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Immunolocalization of Allatotropin Neuropeptide in the Developing Brain of the Silk Moth *Bombyx mori*

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Polyclonal antiserum against *Manduca sexta* allatotropin has been utilized to investigate the localization of allatotropin-immunoreactivity in the brain of the silk moth *Bombyx mori*. *Manduca sexta* allatotropin-immunoreactive (Mas-AT-IR) neurons were found in all larval brains investigated, but not in prepupal, pupal and adult brains. In the larval stages, first appearance of Mas-AT-immunoreactivity was shown in the brain of first instar larvae, which contains four pairs of bilateral Mas-AT-IR cell bodies. Labeled neurons increased to six pairs in the second instar larval brain, including two pairs of median neurosecretory cells in the pars intercerebralis. In the third and fourth instar larvae, five pairs of labeled cell bodies were distributed throughout each brain. In the fifth instar, there were about ten pairs of bilateral cell bodies in the day-1 brain, about seven pairs in the day-3 brains, and five pairs in the day-5 brains, respectively. Mas-AT-labeling was observed in both axons within nervi corpora cardiaci (NCC) I+II and corpora allata. This suggests that the Mas-AT produced from the brain neurons is transported via some axons of the NCC I+II and nervi corpora allati I to the corpora allata, which appears to be a main accumulation site for the Mas-AT neuropeptide in some brain neurons produced in *B. mori*.

Juvenile hormone (JH), a sesquiterpenoid hormone, is released from the corpora allata of insect and plays a vital role in metamorphosis, reproduction and adult sexual maturation (Engelmann, 1970). JH titer in hemolymph changes throughout the development in *M. sexta* (Baker et al., 1987) and is also controlled by either a stimulatory neuropeptide (allatotropin, AT) or an inhibitory neuropeptide (allatostatin, AS) produced in the brain. AT stimulates biosynthesis of JH by the corpora allata (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), whereas AS inhibits it (Kramer et al., 1991).

In *M. sexta*, AT that is synthesized by specific neurons of abdominal ganglia has a function of a cardioacceleratory peptide on the heart of pharate adult (Veenstra et al., 1994). AT that is produced in specific neurons of frontal ganglion shows a strong stimulatory action on the foregut of *Lacanobia oleracea* (Duve et al., 2000). *M. sexta* AT (Mas-AT), which has been purified and characterized from the head of pharate adult, is a 13-residue amidated tridecapeptide (Kataoka et al., 1989). AT has been also identified recently in *L. oleracea* (Audsley et al., 2000) and *Spodoptera frugiperda* (Oeh et al., 2000).

Mas-AT mRNA has been detected in different neurons of brain, terminal abdominal ganglia and frontal ganglion of *M. sexta* by *in situ* hybridization (Taylor et al., 1996; Bhatt and Horodyski, 1999). Using a polyclonal antibody to Mas-AT, AT-immunolocalization has been demonstrated in the brain, ventral ganglia and midgut of *M. sexta* and *Drosophila melanogaster* (Zitnan et al., 1993, 1995; Taylor et al., 1996; Duve et al., 2000). Immunoreactivity has been also detected in the abdominal ganglia of pharate adults and Mas-AT levels in the brain, ventral nerve cord, and corpora cardiaca and corpora allata have been also quantified by ELISA tests (Veenstra and Hagedorn, 1993; Veenstra et al., 1994).

It has been reported that the prothoracicotropic hormone (PTTH) is produced by two pairs of dorso-lateral neurosecretory cells in the brain and then transported to the corpora allata via NCC II and NCA I in *B. mori* (Mizoguchi et al., 1990). In *M. sexta*, some of Mas-AT-producing neurons shows immunoreactivity to *Bombyx* PTTH in the brain of parasitized larvae (Zitnan et al., 1995).

Production of AT in specific brain neurons has not been demonstrated with the data related to different developmental stages of an insect. Whether Mas-AT is colocalized with *Bombyx* PTTH in specific neurons of a normal larval brain is also needed to be investigated.

In this paper, therefore, immunolocalization of AT is

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described in the brain of 1st, 2nd, 3rd, 4th, 5th (day-1, day-3 and day-5) instar larvae, prepupae, day-3 and day-5 pupae, and day-1 adults of the silk moth *B. mori*, including the projection of Mas-AT-IR brain neurons into the corpora allata. We also show that Mas-AT is not colocalized with PTTH in the larval brain neurons.

Materials and Methods

Animals

Cold-treated eggs from the silk moth *Bombyx mori* (supplied from the National Institute of Agriculture, Science and Technology) were hatched to 1st instar larvae about 10 d after incubation at 27-28℃ and relative humidity of 60-70%. Larvae were reared on an artificial diet (purchased from Korean Society for Sericulture; including mulberry leaves, essential minerals, etc.) under a long-day photoperiod regimen (17 h light-7 h dark). Insects used were the first, second, third, fourth and fifth instar larvae, prepupae, day-3 and day-5 pupae, and day-1 adults. The fifth instar stage was subdivided into three stages of day-1, day-3, and day-5. Seven insect individuals were used for the detection of Mas-AT-immunoreactivity at each of eleven post embryonic stages.

Wholemount immunocytochemistry

Tissue preparaton and wholemount immunocytochemistry were performed according to the methods of Lee et al. (1998) and Kim et al. (1998). Following anaesthetic treatment of the above-mentioned insects at 4℃ for 1 h, brain-corpora cardiaca and allata from each developing stage were dissected in 0.1 M sodium phosphate buffer (pH 7.4; PB) and fixed in 4% paraformaldehyde (PFA) in 0.1 M PB for 5-9 h at 4℃, depending on the size of tissues. Fixed tissues were immersed in 0.01 M phosphate-buffered saline (PBS) with 1% Triton X-100 at 4℃ overnight and then 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, Veenstra et al., 1994), diluted to 1:1,500 in dilution buffer (0.01 M PBS with 1% Triton X-100 and 10% normal goat serum), for 4-5 d with gentle shaking. Anti-Mas-AT has been demonstrated to show interspecies cross-reactivity in the fall armyworm *Spodoptera frugiperda* (Oeh et al., 2000). 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 d at 4℃. Following preincubation in 0.03% diaminobenzidine (DAB, Sigma) in 0.05 M Tris-HCl buffer for 1 h at 4℃, 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 mounted in glycerin, examined and photographed with a Zeiss interference microscope. As a specificity control, immunocytochemistry was performed on whole brain-corpora cardiaca and allata of larvae, pupae and adults with anti-Mas-AT preincubated with 50 nmol synthetic Mas-AT per ml of diluted antiserum (diluted to 1:1,500) for 24 h.

Immunocytochemistry of brain sections

Isolated brains were fixed in 4% PFA for 4 h at 4℃. Tissues were rinsed in 0.1 M PB (pH 7.4) and treated in 0.01 M PBS with 1% Triton X-100 at 4℃ overnight. Tissues were embedded in 5% agarose and then serially sectioned on a vibrating microtome in 50 μm thickness. Brain sections were rinsed with 0.01 M PBS with 1% Triton X-100 several times and incubated with anti-Mas-AT antisera (diluted to 1:1,000 in 0.01 M PBS with 1% Triton X-100 and 10% normal goat serum) for 2 d at room temperature. Additional rinsing of the sections in 0.0l M PBS with 1% Triton X-100 for 1 h at room temperature was followed by incubation with swine anti-rabbit IgG conjugated with horseradish-peroxidase (HRP) for 2 h at room temperature. The peroxidase reaction was carried out in 0.05% DAB and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer. The brain sections were thoroughly washed in 0.05 M Tris-HCl buffer, mounted in glycerin, and examined and photographed with the interference microscope.

Fluorescence immunocytochemistry

Brain-corpora cardiaca and allata were isolated in 0.1 M PB, fixed in 4% PFA for 4 h at 4℃, and washed with 80% ethanol (8 x 10 min). Additional washes with 0.01 M PBS with 1% Triton X-100 (4 x 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 goat serum) overnight at room temperature. Tissues were rinsed in 0.01 M PBS with 1% Triton X-100 (5 x 10 min) and then incubated with swine anti-rabbit IgG conjugated with FITC for 4 h at room temperature in the dark room. Tissues were finally washed in 0.01 M PBST (3 x 10 min), mounted in glycerin, examined and photographed with a fluorescence microscope.

Double-label immunofluorescence

Double-label immunofluorescence was performed according to a procedure modified slightly from the method of Nässel (1993). Brains of day-1 fifth instar larvae were isolated in 0.1 M PB, fixed in 4% PFA for 4 h at 4℃, and washed in 80% ethanol (5 x 10 min). After washes in 0.01 M PBS with 1% Triton X-100, tissues were incubated with anti-*Bombyx* PTTH and anti-Mas-AT, diluted to 1:1,000 in 0.01 M PBS with 1% Triton X-100 and 10% normal goat serum at 4℃ for 2 d. Tissues were rinsed in 0.01 M PBS with 1% Triton

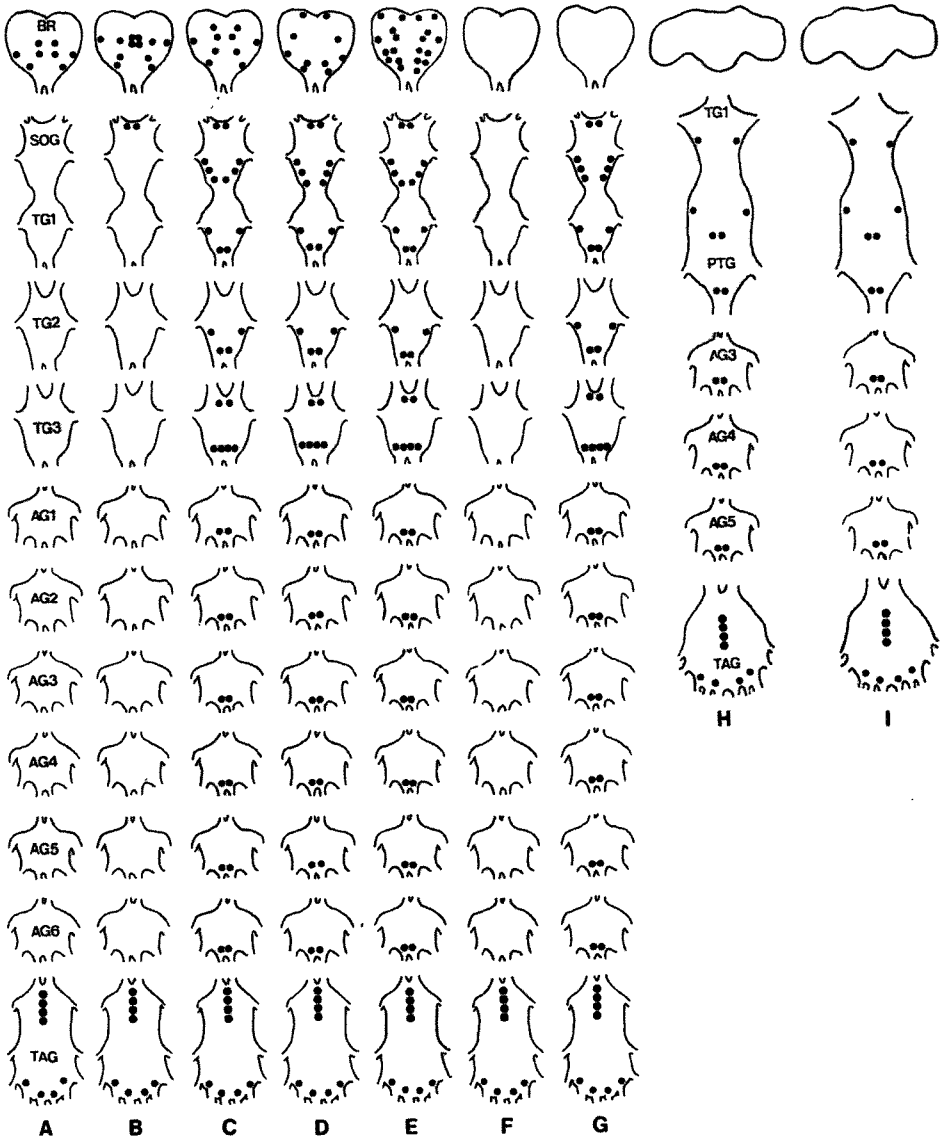

Fig. 1. Schematic drawings for the localization of Mas-AT-IR neuronal cell bodies in the brains (Br), suboesophageal ganglia (SOG), thoracic ganglia (TG), abdominal ganglia (AG) and terminal abdominal ganglia (TAG) of larvae, pupae and adults of *B. mori*. All drawings have been made from whole-mounts and only consistently labeled neuronal cell bodies have been drawn. A, First instar larva. B, Second instar larva. C, Third instar larva. D, Fourth instar larva. E, Day-1 fifth instar larva. F, Prepupa. G, day-3 pupa. H, 5-day-old pupa. I, day-1 adult. In the first instar larva to day-3 pupa, there are TAG formed by fusion of AG 7 and 8. In the day-5 pupa to day-1 adult, however, there are two larger ganglia in the thoracic and abdominal segment: pterothoracic ganglia (PTG, formed by fusion of TG 2 and 3 with AG 1 and 2) and TAG (formed by fusion of AG 6 with AG 7/8). Note that the number of immunolabeled cell bodies increases not only in the brains of the larval stages but also in SOG, TG 1-3, and AG 1-8 of the 3rd, 4th and day-1 5th instar larvae, 3- and day-5 pupae, and day-1 adults.

X-100 (5 x 10 min) and then incubated with swine anti-rabbit IgG coupled to the fluorescein isothiocyanate (FITC) for 1 d at 4℃. Rinses with 0.01 M PBS including 1% Triton X-100 (5 x 10 min) and then incubation with goat anti-mouse IgG conjugated with 7-amino-4-methylmoumanrin-3-acetic acid (AMCA) for 1 d were followed. Finally, tissues were washed in 0.01 M PBS with 1% Triton X-100 (3 x 10 min), mounted in glycerin, examined and photographed with a Nikon fluorescence microscope.

Results

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

Mas-AT-immunoreactivity in the brain and corpora allata of the first instar larvae is shown in Fig. 1. The brain contained four pairs of bilateral Mas-AT-IR cell bodies and corpora allata showed immunoreactivity. Mas-AT-immunoreactivity in the corpora allata of the

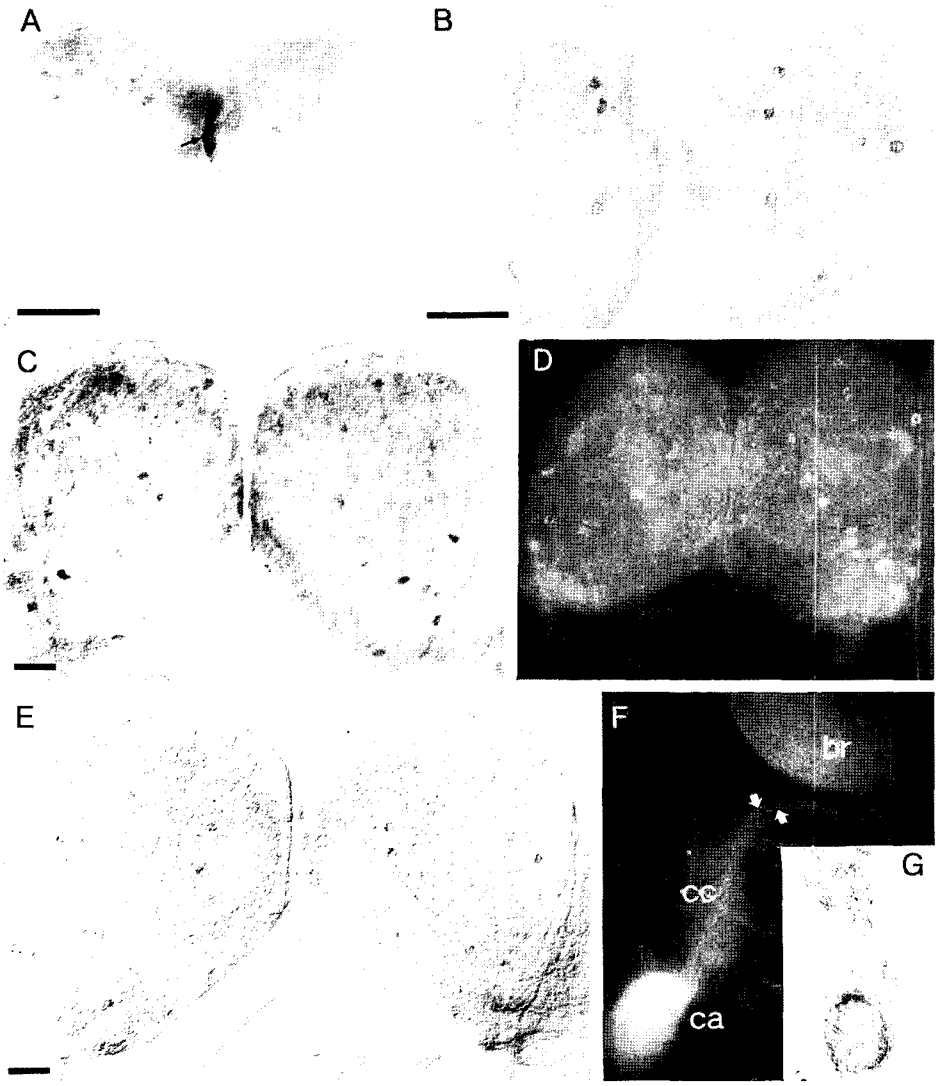

Fig. 2. Mas-AT-immunoreactivity in the larval brains and corpopra allata (wholemount preparation). A, Second instar larval brain. In addition to bilateral cell bodies, a cluster of immunolabeling (arrowhead; seen as a few intensively-labeled cell bodies) is found in the pars intercerebralis of protocerebrum. B, Third instar larval brain. Several labeled pairs of cell bodies could be seen in both cerebral hemispheres. C, Day-1 fifth instar larval brain. About ten pairs of labeled cell bodies could be seen. D, Day-1 fifth instar larva brain stained by an immunofluorescent method. E, Day-5 fifth instar larva brain. A few Mas-AT-IR cell bodies are clearly stained. F, Brain(br)-corpora cardiaca(cc)-corpora cardiaca(ca) of day-1 fifth instar larva by an immunofluorescent method. Labeling can be clearly seen in both ca and axons (arrows) of NCC I and NCA I with no labeling in brain and cc. G, Mas-AT immunoreactivity in the ca of day-1 fifth instar larva. Scale bars=50 μm

1st instar larvae could be found until the day-1 of fifth instars. Mas-AT-labeled cell bodies increased to six pairs in the second instar larval brain (Fig. 2A), including two pairs (arrow) in the pars intercerebralis. It appears that these cells are median neurosecretory cells, although their axons could not be traced. These cell bodies were not apparent in the third instars onwards. In the brain of third instar larvae, five pairs of labeled cell bodies were distributed throughout the brain (Fig. 2B). There was the same number of labeled cell bodies in the fourth instar larval brain. As seen in Fig. 1, however, their localization was slightly different between the third and the fourth instars. In day-1 fifth instar brains, there were about ten pairs of small neuronal cell bodies that showed bilateral localization (Fig. 2C, D). About seven pairs of labeled small cells were also found in the day-3 fifth instar brains (data not shown), whereas there was five pairs in the day-5 fifth instar brains (Fig. 2E).

Mas-AT-labeling could be seen in both axons (white arrows) within NCC I+II and corpora allata from day-1 fifth instars, as shown in Fig. 2F and 2G. In paticular, corpora allata contained abundant Mas-AT (Fig. 2F). This suggests that Mas-AT produced in the brain neurons are transported via the axons within the NCC I+II and NCA I to the corpora allata that appears to be a main accumulation and release site for the Mas-AT in some brain cell bodies produced (Fig. 2F, G).

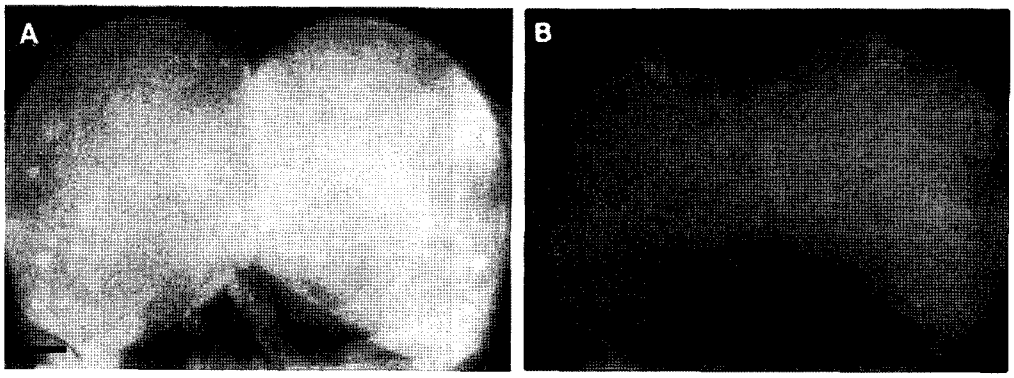

Fig. 3. Double-label immunofluorescence of a brain from fourth instar larva for detection of both Mas-AT-IR and PTTH-immunoreactive cell bodies. A, Mas-AT-IR cell bodies. B, PTTH- immunoreactive cell bodies. Note that there is no doubly labeled cell body in the brains. It suggests that in this stage any of Mas-AT-IR neurons has no activity to produce the PTTH. Scale bars=50 μm.

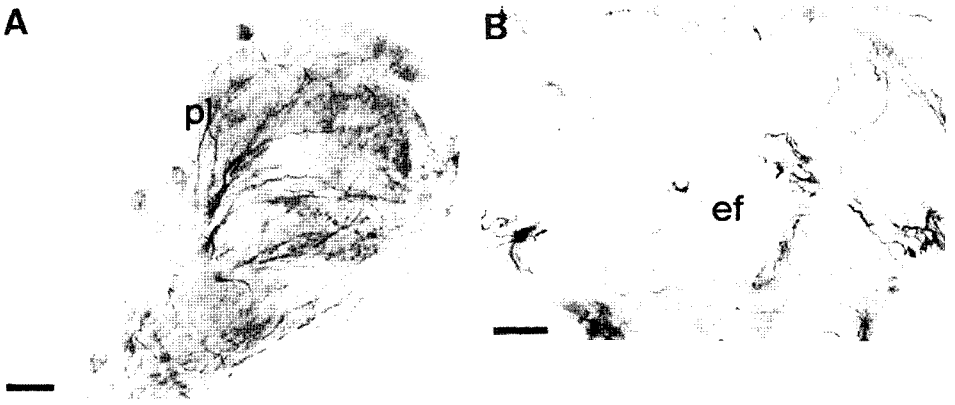

Fig. 4. Sectioned brains of the larva and the adult. A, Left hemisphere of day-1 fifth instar larval brain. It has Mas-AT-IR cell bodies mostly in the pars lateralis of protocerebrum (PL), but it does not contain Mas- AT-IR processes. B, One-day-old adult brain. Note that there is no Mas-AT-IR cell body. EF, esophageal foramen. Scale bar=50 μm.

Mas-AT-IR cell bodies colocalized with *Bombyx* PTTH were not found in the brains of day-1 fifth instar larvae (Fig. 3A, B). Although there were two pairs of *Bombyx* PTTH-immunoreactive neurons in the pars lateralis of the protocerebrum (Fig. 3B), they were not colocalized with the Mas-AT-IR cells (Fig. 3A).

In the sectioned brain of 5th instars, labeled cell bodies were found, but labeled processes could not be detected (Fig. 4A). The origins of the labeled axon terminals could not be traced. However, it is possible that they might project from Mas-AT-IR neurons in the brain. No Mas-AT-immunoreactivity was detected in the sectioned brains of the prepupa to adult (Fig. 4B).

Discussion

Four to ten pairs of Mas-AT-labeled neurons have been demonstrated in the brains of the 1st to the 5th instar larvae from *B. mori*. These labeled neuronal cell bodies were widely distributed throughout the brains. However, there was no Mas-AT-immunoreactivity in the brains from prepupae to adults. This suggests that there is little or no JH biosynthesis regulated by the brain AT in these stages. The possibility can not be still excluded that the Mas-AT might be released rapidly during this period and not appear in the cells. It has been shown that there is no expression of Mas-AT mRNA in the prepupal, pupal, or pharate adult brains in *M. sexta* (Bhatt and Horodyski, 1999).

AT-immunoreactivity in the corpora cardiaca and/or corpora allata varied with different insect species. In the retrocerebral complex of *B. mori*, corpora allata showed abundant labeling, whereas corpora cardiaca did not. In addition, Mas-AT- immunoreactivity could be also detected in some axons of the NCC I+II as well as in some axons of the NCA I. In *M. sexta* Mas-AT immunoreactivity has been described in both the corpora cardiaca and the corpora allata of the fifth instar larvae (Zitnan et al., 1995), despite a mention of the absence of Mas-AT mRNA in the corpora allata (Bhatt and Horodyski, 1999). In *G. mellonella*, it has been suggested that AT produced in the median neurosecretory cells of the pars intercerebralis is transported via axons to the corpora cardiaca which accumulate the AT (Bogus and Scheller, 1996). AT is released from CC into hemolymph and acts via a

humoral route. These observations suggests that accumulation and release of brain AT in the retrocerebral complex are clearly different among various species.

It has been mentioned that in *M. sexta* some of the Mas-AT-IR cells are colocalized with *Bombyx* PTTH-immunoreactive cells in the brain of the parasitized larvae (Zitnan et al., 1995). Although we repeated this experiment extensively, we have never observed the colocalization of double-labeled immunofluorescence using anti-Mas-AT and anti-*Bombyx* PTTH in the brains of unparasitized (normal) fifth instar larvae. However, it could not be excluded that Mas-AT peptide was present in the *Bombyx* PTTH-immunoreactive cells below the detectable level.

A cluster of about four Mas-AT-IR cell bodies was seen in the pars intercerebralis of *B. mori* larval brain, although they could be detected only in the second instar larvae. This result has been also described in some other insect species (Granger and Sehnal, 1974; Sehnal and Rembold, 1985; Bogus and Scheller, 1994). In *G. mellonella* larvae have also two pairs of AT-immunoreactive median neuroscretory cells in the brain (Bogus and Scheller, 1994). These neurons of *G. mellonella* larval brain project their axons to the corpora cardiaca, apparently to stimlate JH biosynthesis by the corpora allata. However, in *B. mori*, the projection of these two pairs of median neurosecretory cells into the retrocerebral complex remained to be traced.

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References

Audsley N, Weaver RJ, and Edwards JP (2000) Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. *Insect Biochem Mol Biol* 30: 681-689.

Baker FC, Tsai LW, Reuter CC, and Schooley DA (1987) In vitro fluctuations on JH, JH acid and ecdysteroid titer, and JH esterase activity, during development of fifth statium *Manduca sexta*. *Insect Biochem* 17: 989-996.

Bhatt TR and Horodyski FM (1999) Expression of the *Manduca sexta* allatotropin gene in cells of the central and enteric nervous systems. *J Comp Neurol* 403: 407-420.

Bogus MI and Scheller K (1994) Identification of allatotropin-secreting cells in the brain of an insect larva. *Naturwissenschaften* 81: 87-89.

Bogus M and Scheller K (1996) Allatotropin released by the brain controls larval molting in *Galleria mellonella* by affecting juvenile hormone synthesis. *Int J Dev Biol* 40: 205-210.

Duve H, Audsley N, Weaver RJ, and Thorpe A (2000) Triple co-localization of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran *Lacanobia oleracea* (Noctuidae): innervation and action on the foregut. *Cell Tissue Res* 300: 153-163.

Engelmann F (1970) The Physiology of Insect Reproduction. Pergamon Press, Oxford.

Granger NA and Sehnal F (1974) Regulation of larval corpora allata in *Galleria mellonella*. *Nature* 251: 415-417.

Hebda C, Yu J-H, Bhaskaran G, and Dahm KH (1994) Reactivation of corpora allata in pharate adult *Manduca sexta*. *J Insect Physiol* 40: 849-858.

Kataoka H, Toschi A, Li JP, Carney RL, Schooley DA, and Kramer SJ (1989) Identification of an allatotropin from adult *Manduca sexta*. *Science* 243: 1481-1483.

Kim MY, Lee BH, Kwon D, Kang H, and Nässel DR (1998) Distribution of tachykinin-related neuropeptide in the developing central nervous system of the moth *Spodoptera litura*. *Cell Tissue Res* 294: 351-365.

Kramer SJ, Toschi A, Miller CA, Kataoka H, Quistad GB, Li JP, Carney RL, and Schooley DA (1991) Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. *Proc Natl Acad Sci USA* 88: 9458-9462.

Lee BH, Kang H, Kwon D, Park CI, Kim WK, and Kim MY (1998) Postembryonic development of leucokinin-like immunoreactive neurons in the moth *Spodoptera litura*. *Tissue Cell* 30: 74-85.

Mizoguchi A, Oka T, Kataoka H, Nagasawa H, Suzuki A, and Ishizaki H (1990) Immunohistochemical localization of prothoracicotropic hormone-producing neurosecretory cells in the brain of *Bomby mori*. *Dev Growth Differ* 32: 591-598.

Nässel DR (1993) Insect myotropic peptides: differential distribution of locustatachykinin- and leucokinin-like immunoreactive neurons in the locust brain. *Cell Tissue Res* 274: 27-40.

Oeh U, Lorenz MW, Dyker H, Losel P, and Hoffmann KH (2000) Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. *Insect Biochem Mol Biol* 30: 719-727.

Riddiford LM (1994) Cellular and molecular actions of juvenile hormone. I. General considerations and premetamorphic actions. *Adv Insect Physiol* 24: 213-274.

Sehnal F and Rembold H (1985) Brain stimulation of juvenile hormone production in insect larvae. *Experientia* 41: 684-685.

Stay B and Woodhead AP (1993) Neuropeptide regulators of the corpora allata. *Am Zool* 33: 357-364.

Stay B, Tobe SS, and Bendena WG (1994) Allatostatins: identification, primary structures, functions and distribution. *Adv Insect Physiol* 25: 267-337.

Taylor PA, Bhatt TR, and Horodyski FM (1996) Molecular characterization and expression analysis of *Manduca sexta* allatotropin. *Eur J Biochem* 239: 588-596.

Unni BG, Bhaskaran G, Dahm KH, and Hayes TK (1991) Stimulation of juvenile hormone biosynthesis by analogues of a *Manduca sexta* allatotropin: in vitro studies. *Arch Insect Biochem Physiol* 17: 129-142.

Veenstra JA and Hagedorn HH (1993) A sensitive enzyme immunoassay for *Manduca* allatotropin and the existence of an allatotropin-immunoreactive peptide in *Periplaneta americana*. *Arch Insect Biochem Physiol* 23: 99-109.

Veenstra JA, Lehman HK, and Davis NT (1994) Allatotropin is a cardioacceleratory peptides in *Manduca sexta*. *J Exp Biol* 188: 347-354.

Wyatt GR and Davey KG (1996) Cell and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv Insect Physiol* 26: 1-155.

Zitnan D, Sehnal F, and Bryant P (1993) Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed *Drosophila*. *Dev Biol* 156: 117-135.

Zitnan D, Kingan TG, Kramer SJ, and Beckage NE (1995) Accumulation of neuropeptides in cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J Comp Neurol* 356: 83-100.

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