Eur. J. Entomol. **95**: 185–196, 1998
ISSN 1210 – 5759

Immunohistochemical demonstration of mammalian- and FMRFamide-like peptides in the gut innervation and endocrine cells of the wild silkmoth, *Antheraea yamamai* (Lepidoptera: Saturniidae) during diapause and post-diapause of pharate first-instar larvae

YING AN [1], TAKAYUKI NAKAJIMA[2] and KOICHI SUZUKI [1]*

[1] Department of Applied Biology and [2] Department of Veterinary Anatomy, Faculty of Agriculture, Iwate University, Morioka 020, Japan

Immunohistochemistry, peptide hormones, pharate first-instar larvae, diapause, silkmoth

Abstract. FMRFamide-, vasoactive intestinal polypeptide (VIP)-, somatostatin-, substance P(SP)-, cholecystokinin octapeptide (CCK-8)- and pancreatic polypeptide (PP)-like materials were immunohistochemically detected in the endocrine cells and the gut innervation of diapausing pharate and newly-hatched first-instar larvae of *Antheraea yamamai*. SP- CCK-8-, PP- and FMRFamide-like immunoreactive cells were distributed unequally in different midgut regions; no cells reacting with antisera against VIP and somatostatin were found. Innervation of the anterior region of midgut, close to the foregut-midgut boundary, included 90–100 FMRFamide-like bipolar neurons that project along the midgut longitudinal muscles. No immunoreactive cell bodies were found in the foregut and hindgut, but nerve fibers with FMRFamide-like material were detected in hindgut muscles and extended over the posterior midgut. No changes in the distribution and intensity of immunostaining of both the endocrine cells and the gut innervation were found in four developmental stages between pre-diapause and hatching. SP-, CCK-8-, PP- and FMRFamide-like immunoreactive cells were also found in the CNS, indicating that the corresponding antigens belong to brain-gut regulatory peptides.

INTRODUCTION

The wild silkmoth *Antheraea yamamai* enters diapause as pharate first-instar larvae. This stage is reached about 10 days after oviposition. Suzuki et al. (1990, 1991) suggested that two factors regulate this pre-larval diapause: a repressive factor (RF), which is produced somewhere in the mesothorax, inhibits production of a maturation factor (MF), originating in the second to fifth abdominal segments and inducing post-diapause development. Juvenile hormones, ecdysteroids and eclosion hormone are not involved in the diapause control (Suzuki et al., 1991; Naya et al., 1994a). Previous results indicated that the two factors are products of an endocrine system that is independent of the central nervous system (CNS), and that MF is a peptidic compound (Suzuki et al., 1990; Naya et al., 1994b). However, neither of the two factors have been isolated.

The gut is a very important endocrine organ of insects. A number of invertebrate and vertebrate bioactive peptides were immunochemically detected in the epithelial endocrine cells and in the innervation of the midgut (Fujita et al., 1981; Andries & Tramu, 1985; Brown et al., 1986; Jenkins et al., 1989; Sehnal & Žitňan, 1990, 1996; Žitňan et al., 1993, 1995; Yu et al., 1995; East et al., 1995). Numerous immunoreactive cells apparently exert important paracrine and/or endocrine functions (Schols et al., 1987). In the corn earworm

* To whom all correspondence should be addressed; e-mail: koichi@iwate-u.ac.jp.

Heliothis zea, FMRFamide-related material is released from the midgut endocrine cells into the hemolymph following food ingestion and gut-emptying (Jenkins et al., 1989). Accumulation of FMRFamide-like antigens in the gut innervation and the endocrine cells is associated with developmental arrest of parasitized *Manduca sexta* larvae; it does not occur in larvae whose development is extended by starvation (Žitňan et al., 1995). A midgut hormone discovered in liver-fed *Phormia regina* adult females acts on the brain to initiate oogenesis (Yin et al., 1994). To summarize, the secretory products of the gut innervation and the midgut endocrine cells of insects appear to control a variety of vital functions (Žitňan et al., 1993).

In search for the endocrine center regulating the diapause in pharate first-instar *A. yamamai*, we examined endocrine cells and the innervation of the midgut, which is a very conspicuous organ extending from the mesothorax to the 5th abdominal segment. We verified the presence of gut innervation and gut endocrine cells and tried to detect changes in the contents of hormone-like materials during diapause and its termination. We report that gut innervation and gut endocrine cells are concentrated in two discrete regions, similarly as found for the two factors that regulate diapause.

MATERIAL AND METHODS

Animals

The eggs of *A. yamamai* (Lepidoptera: Saturniidae) were obtained as described by Suzuki et al. (1990). Immunohistochemistry was applied to pre-diapausing pharate first-instar larvae (8 days after oviposition, before swallowing yolk cells), diapausing pharate first-instar larvae (one month at 25°C), pharate first-instar larvae (48 to 24 h before hatching), and newly hatched larvae (within 2 h of eclosing) (Naya et al., 1994a).

Dissection and fixation

Animals were anesthetized over dry ice and secured dorsal side up (for paraffin sections) or ventral side up (for whole mounts) on a wax-lined dish. Dissections were carried out under cold *Bombyx* saline (Narahashi, 1963). The integument was cut open along the longitudinal body axis and pinned to the wax. To prevent curling, the flattened animal with an exposed gut was fixed with a droplet of Bouin's fluid for 5 s and immediately washed 3 times with saline. Then, the gut wall was cut along the longitudinal axis and gut contents were removed. For paraffin sections, the preparation was transferred to Bouin's fixative for 6–12 h, then dehydrated, cleared and embedded. Sections were cut sagittally at 7 μm, mounted on those slides and dried for 20–24 h at 37°C. For the whole mount immunostaining, the opened and cleaned gut was dissected from the animal in cold saline, fixed in Bouin's fixative for 4 h at room temperature, and then washed with cold 70% ethanol overnight.

Immunohistochemistry

Paraffin sections and whole mounts were processed for immunostaining with the avidin-biotin peroxidase complex (ABC) method. Rabbit antisera against FMRFamide (1 : 1,500, Affiniti Research Products, Exeter, United Kingdom), substance P (SP) (1 : 24,000, Incstar, Stillwater, USA), CCK-8 (1 : 20,000, Incstar), PP (1 : 2,000, Peninsula Laboratories, Belmont, USA), VIP (1 : 1,000, Incstar) and Somatostatin (1 : 1,000, Funakoshi, Tokyo, Japan), were used as primary antibodies.

Whole mount staining: Tissues were washed in 15-min intervals in 70% ethanol, 95% ethanol with 0.1% H_2O_2, and 70% ethanol, and then rinsed several times in cold phosphate-buffered saline (PBS) (pH 7.25) containing 1% Triton X-100 (PBST), until the yellow fixative was completely removed. They were then pre-incubated in 10% normal goat serum in PBST for 1 h at 32°C, washed three times for 15-min in PBST, and incubated with a primary rabbit antiserum for 24–48 h at 4°C. After three 15-min washings with cold PBST, the biotinylated goat anti-rabbit IgG (dilution, 1 : 600, Vector, Burlingame, USA) was applied for 1 h at 32°C. Afterwards, the tissues were rinsed again 3 times with cold PBST (15 min each time) and incubated with ABC for 1 h at 32°C. Three final washings (15 min each time) were done with

cold PBS and once with 0.05 M Tris-HCl (pH 7.6) at room temperature. Staining was done with 0.05 M Tris-HCl (pH 7.6) containing 0.05% 3-3′ diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 for 10 min. The staining reaction was stopped by PBS; the tissues were then dehydrated, cleaned and mounted.

Paraffin sections: The immunostaining steps were identical with the whole mount staining, but incubations were shortened to 40 min and washings to 5 min. Staining was done with 0.05 M Tris-HCl (pH 7.6) containing 0.01% DAB and 0.003% H_2O_2 for 10 min.

The specificity of positive staining was verified by replacing specific antisera with normal rabbit serum (Andries & Tramu, 1985) or with PBS.

Vital staining

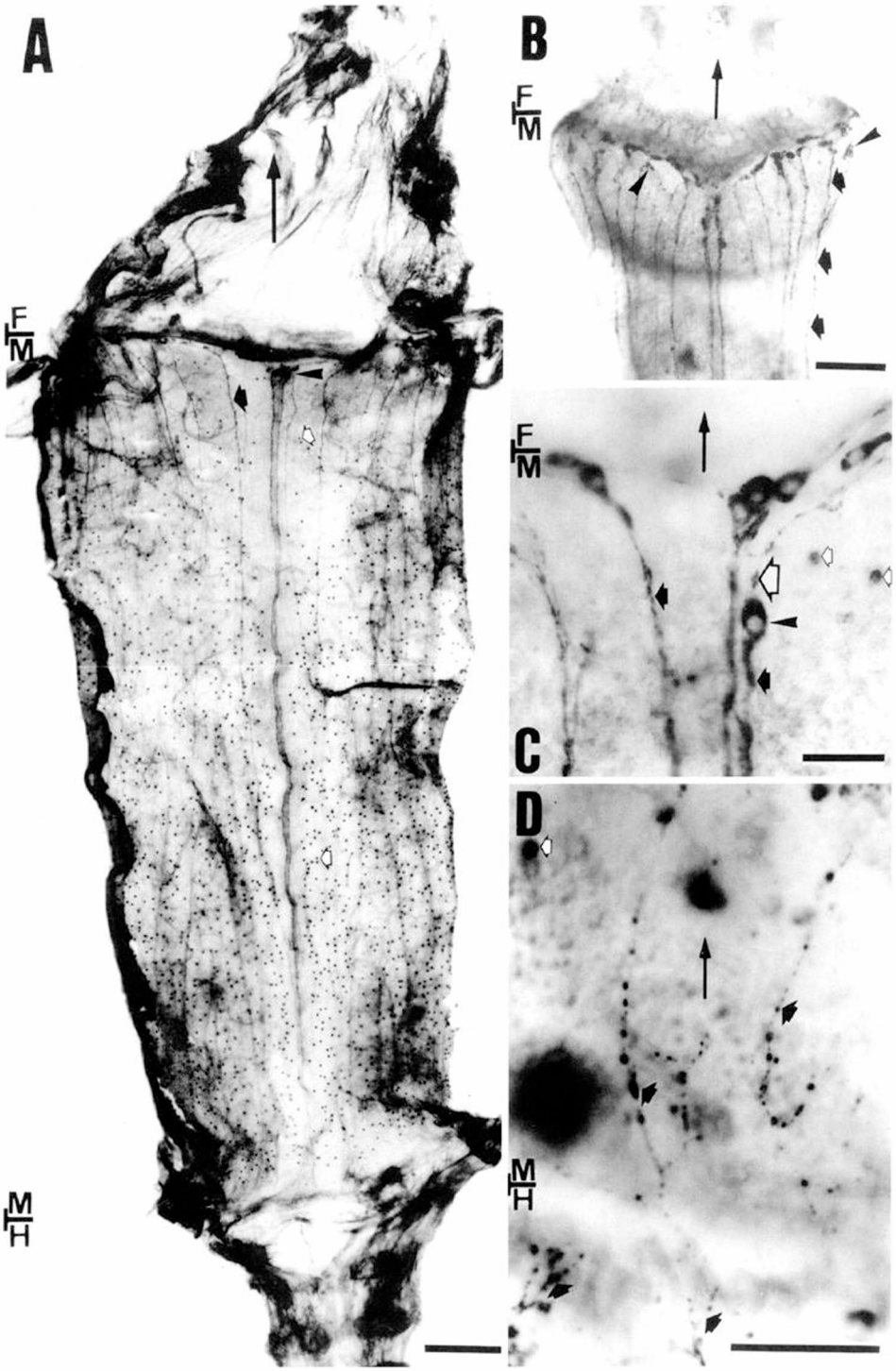
The enteric nervous system of pharate first-instar larvae was stained by intra-vital injection of 0.4% methylene blue in *Bombyx* saline. Glass needles were prepared from micro-pipette capillaries (Drummond Scientific Co., USA) and inserted laterally between the seventh and eighth abdominal segments; 2.5 µl of 0.4% methylene blue per individual was injected into the coelom. The injected animal was immediately ligated with dental floss across the seventh abdominal segment and stained for 8 h at room temperature. Dissection of the gut from the ventrum was carried out as described above, but was performed under 4% ammonium molybdate solution. The cleaned and flattened gut wall was fixed in fresh 4% ammonium molybdate for 4 h at room temperature, dehydrated, cleared and mounted.

RESULTS

We first examined the distribution of FMRFamide-like peptides in the gut innervation and the endocrine cells of diapausing pharate, and post-diapausing newly-hatched first-instar larvae. No immunopositive cell bodies and fibres were found in the foregut (Fig. 1A–C). The anterior region of the midgut near the foregut-midgut boundary, however, contained 90–100 FMRFamide-like immunoreactive bipolar neurons that send axons along the surface of the midgut longitudinal muscles (Figs. 1A–C and 2A, B arrowheads). Axons of the dorso- and ventro-medial gastric nerves ran along the length of the midgut to a neurohaemal area at the midgut-hindgut boundary (Fig. 1A). On the other hand, the axons of the dorso- and ventro-lateral nerves terminated on the surface of muscles in the central part of the midgut. A few FMRFamide-like immunopositive nerve fibers extended from hindgut muscles over the posterior midgut (Fig. 1D). No change in the distribution and the degree of immunostaining were found by comparing developmental stages from diapause initiation to hatching (Figs 1A, B and 2). This result suggests that gut innervation is formed before diapause initiation (8 days after oviposition, before swallowing yolk cells).

FMRFamide-like immunoreactive cells, were present throughout the midgut epithelium but appeared to be more numerous in the middle and posterior parts of the midgut; only a few were detected in the anterior part (Fig. 1A, C, small white arrows). The FMRFamide-like immunoreactive cells were small, with rounded or pyramidal-shaped cytoplasm around the basal nucleus, and most had a long thin extension to the gut lumen (Fig. 4A).

SP-, CCK-8- and PP-like immunoreactive cells, but no cells or nerve fibers reacting with the polyclonal antibodies against VIP and somatostatin, were detected in the whole mounts of midgut. The distribution pattern of CCK-8-, PP-like immunoreactive cells was similar to that observed for FMRFamide-like immunoreactive cells (Fig. 3A, the distribution pattern of CCK-8-like immunoreactive cells not shown). Localization of the SP-like immunoreactive cells was different; the cells appeared to be more numerous near the foregut-midgut boundary and in the posterior part of the midgut (Fig. 3B).


A
FM
MH
B
FM
C
FM
D
MH

Paraffin section revealed the presence of open-type endocrine cells which extended from the basal lamina to the lumen, and were dispersed in the midgut epithelium (Fig. 4A, B, D). Both the whole mounts and sections showed that immunoreactive endocrine cells and neurons occurred mostly in the midgut region where the MF factor controlling post-diapause seems to be produced. By contrast, no immunopositive cell bodies and fibers were observed on the foregut surface, i.e. in body region where the RF factor is localized (Fig. 5). When the gut was stained by methylene blue, however, an enteric plexus of nerves originating from the frontal ganglion was observed on the foregut surface (Fig. 5).

No changes in the distribution and the degree of staining of immunoreactive cells and midgut innervation were found from diapause to hatching (data not shown). SP-, CCK-8-, PP- and FMRFamide-like immunoreactive cells were also detected in the abdominal ganglion of *A. yamamai* (Fig. 4A, C, white arrows, the data of SP-, CCK-8-like materials not shown). This supports the assertion that the examined antigens deserve to be called brain-gut peptides.

DISCUSSION

It has been amply demonstrated that endocrine cells localized in the insect midgut produce antibodies that react to a number of vertebrate and invertebrate bioactive peptides (Sehnal & Žitňan, 1990). In the present study, we reported that the PP-, CCK-8- and FMRFamide-like immunoreactive cells in the midgut of *A. yamamai* are unevenly distributed (Figs 1, 3 and 4). Their accumulation in the middle and posterior midgut sections resembles the distribution of midgut endocrine cells in *Aeshna cyanea* (Odonata) (Andries & Tramu, 1985) and some other insects (Žitňan et al., 1993; Sehnal & Žitňan, 1996). It is noteworthy that endocrine cells in *A. yamamai* midgut are most numerous in the region of the 2nd to 5th abdominal segments, i.e. in the body section where the MF controlling post-diapause is produced (Suzuki et al., 1991).

When we observed serial sections, we unexpectedly found the PP-, FMRFamide-like immunoreactivity in both the midgut epithelia and the 3rd abdominal ganglion (Fig. 4A, C). This observation guided us to other parts of CNS of *A. yamamai*. The results showed that the four kinds of immunoreactive materials distributed in the midgut are also common in the CNS (data not shown). Hence, the pharate first-instar larvae of *A. yamamai* display a similar brain-gut distribution of regulatory peptides as was shown in the blowfly *Calliphora vomitoria* (Duve & Thorpe, 1982), the mosquito *Aedes aegypti* (Brown & Lea, 1988), the blood-feeding bug *Rhodnius prolixus* (Tsang & Orchard, 1991) and other insects. The term brain/gut peptides is fully justified (East et al., 1995).

Fig. 1. FMRFamide-like immunoreactivity in the surface view of whole-mounted gut of pre-diapausing and diapausing pharate first-instar larvae of *A. yamamai*. A – the gut of diapausing pharate first-instar larvae; B – the foregut-midgut boundary of pre-diapausing pharate first-instar larvae, before swallowing extra-embryonic yolk cells; C – expanded from the ventral side of B; D – the immunoreactive nerve fibers on the surface of the midgut-hindgut boundary of diapausing pharate first-instar larvae. F/M indicates the foregut-midgut, and M/H indicates the midgut-hindgut boundaries. Long arrows show a longitudinal body axis. Arrowheads indicate the FMRFamide-like immunoreactive bipolar neurons at the anterior part of the midgut near the foregut-midgut boundary. Black arrows indicate axons, and small white arrows the FMRFamide-like immunoreactive endocrine cells in the midgut epithelium. Scale bars: A, D – 265 μm; B – 145 μm; C – 200 μm.

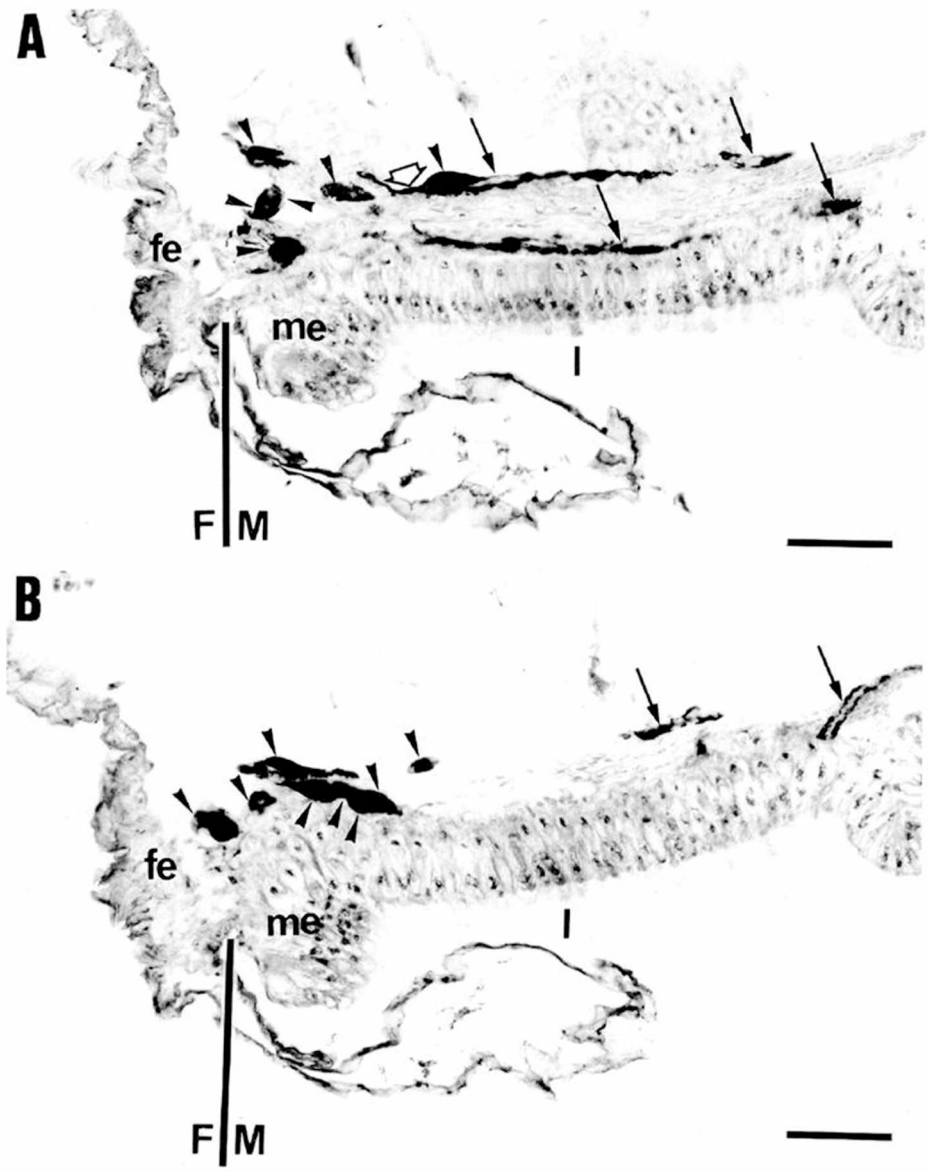


Fig. 2. FMRFamide-like immunoreactive bipolar neurons in the foregut-midgut boundary of a newly hatched first-instar larvae. A and B are serial sections. F/M indicates the foregut-midgut boundary. Arrowheads indicate immunoreactive bipolar neurons. White arrow indicates dendrite (in A). Arrows indicate axons. fe – foregut epithelium; me – midgut epithelium; lu – lumen; 8 μm sections counterstained with methylene blue. Scale bars – 60 μm.

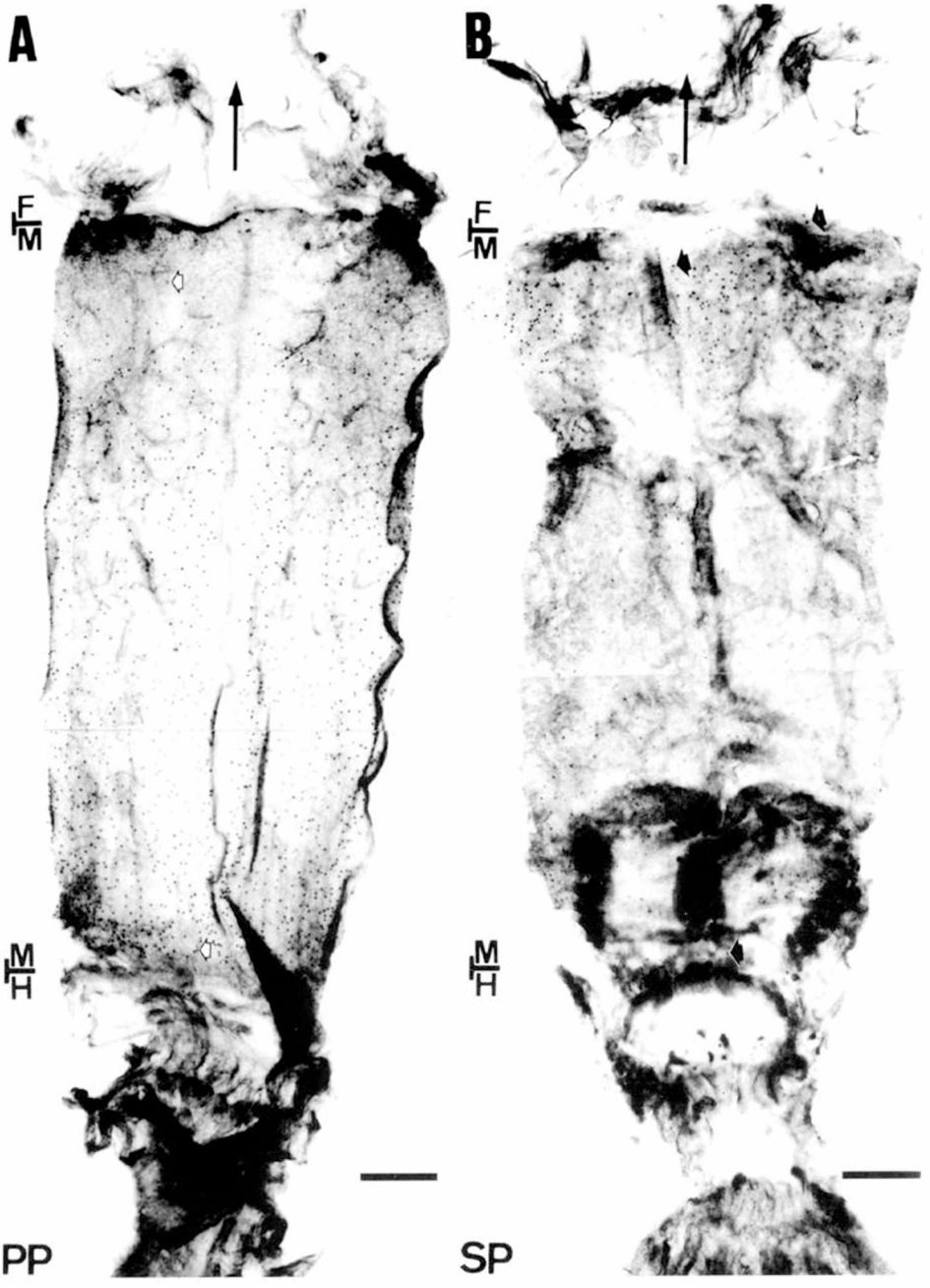


Fig. 3. Distribution pattern of PP- and SP-like immunoreactive midgut cells in the surface view of whole-mounted gut of diapausing pharate first-instar larvae. F/M indicates the foregut-midgut boundary. M/H indicates the midgut-hindgut boundary. Long arrows indicate the longitudinal body axis. White (A) and dark (B) arrows show PP-like and SP-like immunoreactive midgut endocrine cells, respectively. Scale bars – 265 μm.

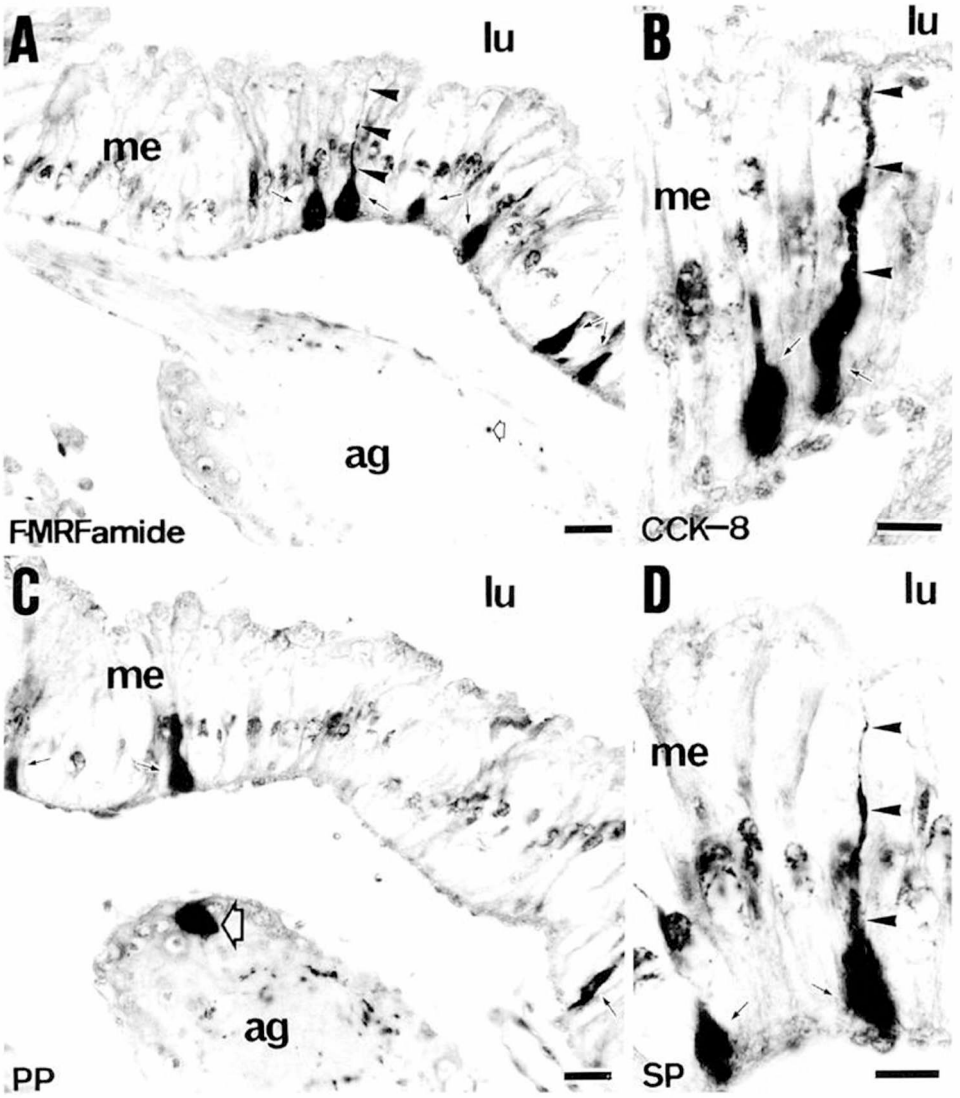


Fig. 4. FMRFamide-, CCK-8-, PP- and SP-like immunoreactivities in the midgut epithelium of the newly hatched first-instar larvae of *A. yamamai*. A – FMRFamide-; B – CCK-8-; C – PP-; D – SP-like immunoreactivity. Arrows show the midgut immunoreactive cells. Arrowheads show the part of midgut endocrine cell's long and thin extension toward to lumen (in A, B and D). The small white arrow shows FMRFamide-like immunoreactive materials in the 3rd abdominal ganglion (in A). Big white arrow shows PP-like immunoreactive cell in the 3rd abdominal ganglion (in C). me – midgut epithelium; lu – lumen; ag – abdominal ganglion; 8 μm sections counterstained with methylene blue. Scale bars: A, C – 20 μm; B, D –10 μm.

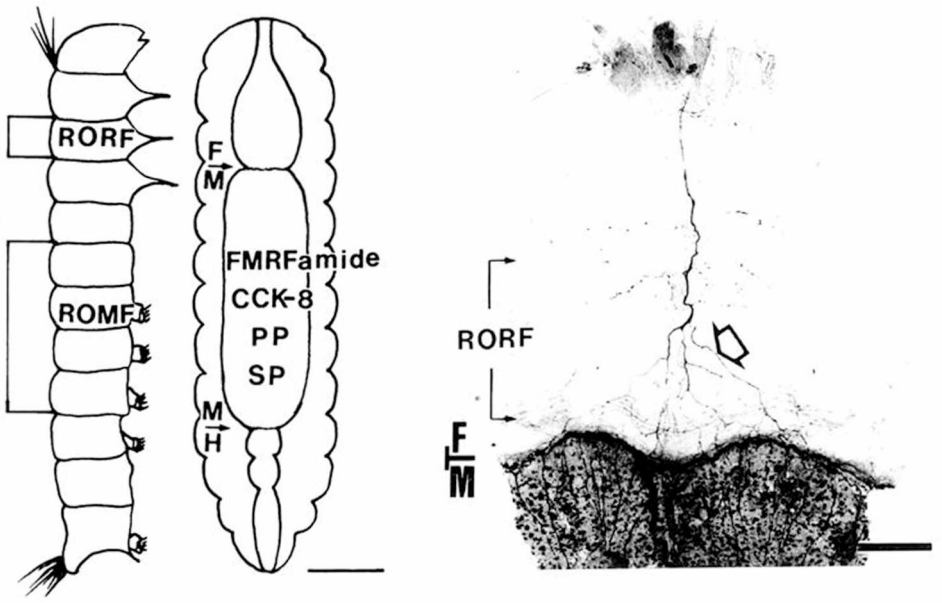


Fig. 5. Schemes of the regions where the RF and MF factors originate (left – a body profile; center – a dorsal view with gut outline and the foregut innervation in the region of RF; right – the frontal ganglion and the enteric nervous system are stained by intra-vital injection of methylene blue). The midgut from the 2nd to 5th abdominal segments, where the MF is generated, is a rich source of peptide hormones FMRFamide, CCK, PP, and SP. RORF indicates the region of RF, ROMF the region of MF. F/M marks the foregut-midgut boundary and M/H the midgut-hindgut boundary. A white arrow indicates the enteric plexus in the region of RF. Scale bars: 1 mm in the schemes; 265 μm in the histological preparation (right).

A midgut hormone of unknown structure is released from the midgut of liver-fed *Phormia regina* adult females and stimulates brain neurosecretory cells that control the beginning of oogenesis (Yin et al., 1994). Yu et al. (1995) suggested that cockroach midgut serves as a supplementary source of allatostatins, which are released in dependence on the nutritional status.

Immunostained midgut cells of *A. yamamai* are adjacent to the basal lamina and exhibit apical extensions toward the lumen (Fig. 4A, B, D). This feature is similar to that described for open type midgut endocrine cells in other insect species (Endo & Nishiitsutsuji-Uwo, 1981; Iwanaga et al., 1981; Žitňan et al., 1993; Yu et al., 1995). Our present results indicate that the pharate first-instar larval midgut is a rich source of peptide hormones, which are produced in both open type and closed type endocrine cells.

FMRFamide was originally isolated from the clam *Macrocallista nimbosa* (Price & Greenburg, 1977), but immunohistochemical and analytical studies showed that FMRFamide-like peptides are common in the central nervous system as well as in the endocrine cells and innervation of insect midgut (Brown & Lea, 1988; Sehnal & Žitňan, 1990; Žitňan et al. 1995). Veenstra & Lambrou (1995) successfully isolated and

sequenced the major (FM)RFamide-immunoreactive peptide from the midgut of the American cockroach *Periplaneta americana*. We observed FMRFamide-like immunoreactivity in the innervation of the midgut and the hindgut (Fig. 1), suggesting that it is generated both in the enteric and in the proctodeal nervous systems. The distribution pattern of FMRFamide-like immunoreactive neurons in the midgut innervation of *A. yamamai* is similar to that in *M. sexta* (Žitňan et al., 1995). A high density of FMRFamide-like bipolar neurons at the foregut-midgut boundary is reminiscent of enteric nervous system development in the embryos of *M. sexta* (Copenhaver & Taghert, 1989a, b). However, we did not observe immunoreactive fibers originating from the frontal and hypocerebral ganglia. Our immunohistochemical investigation in the midgut of *A. yamamai* offers support for Penzlin's view that midgut innervation emanates from the stomodeal nervous system, the arrangement of which is characteristic for insect orders or sub-orders (Penzlin, 1985).

Žitňan et al. (1995) indicated that developmental arrest of parasitized *M. sexta* larvas is associated with accumulation of the FMRFamide-like peptide in the gut nervous and endocrine systems. Our immunoreactive data did not reveal any accumulation of the FMRFamide-like peptides during diapause. We showed previously that the content of eclosion-like material is also unaffected by diapause (Naya et al., 1994a). This indicates that MF in *A. yamamai* is not identical with the detected FMRFamide-like material.

Pharate first-instar larvae of *A. yamamai* are fully formed 8 days after oviposition. The next day they swallow the extra-embryonic yolk cells and their further development is blocked (Sakate, 1984; Suzuki et al., 1990). The disappearance of extra-embryonic yolk cells may be one of the primary physiological changes associated with *A. yamamai* diapause, and it is completely different from the gypsy moth, *Lymantria dispar* (Suzuki et al., 1993; Lee & Denlinger, 1996). Diapause-related proteins in the midgut and hindgut of pharate first instar larvae of the gypsy moth were described (Lee & Denlinger, 1996, 1997).

Our present results indicate that the midgut section located in the 2nd to 5th abdominal segments, where a maturation factor (MF) is produced (Suzuki et al., 1990), is a rich source of peptide hormones. An abundance of SP-, PP-, CCK-8- and FMRFamide-like peptides in the midgut suggests that they play an important function in digestion throughout post-diapause development. Their relationship to the diapause-regulating factors RF and MF is unclear because the factors have not yet been isolated. In future examination, we will continuously investigate the gut innervation and endocrine system in the segments that produce RF and MF (Fig. 5).

ACKNOWLEDGEMENTS. We are grateful to F. Sehnal of the Institute of Entomology, Czech Academy of Sciences, for the critical reading of the manuscript. We also thank K. Taniguchi and O. Ogawa, Department of Veterinary Anatomy, for many helpful discussions and advices. This work was supported by Grants-in-Aids for Scientific Research (Nos. 09265202 and 08556010) from the Ministry of Education, Science and Culture of Japan and Grants-in-Aid of Integrated research program on the development of insect technology from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

REFERENCES

ANDRIES J.C. & TRAMU G. 1985: Distribution patterns of mammalian-like peptide immunoreactive cells in the midgut of Aeshna cyanea (Insecta, Odonata). *Experientia* **41**: 500–503.

BROWN M.R. & LEA A.O. 1988: FMRFamide- and adipokinetic hormone-like immunoreactivity in the nervous system of the mosquito, Aedes aegypti. *J. Comp. Neurol.* **270**: 606–614.

BROWN M.R., CRIM J.W. & LEA A.O. 1986: FMRFamide- and pancreatic polypeptide-like immunoreactivity of endocrine cells in the midgut of a mosquito. *Tissue Cell* **18**: 419–428.

COPENHAVER P.F. & TAGHERT P.H. 1989a: Development of the enteric nervous system in the moth. I. Diversity of cell types and the embryonic expression of FMRFamide-related neuropeptides. *Dev. Biol.* **131**: 70–84.

COPENHAVER P.F. & TAGHERT P.H. 1989b: Development of the enteric nervous system in the moth. II. Stereotyped cell migration precedes the differentiation of embryonic neurons. *Dev. Biol.* **131**: 85–101.

DUVE H. & THORPE A. 1982: The distribution of pancreatic polypeptide in the nervous system and gut of the blowfly, Calliphora vomitoria (Diptera). *Cell Tissue Res.* **227**: 67–77.

EAST P.D., THORPE A. & DUVE H. 1995: Leu-callatostatin gene expression in the blowflies Calliphora vomitoria and Lucilia cuprina studied by in situ hybridisation: comparison with Leu-callatostatin confocal laser scanning immunocytochemistry. *Cell Tissue Res.* **280**: 355–364.

ENDO Y. & NISHIITSUTSUJI-UWO J. 1981: Gut endocrine cells in insect: The ultrastructure of the gut endocrine cells of the lepidopterous species. *Biomed. Res.* **2**: 270–280.

FUJITA T., YUI R., IWANAGA T., NISHIITSUTSUJI-UWO J., ENDO Y. & YANAIHARA N. 1981: Evolutionary aspects of "brain-gut peptides": An immunohistochemical study. *Peptides (Suppl. 2)* **2**: 123–131.

IWANAGA T., FUJITA T., NISHIITSUTSUJI-UWO J. & ENDO Y. 1981: Immunohistochemical demonstration of PP-somatostatin-, enteroglucagon- and VIP-like immunoreactivities in the cockroach midgut. *Biomed. Res.* **2**: 202–207.

JENKINS A.C., BROWN M.R. & CRIM J.W. 1989: FMRFamide immunoreactivity and the midgut of the corn earworm (Heliothis zea). *J. Exp. Zool.* **252**: 71–78.

LEE K.Y. & DENLINGER D.L. 1996: Diapause-regulated proteins in the gut of pharate first instar larvae of the gypsy moth, Lymantria dispar, and the effect of KK-42 and neck ligation on expression. *J. Insect Physiol.* **42**: 423–431.

LEE K.Y. & DENLINGER D.L. 1997: A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, Lymantria dispar. *J. Insect Physiol.* **43**: 289–296.

NARAHASHI T. 1963: The properties of insect axones. *Adv. Insect Physiol.* **1**: 175–256.

NAYA S., SUZUKI K., FUGO H. & SEHNAL F. 1994a: Eclosion hormone-like immunoreactivity in the nervous system of Bombyx mori (Lepidoptera: Bombycidae) and Antheraea yamamai (Lepidoptera: Saturniidae) before and after hatching . *Eur. J. Entomol.* **91**: 189–196.

NAYA S., SUGISAKA A. & SUZUKI K. 1994b: Identification of a maturation factor inducing post-diapause development in pharate first-instar larvae of the wild silkmoth, Antheraea yamamai. *Int. J. Wild Silkmoth Silk* **1**: 195–200.

PENZLIN H. 1985: Stomatogastric nervous system. In Kerkut G.A. & Gilbert L.I. (eds): *Comprehensive Insect Physiology, Biochemistry and Pharmacology. Vol. 5.* Pergamon Press, Oxford, pp. 371–406.

PRICE D.A. & GREENBURG M.J. 1977: Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**: 670–671.

SAKATE S. 1984: Hibernation of the eggs of the Japanese oak silkworm. *Tech. Bull. Seric. Exp. Stn.* **123**: 31–46 (in Japanese).

SCHOLS D., VERHAERT P., HUYBRECHTS R., VAUDRY H., JEGOU S. & DE LOOF A. 1987: Immunocytochemical demonstration of proopiomelanocortin- and other opioid-related substances and a CRF-like peptide in the gut of the American cockroach, Periplaneta americana L. *Histochemistry* **86**: 345–351.

SEHNAL F. & ŽITŇAN D. 1990: Endocrines of insect gut. In Epple A., Scanes C.G. & Stetson M.H. (eds): *Progress in Comparative Endocrinology.* Wiley-Liss, New York, pp. 510–515.

SEHNAL F. & ŽITŇAN D. 1996: Midgut endocrine cells. In Lehane M.J. & Billingsley P.F. (eds): *Biology of the Insect Midgut.* Chapman & Hall, London, pp. 55–85.

SUZUKI K., MINAGAWA T., NAYA S., ENDO Y., OSANAI M. & KUWANO E. 1990: Control mechanism of diapause of the pharate first-instar larvae of the silkmoth Antheraea yamamai. *J. Insect Physiol.* **36**: 855–860.

SUZUKI K., NAYA S., KUMAGAI T., MINAKAWA T., FUJISAWA T. & KUWANO E. 1991: The mode of action of KK-42 on diapause breakdown in pharate first instar larvae of the wild silkmoth, Antheraea yamamai. In Akai H. & Kiuchi M. (eds): *Wild Silkmoths 89' 90'.* International Society for Wild Silkmoths, Tokyo, pp. 73–79.

SUZUKI K., NAKAMURA T., YANBE T., KURIHARA M. & KUWANO E. 1993: Termination of diapause in pharate first-instar larvae of the gypsy moth Lymantria dispar japonica by an imidazole derivative KK-42. *J. Insect Physiol.* **39**: 107–110.

TSANG P.W. & ORCHARD I. 1991: Distribution of FMRFamide-related peptides in the blood-feeding bug, Rhodnius prolixus. *J. Comp. Neurol.* **311**: 17–32.

VEENSTRA J.A. & LAMBROU G. 1995: Isolation of a novel RFamide peptide from the midgut of the American cockroach, Periplaneta americana. *Biochem. Biophys. Res. Commun.* **213**: 519–524.

YIN C.-M., ZOU B.-X., LI M.-F. & STOFFOLANO J.G., JR. 1994: Discovery of a midgut peptide hormone which activates the endocrine cascade leading to oogenesis in Phormia regina (Meigen). *J. Insect Physiol.* **40**: 283–292.

YU C.G., STAY B., DING Q., BENDENA W.G. & TOBE S.S. 1995: Immunochemical identification and expression of allatostatins in the gut of Diploptera punctata. *J. Insect Physiol.* **41**: 1035–1043.

ŽITŇAN D., ŠAUMAN I. & SEHNAL F. 1993: Peptidergic innervation and endocrine cells of insect midgut. *Arch. Insect Biochem. Physiol.* **22**: 113–132.

ŽITŇAN D., KINGAN T.C. & BECKAGE N.E. 1995: Parasitism-induced accumulation of FMRFamide-like peptides in the gut innervation and endocrine cells of Manduca sexta. *Insect Biochem. Molec. Biol.* **25**: 669–678.

Received September 15, 1997; accepted December 12, 1997