupae to adults there was one pair in a similar location of AG 1 to 5. The seventh neuromeres of terminal abdominal ganglia (TAG) from third instar larvae to 3-day-old pupae contained four labeled large cell bodies. In each of AG 1 to 7, these cell bodies showed similar allatotropin-immunoreactivity in appearance. Some labeled axons, projected from Mas-AT-IR cells in each of those AG, were extended to the nerves N 1 and N 2.

Key words : Localization, Allatotropin cells, Ventral nerve cord, Postembryonic development, *Bombyx mori*

Introduction

Allatotropin (AT) neuropeptide stimulates juvenile hormone (JH) biosynthesis by the corpora allata in Lepidoptera (Kataoka *et al.*, 1989; Unni *et al.*, 1991; Stay and Woodhead, 1993; Hebda *et al.*, 1994; Riddiford, 1994; Stay *et al.*, 1994; Wyatt and Davey, 1996; Bhatt and Horodyski, 1999). AT also stimulates reproduction and egg maturation in the adult and has a strong myostimulatory action (Duve *et al.*, 2000). It has been also shown that AT is a cardioacceleratory peptide active on the pharate adult heart (Veenstra *et al.*, 1994).

Allatostatin inhibits JH biosynthesis by the corpora allata (Tobe and Stay, 1985). Various types of allatostatin neuropeptides have been characterized from different insect species, including cockroach (Pratt *et al.*, 1991; Donly *et al.*, 1993: Woodhead *et al.*, 1989, 1993, 1994; Bellés *et al.*, 1994, 1999; Hayes *et al.*, 1994; Weaver *et al.*, 1994; Ding *et al.*, 1995), moth (Kramer *et al.*, 1991; Davis *et al.*, 1997; Duve *et al.*, 1997), and blowfly (Duve *et al.*, 1993, 1994). To date, however, only one type of AT, a 13-residue amidated tridecapeptide (Kataoka *et al.*, 1989), has been purified and characterized from the heads of pharate adults of *Manduca sexta* in different insects. This fact suggested that further studies on characterization and immunolocalization of the AT neuropeptide in different insect species would be useful to reveal more detailed functions, cellular production and gene expression of the AT.

It has been also shown that Mas-AT mRNA is included in the neurons of brain, TAG and frontal ganglion of *M. sexta* (Taylor *et al.*, 1996; Bhatt and Horodyski, 1999). Using polyclonal antibodies to Mas-AT, Žitňan *et al.* (1993, 1995) and Taylor *et al.* (1996) have demonstrated immunoreactivity in the brain and ventral ganglia, as well as in the frontal ganglia, hypocerebral ganglion, enteric plexus and midgut from *M. sexta* and *D. melanogaster.* Immunoreactivity was also detected in AG of pharate adults with antisera raised to Mas-AT, and Mas-AT levels were also quantified by ELISA tests in the brain, ventral nerve cord and corpora cardiaca-corpora allata (Veenstra and Hagedorn, 1993; Veenstra *et al.*, 1994). Using both *in situ* hybridization and immunocytochemistry, Bhatt and Horodyski (1999) have demonstrated that Mas-AT mRNA and immunoreactivity to Mas-AT were detected in the frontal ganglia, TAG and SOG from larval stages, and

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were also found in the AG and TAG during the pupal and pharate adult stages.

Studies using *in situ* hybridization with a Mas-AT RNA probe, and immunocytochemistry with antisera against Mas-AT, revealed extensive distribution of Mas-AT neuropeptide in the central and enteric nervous systems of *M. sexta* (Veenstra and Hagedorn, 1993; Veenstra *et al.*, 1994; Žitňan *et al.*, 1993, 1995; Taylor *et al.*, 1996; Bhatt and Horodyski, 1999).

In this paper, localization of AT neuropeptides is described in developing ventral nerve cord of the silk moth *B. mori,* including projection of axons from the Mas-AT-IR structures of AG 1 to 7 to each N 1 and 2.

Materials and Methods

Animals

Cold-treated eggs from the silk moth *Bombyx mori*, which were supplied from the National Institute of Agricultural Science and Technology (Suwon, Korea), were hatched about 10 days after incubation at 27-28°C with relative humidity of 60-70%. Larvae were reared on an artificial diet (commercially purchased from Korean Society for Sericulture; including mulberry leaves, essential minerals and so on) under a long-day photoperiod regimen (17 hrs light-7 hrs dark). Insects used were first, second, third, fourth and fifth instar larvae, prepupae, 3- and 5-day-old pupae, and 1-day-old adults. The fifth instar stage was subdivided into three stages of day-1, day-3, and day-5. Seven insect individuals were used for detection of Mas-AT-immunoreactivities at each of eleven postembryonic stages.

Wholemount immunocytochemistry

Tissue preparaton and wholemount immunocytochemistry were performed according to Lee *et al.* (1998) and Kim *et al.* (1998). The ventral nerve cord (including SOG, three TG and eight AG) at each developing stage was dissected in 0.1 M sodium phosphate buffer (pH 7.4) (PB) and then fixed in 4% paraformaldehyde (PFA) in 0.1 M PB for 5-9 hrs at 4°C, depending on the size of tissues. The fixed tissues were immersed in 0.01 M phosphate-buffered saline (PBS) with 1% Triton X-100 at 4°C overnight. Blockage of peroxidase activity was performed in 10% methanol with 3% H_2O_2 for 25 min. Washes in 0.1 M Tris-HCl buffer (pH 7.6-8.6) containing 1% Triton X-100 and 4% NaCl were followed by incubation with a primary antiserum anti-Mas-AT [provided kindly by Dr. Jan A. Veenstra (Universite Bordeaux 1, France) diluted to 1 : 1,500 in dilution buffer (0.01 M PBS with 1% Triton X-100 and 10% normal serum), for 4-5 days with gentle shaking. After washes in 0.01 M PBS with 1% Triton X-100, tissues were incubated in peroxidase-conjugated swine anti-rabbit IgG (DAKO), diluted to 1 : 200 for 2 days at 4°C. Following preincubation in 0.03% diaminobenzidine (DAB, Sigma) in 0.05 M Tris-HCl buffer for 1 hr at 4°C, the tissues were treated with 0.03% DAB in 0.05 M Tris-HCl buffer for 5-10 min containing 0.01% H_2O_2. After rinses in 0.05 M Tris-HCl buffer, tissues were embedded in glycerin, examined and photographed with a Zeiss interference microscope. As a specificity control, immunocytochemistry was performed on whole ventral ganglia of larvae, pupae and adults with anti-Mas-AT preincubated with 50 nmol synthetic Mas-AT per ml diluted antiserum (diluted to 1 : 1,500) for 24 hrs.

Florescence immunocytochemistry

Three TG, seven AG and TAG were isolated in 0.1 M PB, fixed in 4% PFA for 4 hrs at 4°C, and washed with 80% ethanol (8 × 10 min). Additional washes in 0.0l M PBS with 1% Triton X-100 (4 × 10 min) were followed, and tissues were then incubated with anti-Mas-AT (diluted to 1:1,000 in 0.01 M PBS with 1% Triton X-100 and 10% normal serum) overnight at room temperature. Tissues were rinsed in 0.01 M PBS (5 × 10 min) and then incubated with swine anti-rabbit IgG conjugated with FITC for 4 hrs at room temperature in the dark room. Tissues were finally washed in 0.01 M PBS with 1% Triton X-100 (3 × 10 min), embedded in glycerin, examined and photographed with a fluorescence microscope.

Results

Following application of Mas-AT antiserum preincubated with 50 nmol synthetic Mas-AT, there were no immunolabeled structures in the ventral ganglia of larvae, pupae and adults.

Mas-AT-immunoreactivity in ventral nerve cord

Mas-AT-IR cell bodies in the SOG began to appear from the second instar. There was one pair of labeled cell bodies in the anteromedial area of SOG (Fig. 1a, arrowheads). These cells were apparent until the 3-day-old pupal stage. In the third instar larva to the 3-day-old pupa (excluding prepupa), Mas-AT-IR cell bodies were localized in the same locations in the SOG. In addition to one pair in the anteromedial portion of the SOG, there were three immunostained pairs in bilateral positions of the middle and posteromedial portions (Fig. 1b, c, d).

In the case of labeling of cells in the three TG, immunoreactive cells first appeared in third instar larvae. In TG 1 of third instar larvae, there was one pair of labeled cell

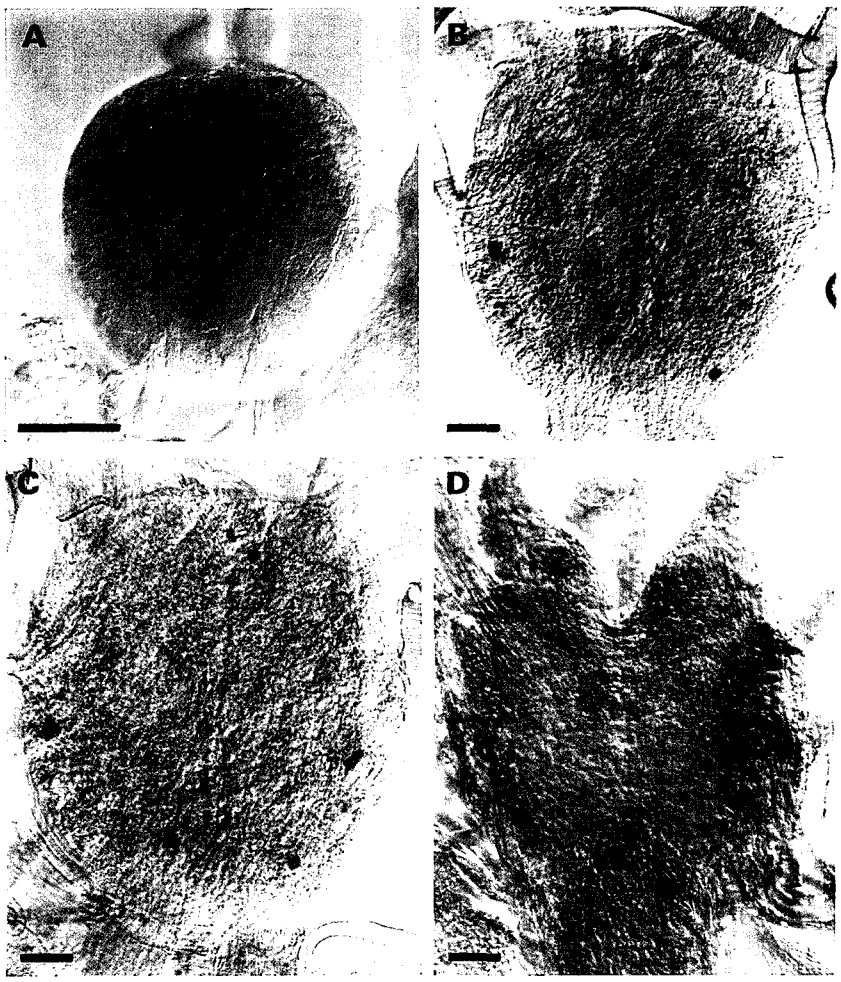

Fig. 1. Mas-AT-IR cell bodies in the larval and pupal SOG (whole-mount preparations). **A**, Second instar larva. One weakly labeled pair of cell bodies (arrowheads) is bilaterally localized in the anteromedial portion. **B**, Day-1 fifth instar larva. Mas-AT-IR cell bodies are seen in middle and posteromedial portion, as well as anteromedial portion. **C**, Day-5 fifth instar larva. Note that number of labeled cell bodies in SOG is the same as in day-1 and day-3 fifth instar larvae. **D**, 3-day-old pupa. Localization of labeled cell bodies is the same as in the larval stages.

bodies in a bilateral position of middle portion and in the posteromedial portion, respectively. The localization of Mas-AT-labeled cell bodies in the TG 1 was the same as those in the fourth and fifth instar larvae and 3-day-old pupa (Fig. 2a, d). In the TG 2 from third instar larvae to 3-day-old pupae, one pair of labeled cell bodies was localized in a bilateral position of the middle portion and in the posteromedial portion, respectively (Fig. 2b, c). Labeled cell bodies also showed specific localizations in the TG 3 of third instar larva to 3-day-old pupa. There was one pair in the anteromedial portion and four closely-located cell bodies in the posteromedial portion (Fig. 2e). However, there were changes in the position of stained cell bodies in the TG 2 and 3 of 5-day-old pupae, in which PTG were formed (Fig. 2f). PTG showed one pair of cell bodies in a bilateral location of the TG 2 neuromere, another pair in a medial position of their TG 3 neuromere, and third pair in the posteromedial portion of their AG 2 neuromere, respectively. In the 1-day-old adults, PTG contained Mas-AT-labeled cell bodies in the same positions as in the 5-day-old pupae (Fig. 3h).

In the AG of first and second instar larvae, Mas-AT-immunoreactivity was detected only in TAG (data not shown). There were four closely located large cell bodies in the median portion of their AG 7 neuromere. AG 1 to 6 of the third to fifth instar larvae contained one pair of labeled, closely located cell bodies in their posteromedial portions, respectively (Fig. 3a, b, c, d, e, f), and AG 7 had four, closely located large cell bodies in the medial portion (Fig. 3g). Processes from these cell bodies in AG 1 to 7 contributed directly to the formation of similar structures of Mas-AT-immunoreactivity in each ganglionic neuropil,

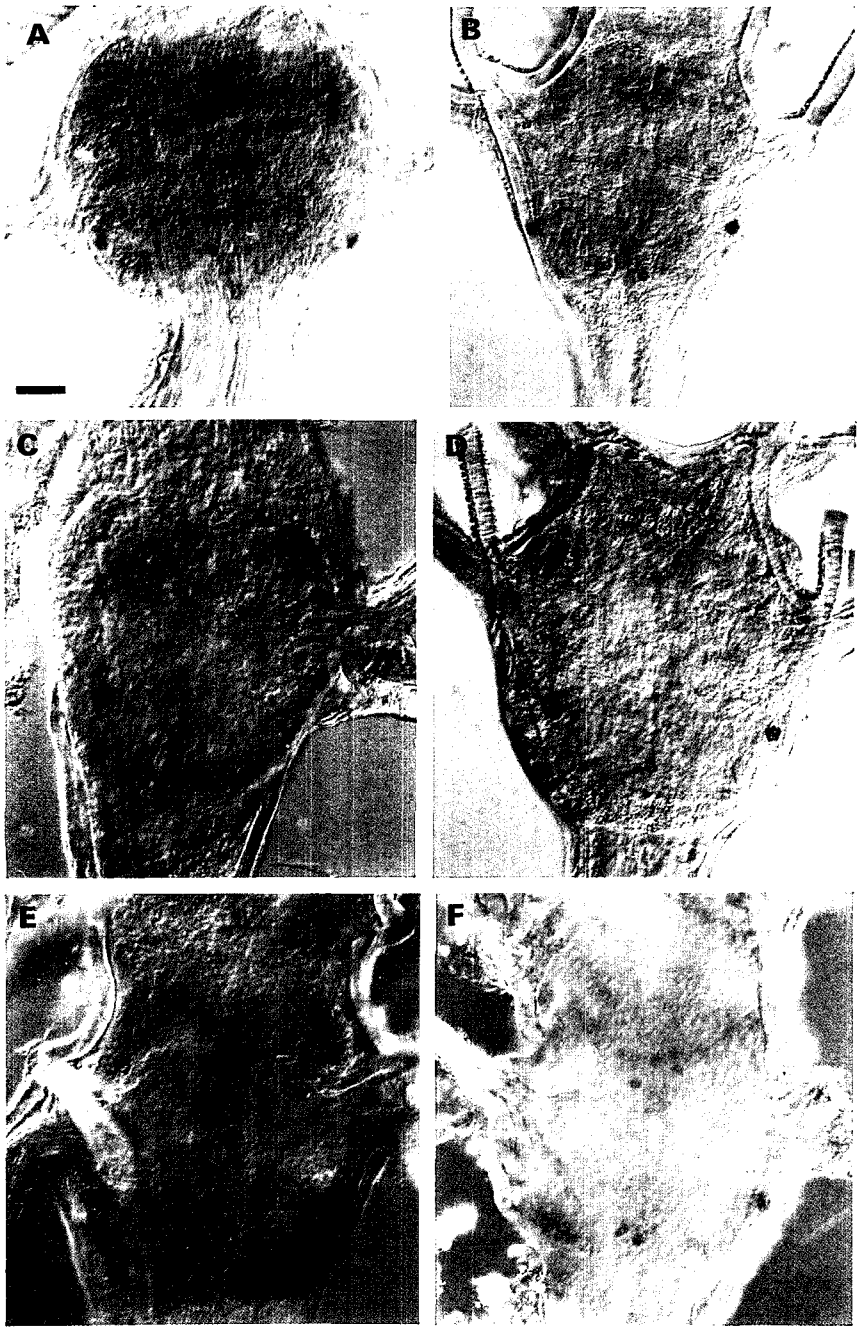

Fig. 2. Mas-AT-immunostained cell bodies in the TG 1-3 of the larval and pupal stages (whole-mount preparations). **A**, TG 1 of day-1 fifth instar larva containing labeled cell bodies in the middle and posterior portions. **B**, TG 2 of day-3 fifth instar larva showing one pair of cell bodies in bilateral position of middle neuromere and another pair in posteromedial portion. **C**, TG 1 of 3-day-old pupa containing one pair of bilateral cell bodies in the middle portion. **D**, TG 2 of 3-day-old pupa. **E**, TG 3 of 3-day-old pupa. It shows two clusters of labeled cell bodies in both anteromedial portion (about two) and posteromedial portion (about four). **F**, PTG of 5-day-old pupa. PTG contain labeled cell bodies in each medial portion of TG 3 and AG 2 neuromeres.

as shown in Fig. 3a (arrowheads). This immunoreactivity was localized in the anterior part of AG 1 to 7 of larvae, pupae and adults but not in the AG 1 to 6 of the prepupae. In the AG 1 to 7 of third instar larvae to 1-day-old adult (except for prepupae), labeled axons projected bilaterally from the anterior and posterior positions of similar structures of Mas-AT-immunoreactivity in each central neuropil and then extended to ipsilateral N1 (Fig. 4a, c, f;

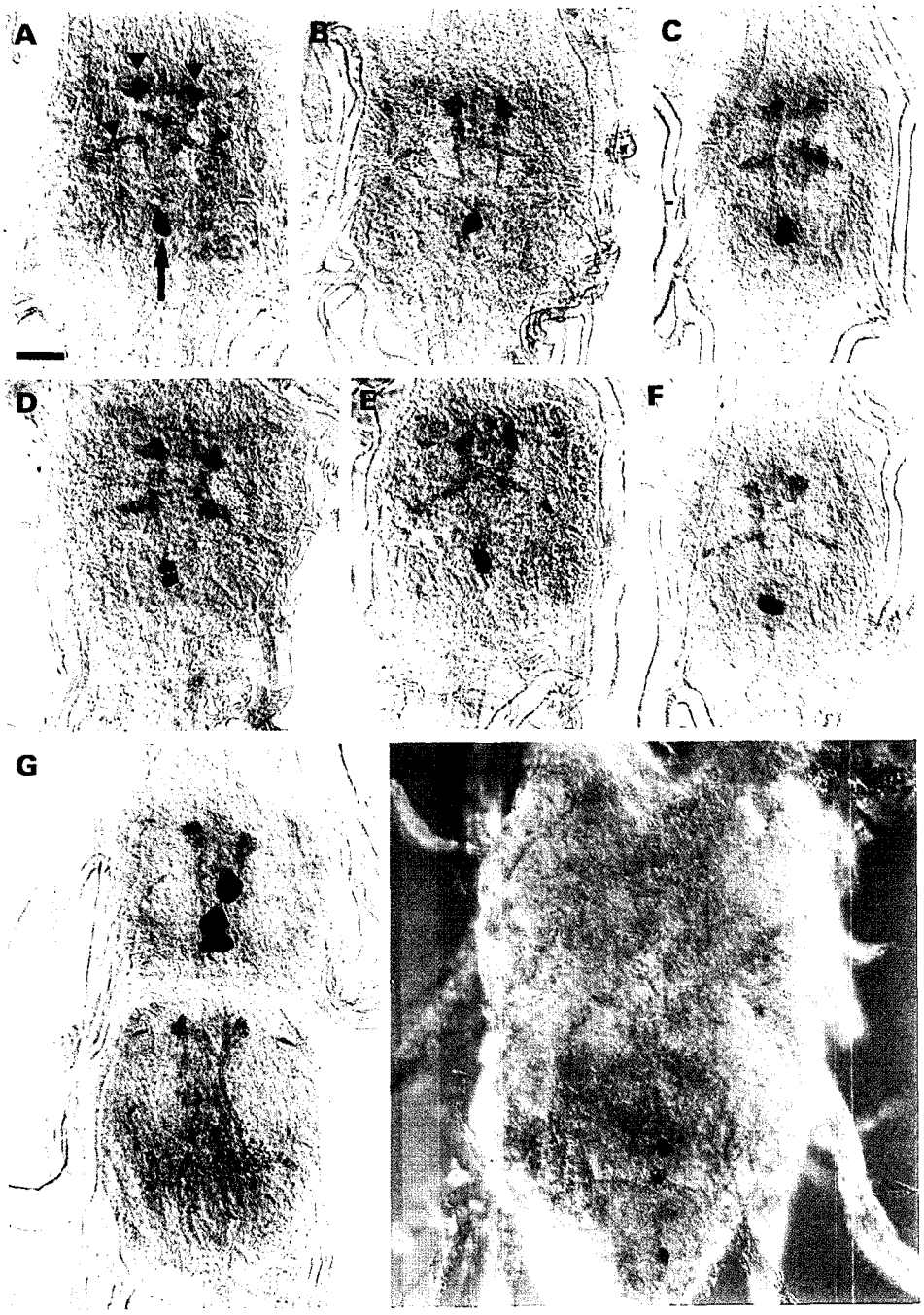

Fig. 3. Mas-AT-IR neuronal cell bodies in the AG 1 to 8 of fifth instar larvae and PTG of adult (whole-mount preparations). Note that AG 1 to 7 contain one pair of labeled cell bodies (arrow in a) in the posteromedial portion, respectively, and similar structure of Mas-AT-immunoreactivity (arrowheads in a) in each central neuropil. **A**, AG 1 of day-5 fifth instar larva. **B**, AG 2 of day-3 fifth instar larva. **C**, AG 3 of day-1 fifth instar larva. **D**, AG 4 of day-5 fifth instar larva. **E**, AG 5 of day-3 fifth instar larva. **F**, AG 6 of day-1 fifth instar larva. **G**, TAG of day-1 fifth instar larva. There are four labeled large cell bodies in the central neuropil of AG 7 neuromere of TAG. Mas-AT-immunoreactivity in AG 8 are structurally different from those in AG 7. **H**, PTG of 1-day-old adult. Labeled cell bodies are localized in TG 3 neuromere and AG 2 neuromere.

arrowheads) or N2 (Fig. 4b, c, d, e; arrows) of the same AG, respectively [see Burrows (1996) for definition of the nerves N 1 and 2]. Labeled axons (arrowhead in Fig. 4f) projected from AG 8 extended to ipsilateral anterior nerves of the same ganglion. Some of four labeled cell bodies in the median portion of AG 7 projected their

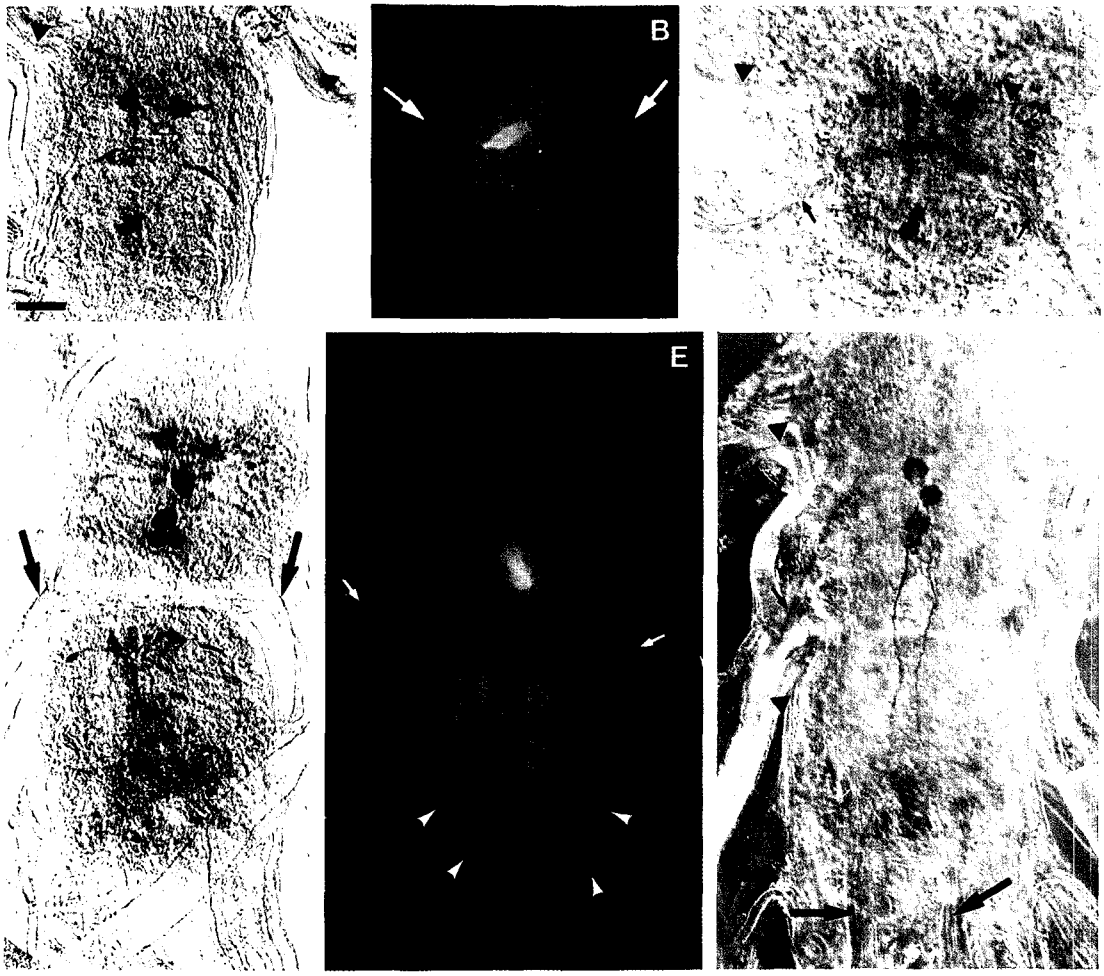

Fig. 4. Mas-AT-immunoreactivity in the central neuropils of the AG 1 to 8 and Mas-AT-labeled axons in the N 1 and N 2 extended from the AG of larvae and pupae (whole-mount preparations). **A**, AG 1 of day-1 fifth instar larva. Note that the ganglion contains labeled cell bodies and Mas-AT-immunoreactivity in the central neuropil and there are also Mas-AT-labeled axons (arrowheads) in the N 1 projected from AG 1. **B**, AG 5 of day-3 fifth instar larva. There are two FITC-labeled cell bodies in the ganglionic posteromedial portion and nerve fibers (arrows) in the N 2 of AG 5. **C**, AG 4 of 5-day-old pupa. Note that localization of labeled cell bodies and structure of Mas-AT-immunoreactivity are the same as those in the larval stages. The N 1 (arrowheads) and N 2 (arrows) extending from the ganglion have also Mas-AT-labeled nerve fibers. **D** and **E**, TAG of day-5 fifth instar larvae stained by PAP method (d) and FITC (e). AG 7 neuromere has not only four large labeled cell bodies in the medial portion, but it has also very similar structure of Mas-AT-immunoreactivities to those in AG 1 to 6. In contrast, AG 8 neuromere contains two bilateral pairs of small cell bodies (white arrowheads) in the posterior portion. Arrows indicate Mas-AT-IR axons in the N 2. **F**, TAG of 5-day-old pupa. These ganglia show same localization of Mas-AT-IR cell bodies and same structure of Mas-AT-immunoreactivities as TAG of larvae. Mas-AT-labeled nerve fibers (arrowheads) are seen in the N 1 (arrowhead) and 2 (arrow) of AG 7. In AG 8, there are also labeled nerve fiber (arrows) in the N 1 and one pair of nerve fibers (larger arrows) extending caudally. One pair of these labeled caudally-extending axons are projected from labeled cell bodies in the AG 7 neuromere.

axons through the central neuropil of AG 8 to one pair of bilateral nerves extending posteriorly towards the caudal peripheral organs (Fig. 4f, larger arrowheads).

Discussion

We have detected Mas-AT-labeled neuronal cell bodies in the SOG of second, third, fourth and fifth instar larvae, and 3-day-old pupae. In *B. mori,* there are Mas-AT-producing cells in a wide range of postembryonic stages whereas in *M. sexta*, there are four pairs of Mas-AT mRNA-containing neurons in the SOG only in day-2 second instars (Bhatt and Horodyski, 1999). It has been also shown that in addition to AT, insect SOG also contains a variety of peptides including vasopressin (Veenstra, 1984;

Davis and Hildebrand, 1992; Tyrer *et al.*, 1993), neurophysin I and II (Camier *et al.*, 1980), leucokinin (Nässel, 1993; Kang and Lee, 1997; Lee *et al.*, 1998), locustatachykinin (Nässel, 1993; Murren *et al.*, 1995; Kang *et al.*, 1997; Kim *et al.*, 1998), and AS (Žitňan *et al.*, 1993; Davis *et al.*, 1997).

Larvae, pupae and adults of *B. mori* also contained immunolabeled neurons in the TG 1, 2 and 3. These Mas-AT-IR neuronal cell bodies were localized to specific regions in the bilateral and/or medial portion of each of the three TG. One pair of labeled cell bodies was localized in the middle and posterior portions of TG 1 and 2. In TG 3, there was one pair and two pairs of labeled cell bodies in the anteromedian and posterior portions, respectively. This localization of labeled cell bodies in the three TG of third instar larvae was the same as those in fourth and fifth instar larvae and 3-day-old pupae. However, the localization of labeled cell bodies in the PTG of 5-day-old pupae was the same as those in 1-day-old pupae. As a result of metamorphic changes in ventral ganglia of the pupal stages, there were distinct differences in the localization of labeled cell bodies between 3-day-old pupae and 5-day-old pupae. It has been demonstrated in *D. melanogaster* that there is one pair of Mas-AT-labeled neurons in each thoracic neuromere of third instar larvae (Žitňan *et al.*, 1993). There is little other evidence for the occurrence of AT-producing neurons in the TG of the insects, excluding *B. mori* and *D. melanogaster.* The function of the of the AT in insect TG remains unknown, excluding a report that in *Locusta migratoria*, some sensory fibers in the TG contain colocalized peptides related to locustatachykinins, FMRFamide-related peptide (s) or Mas-AT (Persson and Nässel, 1999).

In *B. mori*, the AG of larvae, pupae and adults all had one pair of Mas-AT-IR cell bodies in the posteromedian portions of AG 1 to 6, whereas AG 7 contained four labeled cell bodies in the anteromedian portion. There were also two pairs of labeled cell bodies in the AG 8. AG 1 to 7 have Mas-AT-immunoreactivity which were very similar in their external features. This Mas-AT-immunoreactivity in each neuropil of AG 1 to 7 were perhaps composed mostly of processes extended from one pair of labeled cell bodies in the ganglia in which the Mas-AT-immunoreactivity was localized. Although detailed morphology of Mas-AT-immunoreactivity remains to be determined, labeled axons in the nerves N 1 (or dorsal nerve) and 2 (or ventral nerve) extending from AG 1 to 7 projected out to N 1 or 2 through the Mas-AT-immunoreactivity in those AG. It has been suggested that Mas-AT released from these labeled axons might exert an excitatory effect on the heart contraction of pharate adults of *M. sexta* (Veestra *et al.*, 1994).

There was a report from *Leucophaea maderae* and *Periplaneta americana* that some of labeled axons extending from AG 5 and 6 seemed to be terminated in the rectal pads of the hindgut through the nerve N 2 (Rudwall *et al.*, 2000). However, it is concluded that further studies will be needed to determine detailed structural connections of AT-IR neurons both within the ventral nerve cord and between TG and various peripheral organs.

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