FMRFamide-Expressing Efferent Neurons in Eighth Abdominal Ganglion Innervate Hindgut in the Silkworm, Bombyx mori

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FMRFamide-Expressing Efferent Neurons in Eighth Abdominal Ganglion Innervate Hindgut in the Silkworm, *Bombyx mori*

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ABSTRACT—The tetrapeptide FMRFamide is known to affect both neural function and gut contraction in a wide variety of invertebrates and vertebrates, including insect species. This study aimed to find a pattern of innervation of specific FMRFamide-labeled neurons from the abdominal ganglia to the hindgut of the silkworm *Bombyx mori* using the immunocytochemical method. In the 1st to the 7th abdominal ganglia, labeled efferent neurons that would innervate the hindgut could not be found. However, in the 8th abdominal ganglion, three pairs of labeled specific efferent neurons projected axons into the central neuropil to eventually innervate the hindgut. Both axons of two pairs of labeled cell bodies in the lateral rind and axons of one pair of labeled cell bodies in the posterior rind extended to the central neuropil and formed contralateral tracts of a labeled neural tract with a semi-circular shape. These labeled axons ran out to one pair of bilateral cercal nerves that extended out from the posterior end of the 8th abdominal ganglion and finally to the innervated hindgut. These results provide valuable information for detecting the novel function of FMRFamide-related peptides in metamorphic insect species.

Key words: FMRFamide, efferent neurons, 8th abdominal ganglion, hindgut, silkworm

INTRODUCTION

The FMRFamide (Phe-Met-Arg-Phe-NH_2) peptide was originally isolated and characterized as a cardioexcitatory agent from the bivalve mollusc *Macrocallista nimbosa* (Price and Greenberg, 1977). Since that report, many bioactive peptides with the signature "-RFamide" in the C-terminus have been described from nearly all groups of invertebrates and vertebrates (reviewed by Greenberg and Price, 1992; Mercier *et al*., 2003), including coelenterates (Grimmelikhuijzen and Graff, 1985), worms (Maule *et al*., 1996), molluscs (Santama *et al*., 1995), insects (White *et al*., 1986; Schneider *et al*., 1993a; Lange *et al*., 1994; Nichols *et al*., 1999; Taghert, 1999), and other arthropods (Huybrechts *et al*., 2003) and vertebrates (Dockray *et al*., 1983; Perry *et al*., 1997). In fact, experiments using antiserum developed against the molluscan FMRFamide have shown that nearly all animals have different cells that express FMRFamide-related peptides (Schneider and Taghert, 1990).

Price and Greenberg (1989) suggested that peptides with the common sequence "F(M/L/I)Rfamide" were probably homologous to FMRFamide. Later, they modified their suggestion to come up with a stricter criterion that "F(M/L)Rfamide" (Price and Greenberg, 1994).

About 13 FMRFamide-related peptides were predicted to be derived from a polypeptide precursor of the peptide cDNA of *Drosophila melanogaster* (Schneider and Taghert, 1990; Nambu *et al*., 1988). To date, 12 FMRFamide-related peptides have been identified from animal species (Mercier *et al*., 2003). FMRFamide has been regarded as a key member of an extensive family of peptides with diverse bio-

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logical functions. Physiological studies have demonstrated that FMRFamide-related peptides have pleiotropic modulatory effects on a variety of target tissues (Schneider and Taghert, 1990). These peptides have potent myotropic effects on the heart, skeletal and visceral muscles, oviduct, and hindgut (Painter and Greenberg, 1982; Cottrell *et al*., 1983; Li and Calabrese, 1987; Cuthbert and Evans, 1989; OBrien *et al*., 1991; Peeff *et al*., 1993; Schoofs *et al*., 1993; Schneider *et al*., 1993b; Robb and Evans, 1990; Lange and Orchard, 1998; Orchard and Lange, 1998; Lange and Cheung, 1999).

Previous studies on the localization or expression of FMRFamide-related peptides have shown that in insect species they are found in a variety of nerve or glandular tissues, such as brain (Homberg *et al*., 1991; Kingan *et al*., 1990), optic lobe (Nässel *et al*., 1988; Homberg and Hildebrand, 1989), ventral nerve cord (Myers and Evans, 1985; Lange *et al*., 1994), and retrocerebral complex (Verhaert *et al*., 1985).

Earlier studies on molluscs or arthropods suggested the modulatory effects of FMRFamide-related peptides on the contraction of the visceral muscles (Lehman and Greenberg, 1987; Mercier *et al*., 1997). In insect species, it has been reported that FMRFamide derived from the abdominal ganglion (AG) shows myotropic activity on the hindgut of the adult blowfly (Cantera and Nässel, 1991). However, little is known about which of the several AGs in insects and what neurons of a given AG have a modulatory effect on the con-

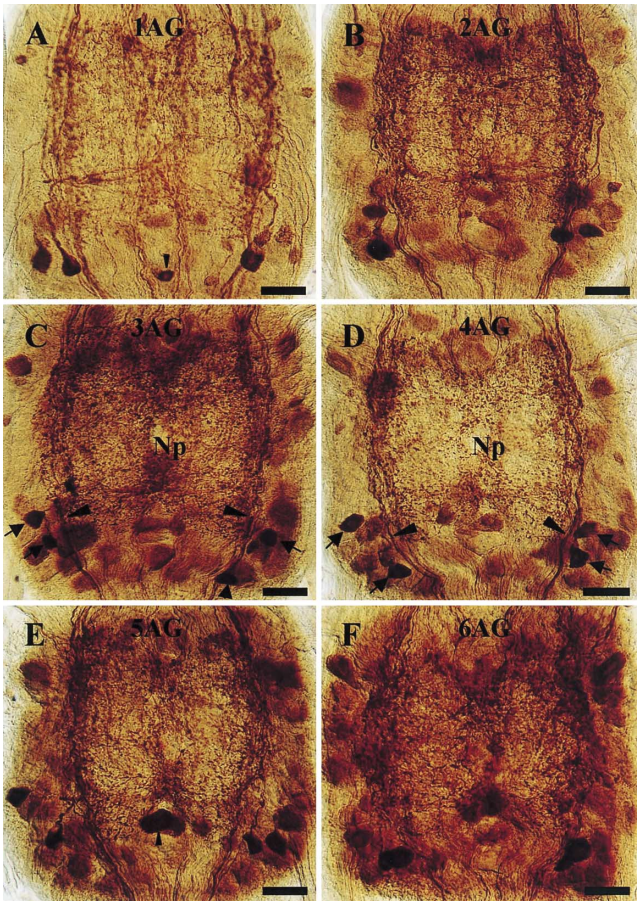


Fig. 1. Photographs of the 1st to the 6th AGs showing the FMRFamide-labeled cell bodies and neurites. From each of the six AGs with similar numbers and locations of FMRFamide-labeled neurons, two pairs of cell bodies (arrows in Figs. 1C for the 3rd AG and 1D for the 4th AG) are strongly stained in the posterior rinds and one pair of bilateral FMRFamide-labeled neurite bundles (larger arrowheads in Figs. 1C for the 3rd AG and 1D for the 4th AG) run from the anterior to the posterior neuropil (Np). All scale bars indicate 30 μm. **A)** 1st AG (1AG) containing a cell body (smaller arrowhead) of a DUM neuron; **B)** 2nd AG (2AG); **C)** 3rd AG (3AG); **D)** 4th AG (4AG); **E)** 5th AG (5AG) including a cell body (smaller arrowhead) of a DUM neuron; **F)** 6th AG (6AG). These six AGs were not the focus of this investigation, because they did not include the nerves between the AGs and the hindgut.

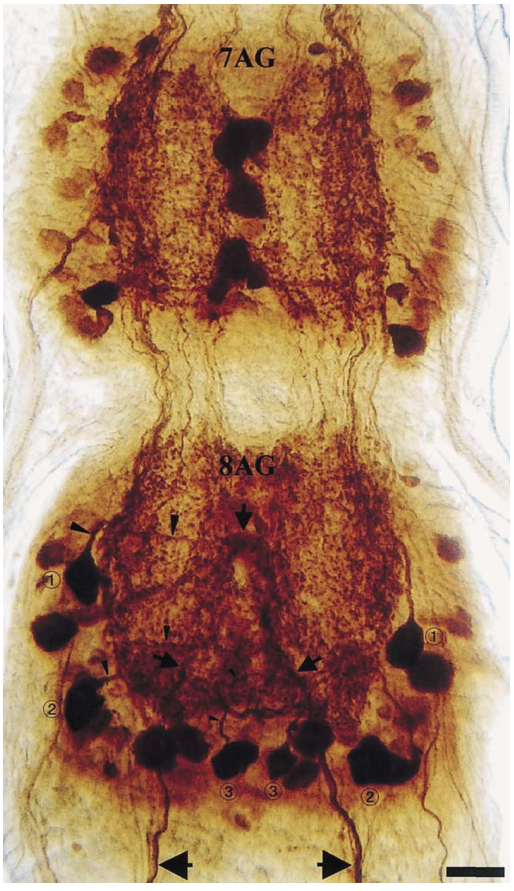


Fig. 2. Photograph of a fused ganglion of the 7th/8th AGs showing projection of FMRFamide-labeled efferent neurons into a neural tract with semi-circular shape in the central neuropil. In the 8th AG (8AG), three pairs of labeled neuronal cell bodies (①, ②, ③) project their axons (two large arrowheads in the axon from a cell body indicated by ①, two medium-sized arrowheads in the axon of a cell body indicated by ②, and two small arrowheads in the axon of a cell body indicated by ③) into the central neuropil, in which the axons from the cell bodies on the left rind extend to the right tract of an FMRFamide-labeled neural tract (three smaller arrows). One pair of the labeled cercal nerves (two larger arrows in lower part), which run toward the hindgut wall, extend from the two ends of the neural tract in the neuropil. None of the neurons in the 7th AG (7AG) was found to contribute to the organization of the labeled neural tract in the central neuropil of the 8th AG. The scale bar indicates 30 μm.

traction of the hindgut.

In this study, evidence of the innervation of three pairs of FMRFamide-labeled efferent neurons in the 8th AG to the hindgut was obtained with the use of an immunohistochemical method in the silkworm, *Bombyx mori*.

MATERIALS AND METHODS

Animals

Cold-treated eggs of the silkworm, *Bombyx mori* (Lepidoptera, Insecta), obtained from the National Institute of Agricultural Science and Technology (NIAST) in Suwon, Korea, were hatched after about 10 d of incubation at 27–28°C with relative humidity of 60–70%. The hatched larvae were reared on fresh mulberry leaves on a long-day photoperiod regimen (17 h light/7 h dark) at 27°C and about 60% humidity. Most of the larvae developed normally into the 5th (final) instar larvae.

Wholemount immunohistochemistry

Tissue preparation and wholemount immunohistochemistry were performed as described previously by Kim *et al*. (1998) and Park *et al*. (2001, 2002). Following incubation of the 5th instar larvae at 4°C for 1 h, eight AGs and the hindgut were isolated in 0.1 M sodium phosphate buffer (pH 7.4) and then fixed in 4% paraform-

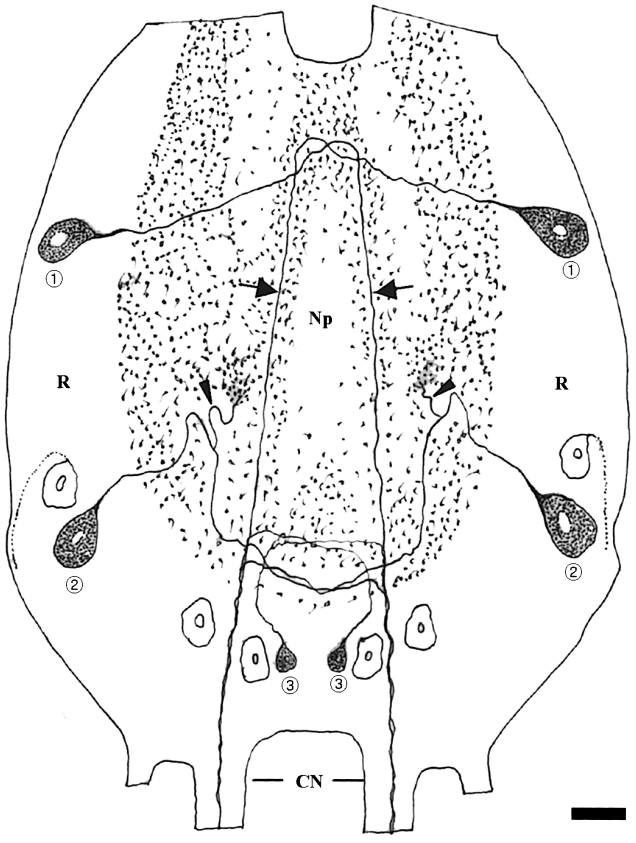


Fig. 3. Schematic drawing of the 8th AG showing the FMRFamide-labeling in three pairs of efferent neurons, a neural tract with semi-circular shape, and one pair of cercal nerves from the 5th instar silkworm. The two pairs of labeled cell bodies (①, ②) in the left or right lateral rind (R) and one pair of labeled cell bodies (③) in the left or right posterior rind project their axons into the central neuropil (Np), in which the labeled axons extend to the contralateral tracts of a FMRFamide-labeled neural tract with semi-circular shape (arrows). One of these three pairs of labeled efferent neurons was found to have dendrites (arrowheads). Axons of these labeled cell bodies on the left or right rind run out of the 8th AG to innervate the hindgut by way of the contralateral cercal nerves (CN) that extend from the posterior end of the 8th AG. A few pairs of empty cell bodies and their neurites, which have the FMRFamide neuropeptide, were not involved in the innervation to the hindgut. The scale bar indicates 20 μm.

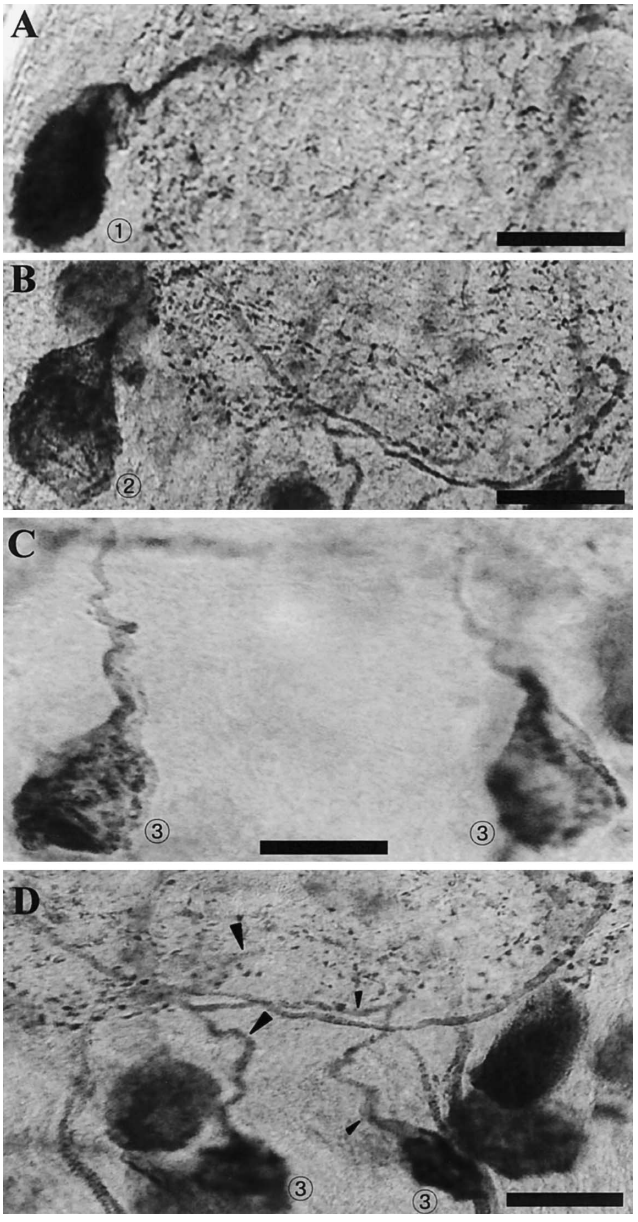
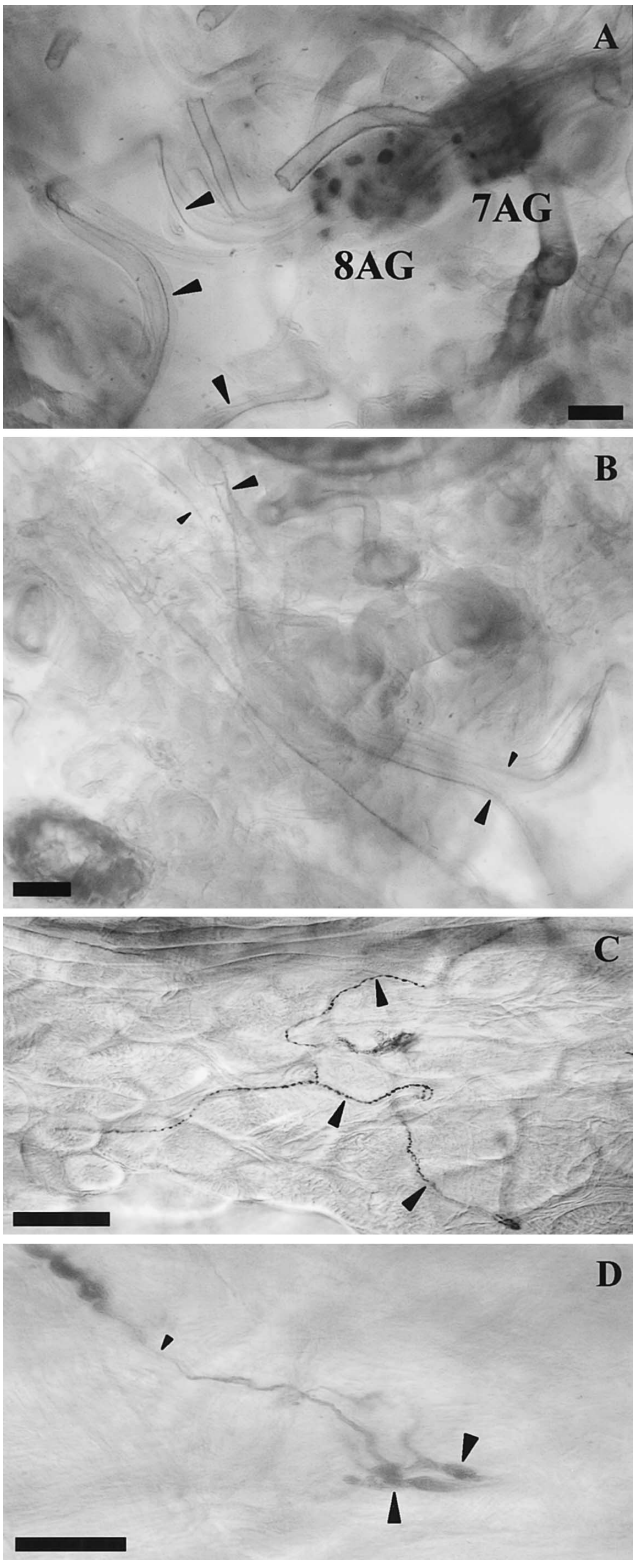


Fig. 4. Magnified photographs of three FMRFamide-labeled efferent neurons that form a neural tract with semi-circular shape in the 8th AG. **A & B)** Two labeled neurons indicated by ① and ② in the 8th AG of Fig. 3. The scale bars indicate 20 μm. **C)** One pair of labeled neurons indicated by ③ in the 8th AG of Fig. 3. The scale bar indicates 15 μm. **D)** The labeled axons (larger or smaller arrowheads) extended from one pair of the labeled cell bodies in the posterior rind of the 8th AG to form the neural tract with semi-circular shape in the neuropil. The labeled axons from the cell bodies show contralateral projections into the posterior neural tract. The scale bar indicates 15 μm.

aldehyde in 0.1 M sodium phosphate buffer for 6 h at 4°C. The isolation and fixation of the eight AGs and hindgut were performed following earlier confirmation in preliminary immunocytochemical experiments that the terminal abdominal ganglion (TAG) had direct nervous connection with the hindgut but there was no connection between the 1st to the 6th AGs and the hindgut. The TAG was a fused large ganglion composed of the 7th and 8th AGs in the 5th instar larva. The hindgut, which was bounded in the malpighian tubules by the midgut, was cleansed with a saline solution immediately after isolation. The fixed eight AGs and hindgut tissues were immersed in 0.01 M phosphate buffer saline with 1% Triton X-100 at 4°C overnight. Blockage of peroxidase activity was performed in 10% methanol in 3% H_2O_2 for 25 min. Washing with 0.1 M Tris-HCl buffer (pH 7.6–8.6) containing 1% Triton X-100 and 4% NaCl was done followed by gentle shaking with a primary antiserum, anti-FMRFamide (Sigma), diluted 1:1000 with dilution buffer (0.01M phosphate buffer saline with 1% Triton X-100 and 10% normal goat serum) for 4–5 d. After washing with 0.01M phosphate buffer saline with 1% Triton X-100, the tissues were incubated in peroxidase-conjugated mouse IgG (DAKO), diluted 1:200 for 2 d at 4°C. Following pre-incubation in 0.03% diaminobenzidine in 0.05 M Tris-HCl buffer for 1 h at 4°C, tissues were treated with 0.03% diaminobenzidine in 0.05 M Tris-HCl buffer containing 0.01% H_2O_2 for 5–10 min. After rinsing with 0.05 M Tris-HCl buffer, tissues were mounted in glycerin, and were examined and photographed with a digital camera attatched to an interference microscope (Zeiss). The FMRFamide-immunolabeled cell bodies and their neurites of the 8th AG were drawn on tracing paper under the microscope with a camera lucida.

To visualize the nerve connections between the AGs and the hindgut, they were exposed by dissection and immersed in the primary antiserum solution for 5 d. The subsequent procedures were the same as described above. Finally, the preparations were examined and photographed using a stereomicroscope equipped a digital camera.



RESULTS

The 1st to the 6th AGs were found to have similar immunoreactivities to FMRFamide, with a slight difference between the 1st and the 6th AGs described later (Fig. 1). There was very clear immunostaining in the two pairs of neuronal cell bodies (arrows in Fig. 1) located in the lateroposterior rinds of all six AGs. In each ganglion, one pair of FMRFamide-labeled neural tracts extended from the anterior to the posterior direction in the boundary between the central neuropils and the rinds (larger arrowheads in Fig. 1). These neural tracts were probably formed in part by neurites projected from two pairs of intensively labeled cell bodies in the lateroposterior rinds (arrows in Fig. 1C, D). However,

Fig. 5. Photographs of FMRFamide-immunoreactive TAG neurons, cercal nerves, their axonal branches and terminals showing projection of 8th AG neurons from TAG to the hindgut wall. These photographs show the transportation of the FMRFamide neuropeptide from the specific neurons of the 8th AG to the hindgut. **A)** Photograph at lower magnification of the 7th AG (7AG)/8th AG (7AG) and the cercal nerve. The FMRFamide-labeled axons (arrowheads), which extend out from the 8th AG neurons, are included in a pair of cercal nerves. The scale bar indicates 80 μm. **B)** FMRFamide-labeled axons in a pair of the cercal nerves. The labeled axons (larger or smaller arrowheads) in the cercal nerves are localized between the 8th AG and the hindgut. The scale bar indicates 80 μm. **C)** FMRFamide-labeled axonal branch (arrowheads) of the cercal nerve on the hindgut wall immediately prior to termination. The scale bar indicates 15 μm. **D)** FMRFamide-labeled axonal branch (smaller arrowhead) of the cercal nerve terminating on the hindgut wall. A few axon terminals (larger arrowheads) are seen in the end of axonal branch. The scale bar indicates 15 μm.

there was also a slight difference in FMRFamide-labelings among these AGs. The 1st and 5th AGs had a strongly labeled neuron in the posterior neuropil regarded as the dorsal unpaired median (DUM) neuron (smaller arrowheads in Figs. 1A, E), whereas this neuron was not found in the 2nd, 3rd, 4th or 6th AGs.

There was no neuronal connection between the 1st to 6th AGs and the hindgut, but all of the six AGs had specific FMRFamide-labeled neuronal cells. This result suggested that none of the 1st to the 6th AGs contribute to the direct myotropic control of the hindgut.

The TAG in the 5th instar larvae of the silkworm consisted of the 7th and 8th AGs. The 7th AG had several intensively labeled neuronal cell bodies that were localized in different patterns from the result described above (Fig. 2). At least one pair of these cell bodies were located in the latero-posterior rind. In particular, one pair of bilateral cell bodies and a few cell bodies that could perhaps be regarded as DUM neurons were localized in the median neuropil. Evidence of FMRFamide-labeled neuronal connection between the 7th AG and the hindgut was not detected.

The 8th AG had specific FMRFamide-labeling that was different from that of the other AGs. As shown in Figs. 2–4, three pairs of efferent neurons, of which cell bodies were located in the 8th AG, formed a morphological connection between the AG and the hindgut. In the 8th AG, two pairs of labeled cell bodies (①, ② in Figs. 2–3) in the lateral rind and one pair (③ in Figs. 2–3) in the posterior rind projected their axons into the contralateral tracts of a labeled neural tract (arrows in Figs. 2–3) with a semi-circular shape in the central neuropil. Dendrites were found from the cell bodies in the middle area of the lateral rind (arrowheads in Fig. 3). The two pairs of efferent cell bodies in the anterior and middle areas of the lateral rind of the 8th AG were medium-sized (about 20 μm in diameter) and intensively labeled (①, ② in Figs. 4A, B). The one pair of efferent cell bodies in the posterior rind were small-sized (about 15 μm in diameter) and moderately labeled (③ in Fig. 4C). Axons of these neurons ran anteriorly to the central neuropil, and then became contralateral tracts of a neural tract in the central neuropil (larger and smaller arrowheads, Fig. 4D). This neural tract was formed only by axons of three pairs of labeled efferent neurons located in the 8th AG.

FMRFamide-labeled axons of the neural tract in the central neuropil projected into one pair of bilateral cercal nerves that extended caudally from the posterior end of the 8th AG (arrowheads in Fig. 5A). Labeled branches of these cercal nerves ran to the hindgut wall (larger and smaller arrowheads in Fig. 5B). Upon approaching the hindgut wall, labeled nerves were divided into axon branches (arrowheads in Fig. 5C), and then each axonal branch formed specific axon terminals that innervated the hindgut wall (Fig. 5D).

DISCUSSION

The AGs, which consist of eight ganglia in the ventral nerve cord, were found to include FMRFamide-labeled cell bodies and neurites. In the 1st to the 6th AGs, each ganglion had two pairs of strongly labeled cell bodies in latero-posterior rinds and one pair of labeled, bilaterally-running neural tracts in the boundary between the neuropil and rind. Therefore, these six AGs all showed a similar pattern in terms of localization of labeled cell bodies and neurites, as shown in Fig. 1. However, a slight difference in labeling could also be traced, that is, only the 1st and the 5th AGs had a DUM cell body that was intensively stained. The 7th AG had specific FMRFamide-labeling that was different from the labeling in the 1st to the 6th AGs (Figs. 1 and 2). In stereomicroscopic observation of nerves between the AGs and the hindgut following immunolabeling of FMRFamide, no labeled nerve was found between the 1st to 7th AGs and the hindgut, suggesting that the movement of *B. mori* hindgut is not directly controlled by nerve fibers projected from efferent neurons in the 1st to 7th AGs.

The 8th AG of TAG had specific FMRFamide-labeling showing that it innervated the hindgut. The morphological and functional connection of AG with the hindgut by FMRFamide-labeled neurons has been reported in the adult blowfly, *Calliphora erythrocephala,* based on a study employing light and electron microscopic immunocytochemistry, using antibody against the FMRFamide (Cantera and Nässel, 1991). The branches from the abdominal nerves include the axons of FMRFamide-like immunoreactive neurons of AG and reach the posterior portion of the gut. These branches eventually innervate the muscle coat of the hindgut with abundant terminals. This FMRFamidergic system derived from the abdominal ganglion uses FMRFamide-like peptide as its transmitter or modulator for contraction control of the visceral muscle in the hindgut. It was suggested that the three pairs of FMRFamide-labeled efferent neurons in the TAG of the silkworm might functionally correspond to the FMRFamide-labeled neurons in the AG that innervate the hindgut wall in the adult blowfly.

Among the eight AGs, only the 8th AG contained three pairs of bilaterally labeled efferent neurons with direct morphological connection to the hindgut, together with other labeled neurons that had no relation to the hindgut (Figs. 2 and 3). These three pairs of labeled efferent neurons formed a labeled neural tract with a semi-circular shape in the central neuropil, in which two bilateral neural tracts were connected with each other, and their labeled axons ran out to the cercal nerves to innervate the hindgut. It has been suggested that the FMRFamide discharged from axon terminals of these specific efferent neurons in the 8th AG might be closely associated with the myotropic control of hindgut, as described in locust (Lange and Cheung, 1999).

Projection of FMRFamide-like peptide-labeled efferent neurons to the hindgut was described earlier in a crustacean. In the crayfish, *Procambarus clarkia*, approximately five axons containing FMRFamide-like immunoreactivity project to the hindgut through the 7th root of the 6th (terminal) AG (Mercier *et al*., 1991). Although these axons could

not be traced to their cell bodies, it is likely that the soma reside in the 6th AG, where the vast majority of hindgut motor neurons originate (Kondoh and Hisada, 1986). It has been demonstrated that crayfish hindgut extracts contain an FMRF-like peptide (pQDVDHVFLRFamide) (Mercier *et al*., 1997) and HPLC fractions containing this peptide enhance spontaneous hindgut contractions (Mercier *et al*., 2003).

In *Locust migratoria*, the TAG and hindgut are also structurally connected by crustacean cardioactive peptide (CCAP)-like immunoreactive neurons of the TAG (Donini *et al*., 2002). The three immunoreactive axons leaving the TAG arrive at the hindgut via the 11th sternal nerve. The CCAP-like substance acts as a neurotransmitter/neuromodulator at the locust hindgut.

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