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In Situ Hybridization Analysis of Leucomyosuppressin mRNA Expression in the Cockroach, *Diploptera punctata*

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ABSTRACT

In the cockroach *Diploptera punctata*, sequencing of the cDNA for the insect myoinhibitory neuropeptide, leucomyosuppressin (LMS), has demonstrated that LMS is the only Phe-Met-Arg-Phe-amide (NH_2) (FMRFamide)-related peptide to be encoded by this gene (Donly et al. [1996] Insect Biochem. Mol. Biol. *26*:627–637). However, in the present study, high performance liquid chromatography analysis of brain extracts showed six discrete FMRFamide-like immunoreactive fractions, one of which co-eluted with LMS. This study compared the distribution of FMRFamide-related peptides visualized by immunohistochemistry with LMS mRNA expression demonstrated by in situ hybridization in *D. punctata*. Immunohistochemistry with a polyclonal antiserum generated against FMRFamide, but which recognizes extended RFamide peptides, demonstrated numerous RFamide-like immunoreactive cells and processes in both nervous and nonnervous tissues. RFamide-like immunoreactivity was found in cells and processes of the brain and optic lobes, the stomatogastric nervous system, including the frontal and ingluvial ganglia, and the suboesophageal ganglion. Immunoreactivity was also present in all ganglia of the ventral nerve cord and in the alimentary canal. Within the alimentary canal, positively stained processes were found in the crop, midgut, and hindgut, and immunoreactive endocrinelike cells were located in the midgut. In situ hybridization with a digoxigenin-labeled RNA probe spanning the entire LMS coding region showed cell bodies containing LMS mRNA in all ganglia studied, other than the ingluvial ganglion. Expression was most abundant in the brain and optic lobes and in the frontal and suboesophageal ganglia. LMS mRNA was also apparent, although less intensely, in all other ganglia of the ventral nerve cord. Within the alimentary canal, LMS mRNA-positive cells were only visible in the anterior portion of the midgut, in the endocrinelike cells. The appearance of LMS mRNA in the central nervous system, stomatogastric nervous system, and midgut suggests that LMS may play a central role in *Diploptera* and may be associated with feeding and digestion. J. Comp. Neurol. 395:328–341, 1998.

Indexing terms: immunohistochemistry; in situ hybridization; myosuppressin; FMRFamide-related peptides; midgut

The tetrapeptide Phe-Met-Arg-Phe-amide (NH_2) (FMRFamide), first isolated from clams (Price and Greenberg, 1977), is only one of a large family of FMRFamide-related peptides (FaRPs). These peptides share the common C-terminus Arg-Phe-NH_2 and occur across a broad range of phyla in the metazoa (Greenberg and Price, 1992). In insects, FaRPs have been shown to affect visceral and skeletal muscle (Walther et al., 1984; Cuthbert and Evans, 1989; Lange et al., 1991; Peeff et al., 1993), neurons (Walther et al., 1984), and glands (Baines and Tyrer, 1989; Yasuyama et al., 1993) and are associated with the central and peripheral nervous systems and with optic lobes, muscle, and visceral tissues (Ohlsson et al., 1989;

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Tsang and Orchard, 1991; Lange et al., 1994; Fusé et al., 1996).

A subfamily of FaRPs, commonly referred to as the myosuppressins (Nachman et al., 1993), includes leucomyosuppressin (LMS; pQDVDHVFLRFamide). LMS was first isolated from the cockroach *Leucophaea maderae* based on its ability to inhibit spontaneously contracting hindgut muscle (Holman et al., 1986). The myosuppressins are decapeptides that share the common amino acid sequence XDVXHXFLRFamide and have since been suggested to have more functions than just the inhibition of visceral muscle contractions (Kingan et al., 1990; Nachman et al., 1996). Members of this family have been found in Orthopterans (Robb et al., 1989; Schoofs et al., 1993; Peeff et al., 1994), Dipterans (Nichols, 1992; Fonagy et al., 1992), Lepidopterans (Kingan et al., 1990; Bendena et al., 1997), and Dictyopterans (Holman et al., 1986; Donly et al., 1996).

In the cockroach *Diploptera punctata*, the first gene found to encode a FaRP myosuppressin has been sequenced (Dip-LMS gene; Donly et al., 1996). Unlike previously characterized FaRP genes or the sulfakinin gene, which have multiple distinct peptides in the deduced prohormone structure, and multiple copies for particular peptide species (Nambu et al., 1988; Nichols et al., 1988; Schneider and Taghert, 1988; Chin et al., 1990; Taghert and Schneider, 1990), the Dip-LMS gene from *D. punctata* has a simple precursor, from which LMS is the only RFamide predicted. Therefore, the significance of the LMS gene is that it produces only the one RFamide, LMS, although it is clear that many more RFamides exist in the animal (Donly et al., 1996). This suggests that more than one type of FaRP gene exists in *Diploptera*. This possibility is of extreme importance in trying to describe the distribution of particular FaRPs, such as LMS, in insects because we can now take advantage of particular transcript sequences to localize individual mRNA expression. Much of the evidence for the presence of FaRPs has been obtained by using immunohistochemical and chromatographic procedures, in which antibodies recognize numerous RFamide-like peptides, including LMS, based on their common antigenic sites. Having previously shown that LMS mRNA in the brain is translated into peptide (Donly et al., 1996), we can take advantage of in situ hybridization to localize LMS mRNA and compare this result with the distribution of other RFamides by using a very general RFamide antiserum (characterized by Tsang and Orchard, 1991).

The present study examines the distribution of LMS mRNA by in situ hybridization and compares it with the distribution of numerous other FaRPs by using immunohistochemistry in tissues of the cockroach, *Diploptera punctata*.

MATERIALS AND METHODS

Insects

The colony of *Diploptera punctata* was maintained on lab chow (Purina) and water at 27°C on a 12:12-hour light:dark cycle, as described previously (Szibbo and Tobe, 1983).

Tissue extraction and HPLC analysis

Four sets of 400 adult mated female *D. punctata* brains (including corpora cardiaca and corpora allata) were dissected under physiological saline and processed as described by Donly et al. (1996). Brain extracts were chromatographed sequentially by using two high performance liquid chromatography (HPLC) columns with different acetonitrile gradients. Fractions were screened for RFamide-positive material by using radioimmunoassay (RIA) with a polyclonal rabbit–anti-FMRFamide antiserum, as previously described (Peeff et al., 1993).

System 1. A Brownlee RP-C_{18} Spheri-5 column (46 mm × 25 cm), with a linear acetonitrile gradient (containing 0.1% triflouroacetic acid [TFA]) of 9–60% was run at 1 ml/minute. This gradient was run over 34 minutes, beginning 2 minutes after sample injection. Two large peaks of RFamide-like immunoreactivity were obtained, and appropriate fractions were pooled as two separate batches and evaporated to dryness for subsequent purification on system 2. Elution times of fractions positive for RFamide-like material were compared with elution times of synthetic LMS (Peninsula Laboratories, Belmont, CA).

System 2. A Brownlee Phenyl Spheri-5 column (46 mm × 25 cm) was used with two different acetonitrile gradients (containing 0.1% TFA). (A) The first gradient was increased from 18 to 60% acetonitrile, over 60 minutes, beginning 2 minutes after sample injection. Fractions were tested by using RIA, and positive fractions were again compared with the elution time of synthetic LMS. Individual peaks were evaporated to dryness for subsequent purification. (B) A second, slower gradient ran from 20 to 29% acetonitrile, over 60 minutes, beginning 2 minutes after injection.

Synthesis of digoxigenin-labeled RNA probe

The plasmid pLMS8 was used for in vitro RNA transcription because it contained the LMS coding region in the recombinant pBluescript plasmid (corresponding to bp 348–719; see Donly et al., 1996). Sense and antisense LMS cRNA probes were synthesized from the T3 and T7 promoters, respectively (Stratagene, La Jolla, CA), and the plasmid was linearized with *Nde*I and *Eco*RI, respectively. Probes were transcribed with a digoxigenin (DIG)-11-UTP labeling mix (Boehringer-Mannheim, Laval, PQ). The resulting transcripts were purified and resuspended in DEPC-treated H_2O.

In situ hybridization

Wholemount in situ hybridization was performed based on the protocol of Taylor et al. (1996). Tissues were dissected from day-5-mated female *D. punctata* under physiological saline (see Elia and Gardiner, 1990) and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 130 mM NaCl/70 mM Na_2HPO_4/3 mM NaH_2PO_4, pH 7.4) for 2 hours and stored in PBS at 4°C for up to 3 days. All steps were performed at room temperature unless stated otherwise. The tissues were incubated in 0.2 N HCl for 20 minutes and then heated to 70°C in 2× standard saline citrate (SSC; sodium chloride:sodium citrate, 0.3:0.03 M) for 30 minutes prior to a 30-minute proteinase K treatment (1 µg/ml in 100 mM Tris/50 mM ethylenediaminetetra-acetic acid [EDTA], pH 8.0) at 37°C. After washing in PBS, tissues were refixed in 4% paraformaldehyde for 20 minutes, washed in PBS, and acetylated in 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine for 10 minutes. The tissues were prehybridized (50% formamide/0.3 M NaCl/20 mM Tris-HCl, pH 7.5/1 mM EDTA, 1 × Denhardt's medium/100 mM 1,4-dithiothreitol/0.5 mg/ml yeast tRNA/0.02% Ficoll/0.02% polyvinylpyrrol-

idone/0.02% bovine serum albumin) for 1 hour. The tissues were hybridized for 24 hours at 50°C, with 50–100 ng/ml of probe in prehybridization solution containing 50% dextran sulfate and then washed in wash buffer (50% formamide/0.3 M NaCl/10 mM $NaPO_4$/10 mM Tris-HCl, pH 6.8/5 mM EDTA, pH 8.0) for 2 hours. To remove unhybridized probe, the samples were incubated in 20 µg/ml RNaseA and T1 RNAase (Boehringer-Mannheim) for 30 minutes at 37°C (0.5 M NaCl/10 mM Tris-HCl, pH 8.0/1 mM EDTA). Final washes included two sets of 30 minutes in 2 × SSC followed by one set of 15 minutes in 0.1 × SSC at 50°C and one set at room temperature. Tissues were washed in PBS with 0.3% Triton X-100 and preincubated in 2% normal goat serum for 1 hour. They were then incubated for 24 hours in peroxidase-conjugated anti-digoxigenin antibody (1:200) with 10% normal goat serum at 4°C. Digoxigenin-labeled RNA was detected in the presence of 3,3′-diaminobenzidene (20 µg/ml) in PBS containing NH_4Cl (0.4 mg/ml) and β-D^+-glucose (2 mg/ml) by using glucose oxidase (3 U/ml) as the catalyst. The reaction was monitored under a dissecting microscope until LMS mRNA-positive cells could be visualized, and the reaction was stopped by flooding with PBS. The tissues were placed on poly-(D-lysine)-coated slides, dehydrated in an EtOH series (with 0.3 M NH_4Ac) and mounted in 66% permount: 34% xylene. Digoxigenin-labeled sense transcripts were used as controls. The preparations were analyzed using a Zeiss compound microscope.

Immunohistochemistry

Tissues were dissected from day-5-mated female *D. punctata* under physiological saline and fixed in 2% paraformaldehyde in PBS and processed as described by Donly et al. (1996). The primary anti-FMRFamide antiserum (Incstar, Stillwater, MN) was visualized by using either fluorescein isothiocyanate-conjugated goat–anti-rabbit or CY3-conjugated sheep–anti-rabbit immunoglobulin G secondary antibodies (Daymar Laboratories, Toronto, ON), diluted 1:200 in PBS containing 10% normal goat or sheep serum, respectively. Tissues were washed in PBS, cleared, and mounted on depression slides in 5% *n*-propyl gallate in 80% glycerol (pH 7.3). Control tissues were incubated and processed as above in antisera preabsorbed with LMS alone (200 µg/ml), a combination of LMS, FMRFamide (Peninsula, Belmont, CA) and Ala-Phe-Ile-Arg-Phe-NH_2 (AFIRFamide; Core Facility for Protein and Peptide Chemistry, Kingston, ON) at 200 µg/ml each, or with bovine pancreatic polypeptide (BPP; Sigma, St. Louis, MO) at 100 µg/ml for 24 hours at 4°C. Two other sets of controls were incubated and processed as above but omitting either primary or secondary antisera. Preparations were analyzed by using a Zeiss Epifluorescent microscope or a Nikon OPTIPHOT-2 microscope with a BioRad ViewScan DVC-250 confocal imaging system.

RESULTS

Reversed-phase HPLC

We performed reversed-phase (RP)-HPLC separations on an extract from 1,600 isolated cockroach brains and analyzed fractions for the presence of RFamide-like material by using RIA. The brain extract was separated by using two sequential HPLC systems through three acetonitrile gradients (see Materials and Methods). The pooled material run through system 1 (C_{18} column) resulted in the resolution of two large peaks of RFamide-like-immunoreactive material (Fig. 1A). Fractions were pooled as two batches, evaporated to dryness, resuspended in HPLC buffer, and run separately through system 2A, which, for clarity, are depicted on one HPLC profile (Fig. 1B). Six groups of RFamide-like-immunoreactive fractions (stippled and filled bars) were then run individually through system 2B at a slower gradient. The slower acetonitrile gradient showed at least six distinct RFamide-like-immunoreactive peaks, one of which co-eluted with LMS (Fig. 1C; arrow). For clarity, the fractions are depicted on one HPLC profile (Fig. 1C).

Overall analysis of FaRP distribution and LMS mRNA expression in *D. punctata*

Expression of FaRPs and LMS mRNA was examined by using immunohistochemical and in situ hybridization techniques, respectively. FaRPs were detected by using a rabbit polyclonal anti-FMRFamide antiserum. This antiserum, although generated against FMRFamide, detects the presence of extended RFamide peptides (Tsang and Orchard, 1991), and Figure 1 shows the HPLC separation of multiple FaRPs from the brain. Multiple FaRPs have similarly been detected in midgut extracts (unpublished observations). Staining is therefore described as RFamide-like immunoreactivity. Expression of LMS mRNA was studied by using a DIG-labeled fragment of RNA as a hybridization probe. Both immunohistochemistry and in situ hybridization demonstrated positive staining in nervous and nonnervous tissues. Composite camera lucida representations of these results are shown in Figures 2, 4, and 6. RFamide-like-immunoreactive neurons were observed in the brain and optic lobes, the frontal ganglion, the ingluvial ganglion, the suboesophageal ganglion (SOG), and the entire ventral nerve cord. Immunoreactive processes were also observed in these tissues and in some associated neurohemal tissues. Processes projecting to the salivary glands, salivary reservoir, and alimentary canal were also immunoreactive, as were endocrinelike cells of the midgut and gastric cecae. No immunoreactivity was observed in Malpighian tubules or ovaries.

LMS mRNA was not detected in nerves but was expressed in a subset of neurons in all the tissues containing RFamide-like-immunoreactive neurons, except in the ingluvial ganglion. No hybridization was detected in the salivary glands or reservoirs, crop, gastric cecae or hindgut, or in the Malpighian tubules or ovaries. Positive staining was detected in endocrinelike cells of the midgut.

Controls

No immunoreactivity was observed in tissues after incubation in primary or secondary antiserum alone, nor were positive results obtained by in situ hybridization in the presence of sense probe. Preabsorption of the antiserum with either LMS or FMRFamide alone did not always fully abolish staining. For instance, whereas both FMRFamide and LMS could fully abolish staining of the SOG, salivary glands, and reservoir, neither fully blocked staining of neurons in the brain or other ganglia or in the alimentary canal. LMS blocking reduced brain neuron staining more than FMRFamide blocking did, but only a combination of FaRPs including LMS, FMRFamide, and AFIRFamide (another insect RFamide) fully abolished immunoreactivity in all tissues.

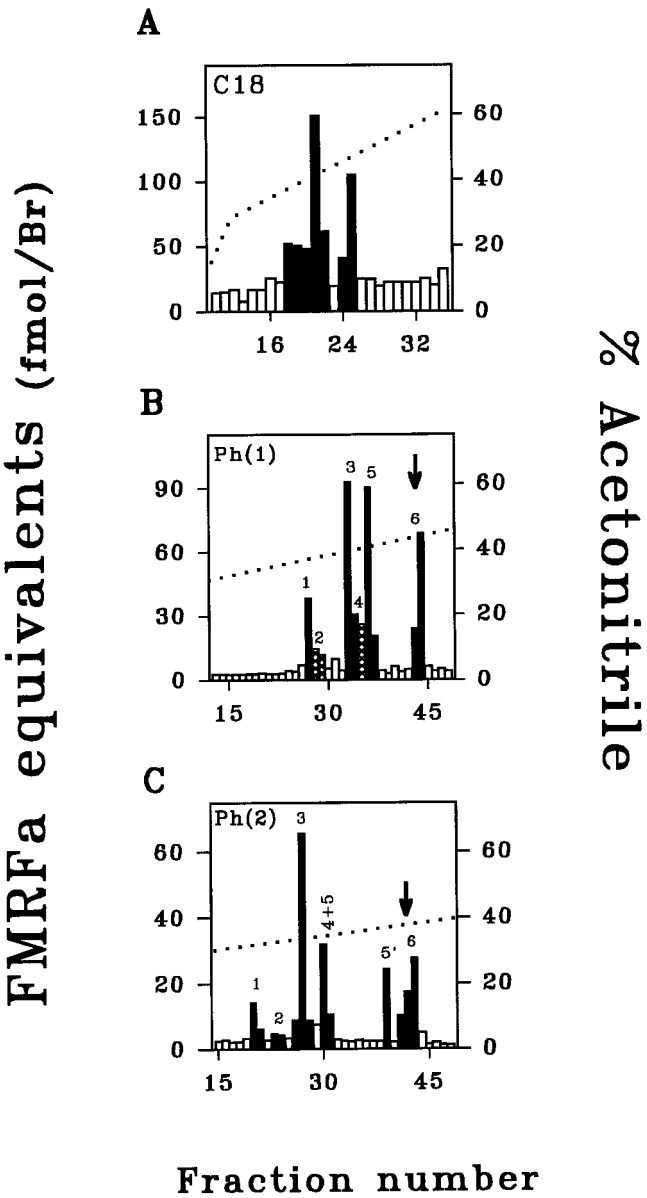

Fig. 1. Reverse-phase high performance liquid chromatography fractionation of RFamide-like material from extracts of 1,600 brains (including corpora cardiaca and corpora allata) of adult mated female *Diploptera punctata*. The values have been converted to single brain equivalents of FMRFamide. Arrows indicate the elution times of synthetic leucomyosuppressin. The broken line is the acetonitrile gradient. **A:** System 1: Separation on a C_{18} column. Two broad peaks of RFamide-like-immunoreactive material (solid bars; 18–22; 24–25) were dried down as two separate batches and run individually on system 2A. **B:** System 2A: Separation of fractions from two runs on system 1 through a Phenyl column, represented on one graph for clarity. Neighboring fractions were pooled (solid bars) or dried separately (stippled bars) for six subsequent phenyl runs on system 2B. **C:** System 2B: The six groups of fractions were individually run on a phenyl column with a slower acetonitrile gradient. Fractions are represented on one graph for clarity. Clear bars represent non-immunoreactive fractions, which were discarded.

Of particular significance was the fact that preabsorption with LMS reduced staining in the anterior midgut but did not affect staining in the posterior region, whereas preabsorption with FMRFamide reduced staining in anterior and posterior regions. Because FMRFamide antisera have often shown cross reactivity to BPP (Myers and Evans, 1985), cross reactivity was checked in this study by preabsorbing the antiserum with BPP. Preabsorption of the antiserum with BPP did not abolish any staining in any tissues except in the posterior region of the midgut. Within the midgut, a subset of normally immunoreactive cells in the posterior region did not stain, whereas the number and intensity of cells in other parts of the gut remained unchanged. The gradient of immunoreactive cells from posterior to anterior regions was still obvious in these preparations (see below for details of this gradient), and blocking with a combination of FaRPs fully abolished immunoreactivity. Therefore, although the FMRFamide antiserum could seemingly cross react with BPP, it was in fact detecting FaRPs and not BPP in the majority of midgut cells.

FMRFamide-related peptides in the brain, retrocerebral complex, and SOG

The brain is composed of the proto-, deuto-, and tritocerebra. Median and lateral neurosecretory cells in the protocerebrum send axons to the corpora cardiaca (CC) via nerves NCC I and NCC II, respectively. The tritocerebrum also sends axons to the CC via NCC III and to the frontal ganglion. Lateral cells of the brain send axons through the CC to the corpora allata (CA), and connections are made to the SOG. The CC connects to the esophageal nerve, which attaches to the frontal ganglion anteriorly. The frontal ganglion projects to an ingluvial ganglion that bifurcates posteriorly at the front region of the crop into a dorsal and a ventral ingluvial nerve. These nerves, comprising numerous neurons, arborize and innervate the crop (reviewed by Penzlin, 1985).

RFamide-like immunoreactivity was detected in the brain and optic lobes (Fig. 2A), the frontal ganglion (Fig. 3A, white arrows), and the ingluvial nerve (Fig. 3D) and in processes connecting the brain to the ingluvial nerves. In the brain, RFamide-like immunoreactivity was most abundant in the protocerebrum and in particular in approximately 35 median neurosecretory cells of the pars intercerebralis, as has already been described by Donly et al. (1996). Although nerve processes could not be traced from the median neurosecretory cells to the CC in the whole-mount preparations, intense immunoreactive fibers were obvious along the path from the medial and lateral neurosecretory cell regions to the CC. Staining was intense within the CC, with immunoreactive processes extending through the CA (Fig. 3A, black arrows), likely to the SOG. Two prominent lateral cells were also immunoreactive in the brain. Three cell bodies were apparent in or near the antennal lobes, along with abundant immunoreactive processes within the antennal neuropil. Strong staining was visible in the tritocerebral neuropil and in the connections to the frontal ganglion. The frontal ganglion had intense immunoreactive processes and six RFamide-like immunoreactive cell bodies. The optic lobes contained numerous RFamide-like immunoreactive cell bodies in clusters in the medulla, with processes spanning the accessory medulla.

The large subset of the neurons described above stained positively for LMS mRNA by in situ hybridization, but no nerve processes were apparent (Fig. 2B). LMS mRNA expression was most abundant in cells of the brain but was also found in the optic lobes and frontal ganglion. Within

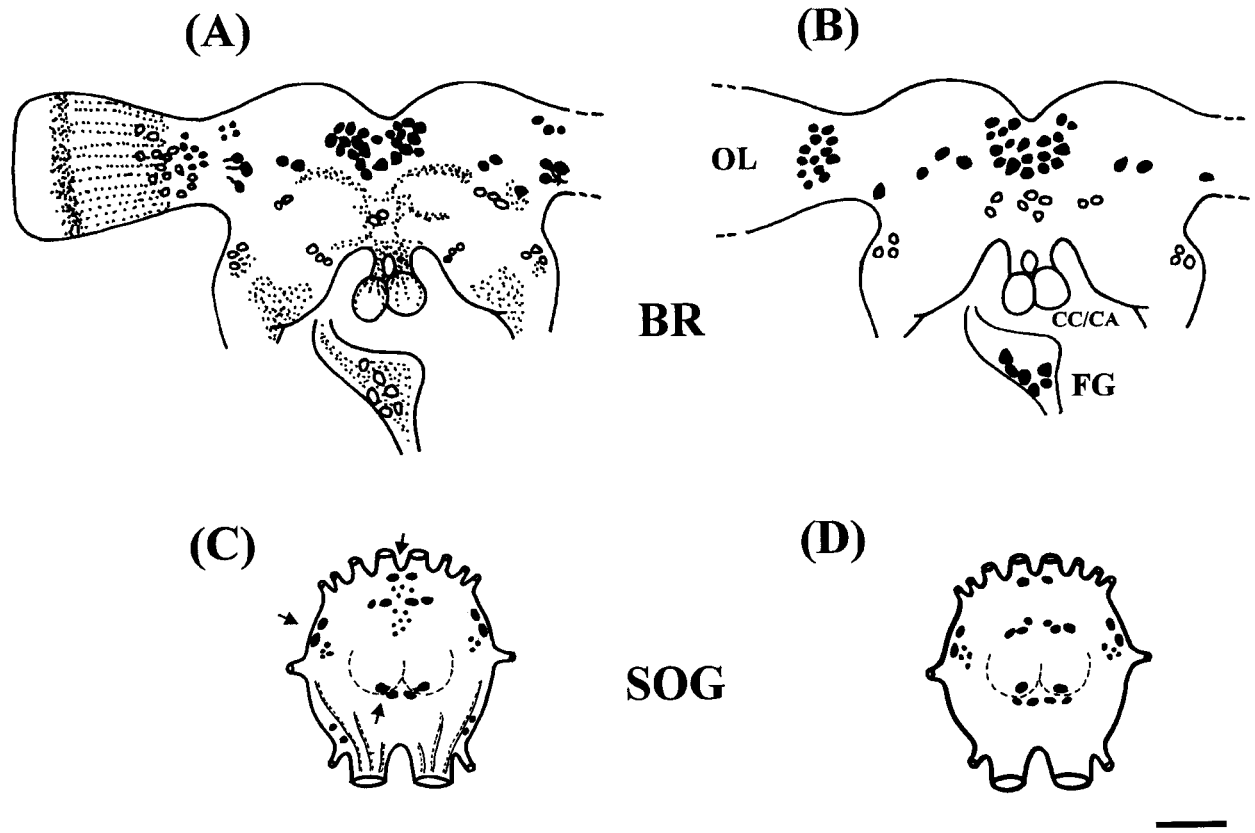

Fig. 2. Camera lucida representations of wholemounts of *D. punctata* brain, optic lobes, and frontal ganglion visualized by immunohistochemistry (**A**) and in situ hybridization (**B**). Similar representations of the subesophageal ganglion are visualized by immunohistochemistry (**C**) and in situ hybridization (**D**). Filled circles represent intensely stained cells and open circles represent faintly stained cells. Dashes denote the location of dorsal connectives of the subesophageal ganglion (SOG). Arrows are discussed in the Results. BR, brain; OL, optic lobes; CC/CA, corpora cardiaca/corpora allata; FG, frontal ganglion. Scale bar = 250 μm.

the brain, approximately 20 median neurosecretory cells stained positively for LMS mRNA. Some lateral cells and cells of the antennal and optic lobes were also LMS mRNA positive. Within the frontal ganglion (Fig. 3B), six LMS mRNA-positive cells were apparent, which, by their positions along the perimeter of the ganglion, appeared to be identical to those detected by immunohistochemistry. A few cells in the brain that were positive for the mRNA probe were not immunoreactive. This discrepancy has been noted by Donly et al. (1996) and may be a reflection of either low levels of peptide synthesis or lower detection capabilities of the antibody for LMS. Stringent wash conditions were used to reduce the possibility of false positive results by in situ hybridization.

Within the SOG were numerous RFamide-like immunoreactive neurons. Ten cells were consistently the most immunoreactive; two pairs located centrally at the origin of the cervical nerves, two pairs located laterally, and one pair located anteriorly (Fig. 2C; arrows point to three of five pairs of intensely staining cells). The central neuropil region was also highly immunoreactive, although it is not depicted in the camera lucida representation. Numerous cells were also LMS mRNA positive in the SOG, again with 10 cells being highly expressed (Fig. 2D).

The salivary glands and reservoirs, which are directly innervated by the SOG in many insects, were also intensely RFamide-like immunoreactive (Fig. 3C), and this immunoreactivity is discussed in greater detail below.

FMRFamide-related peptides in the ventral nerve cord

In *Diploptera*, the ventral nerve cord consists of three thoracic ganglia (pro-, meso-, and metathoracic), one abdominal ganglion fused to the metathoracic ganglion (abdominal ganglion 1), five unfused abdominal ganglia (ganglia 2–6), and a fused terminal abdominal ganglion. RFamide-like immunoreactivity was detected in neurons and various nerve processes of all ganglia of the ventral nerve cord (Fig. 4A). LMS mRNA-positive neurons, but not nerve processes, were also detected by in situ hybridization in the entire ventral nerve cord, occasionally in regions that did not show RFamide-like immunoreactivity (Fig. 4B). Of significance was the very faint appearance of five dorsally located LMS mRNA-positive neurons in the prothoracic ganglion that were not apparent when using immunohistochemistry. These cells were not detected in the meso- or metathoracic ganglia. Hybridization was less intense in the ganglia of the ventral nerve cord in general, which may be the result of lower levels of expression of the LMS mRNA or an indication of the decreased accessibility of probe through the sheath of the ganglia.

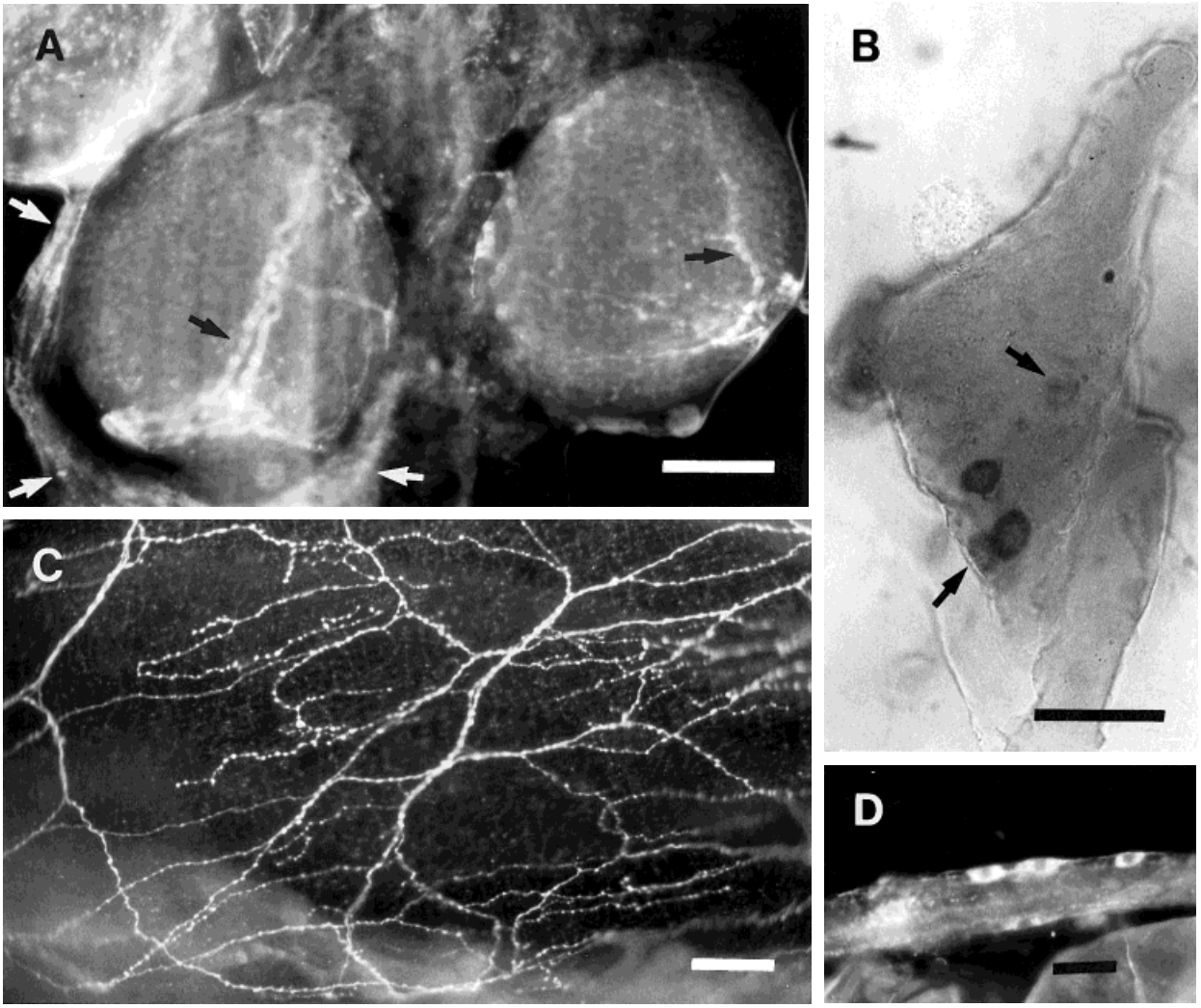

Fig. 3. Photomicrographs of wholemounts of *D. punctata* nervous tissue by using immunohistochemistry and in situ hybridization. **A:** RFamide-like immunoreactivity of corpora allata (black arrows) and frontal ganglia (white arrows). **B:** Four leucomyosuppressin (LMS) mRNA-positive cells in the frontal ganglion. Arrows point to the two faintly stained cells. **C:** RFamide-like-immunoreactive nerves on the salivary reservoir. **D:** Three RFamide-like-immunoreactive neurons and processes of the ingluvial nerve. Scale bars = 50 µm.

In all three thoracic ganglia, two sets of four strongly immunoreactive neurons were located on the mid-ventral surface, sending processes laterally (Fig. 5A, arrows). In situ hybridization demonstrated a single set of similarly located neurons (Fig. 5B, arrow). Two sets of four, very strongly immunoreactive neurons were also located caudolaterally in all three ganglia, but similar cells were only faintly apparent in some in situ preparations of the metathoracic ganglion. One immunoreactive neuron on the dorsal surface was visible within each thoracic ganglion, and this neuron appeared to be the site of origin of the immunoreactivity within the median nerve. The median nerve branched to form the two highly immunoreactive transverse nerves. A dorsal neuron was also observed in all three thoracic ganglia of the in situ preparations.

All abdominal ganglia showed some RFamide-like immunoreactive neurons, as was the case for in situ hybridization (compare Fig. 5C and 5D). The median nerves from abdominal ganglia 2 and 3 showed RFamide-like immunoreactivity, but this was not consistent for the other abdominal ganglia. However, tracing the transverse nerves from the other ganglia farther along always demonstrated some RFamide-like immunoreactive regions (data not shown) suggestive of the neurohemal organs described by Raabe (1989). Each ganglion had an extensive immunoreactive neuropil region that is outlined in Figure 4 and seen clearly in Figure 5C (arrows).

FMRFamide-related peptides in the alimentary canal

RFamide-like immunoreactivity was apparent throughout the alimentary canal (Fig. 6A), but LMS mRNA was only detected in a defined region of the midgut (Fig. 6B). Numerous cell bodies and processes of the ingluvial ganglion and the two ingluvial nerves displayed RFamide-like immunoreactivity along the entire length of the crop (Figs. 3D, 6A). These nerves sent immunoreactive projections

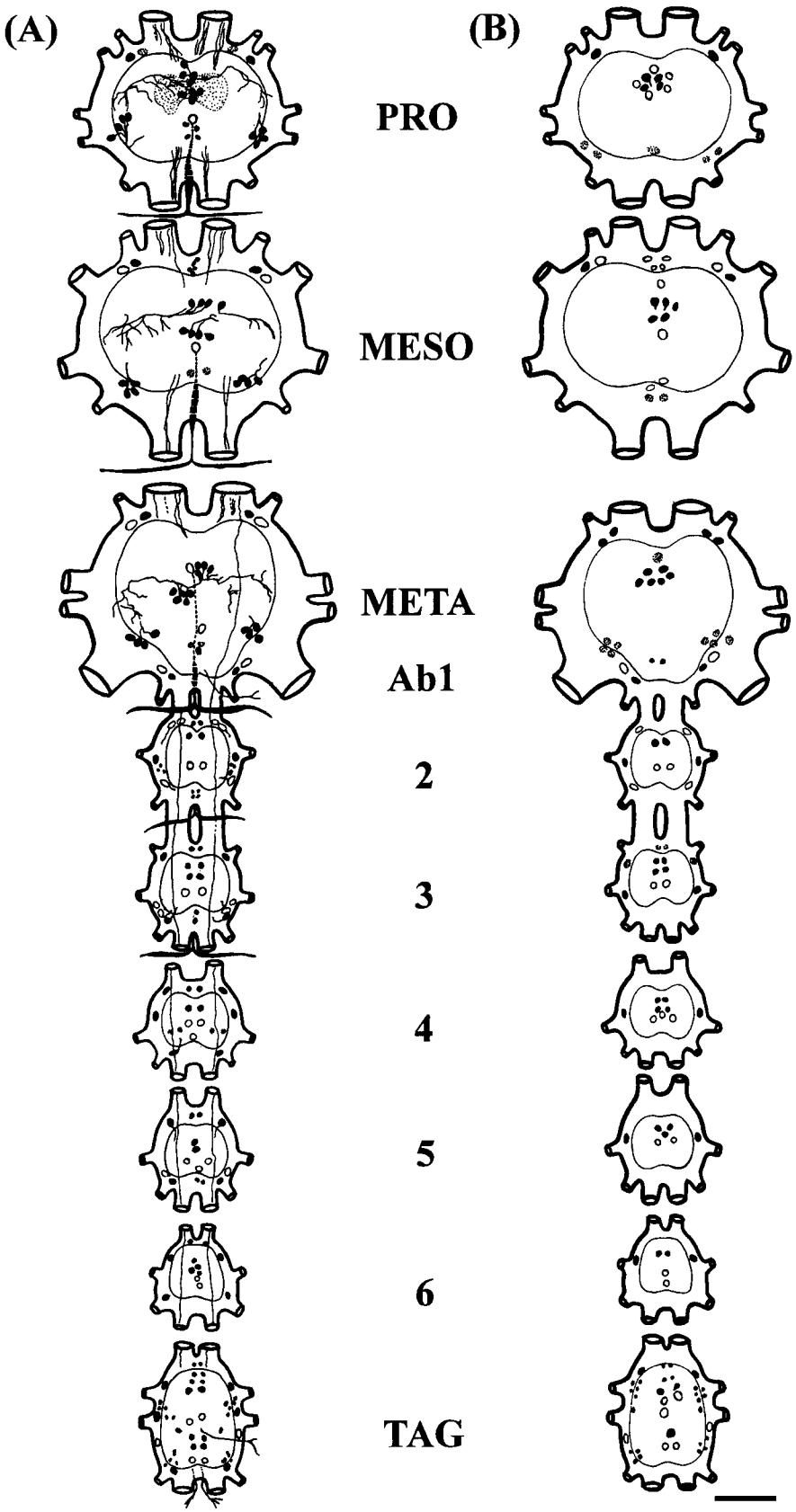

Fig. 4. Composite camera lucida representations of wholemounts of *D. punctata* ventral nerve cord detected by immunohistochemistry (**A**) and in situ hybridization (**B**). Filled circles represent ventral cells, open circles represent dorsal cells, and stippled circles represent faintly stained cells. Ganglia: PRO, prothoracic; MESO, mesothoracic; META, metathoracic; Ab1–6, abdominals 1–6; TAG, terminal abdominal. Scale bar = 250 μm.

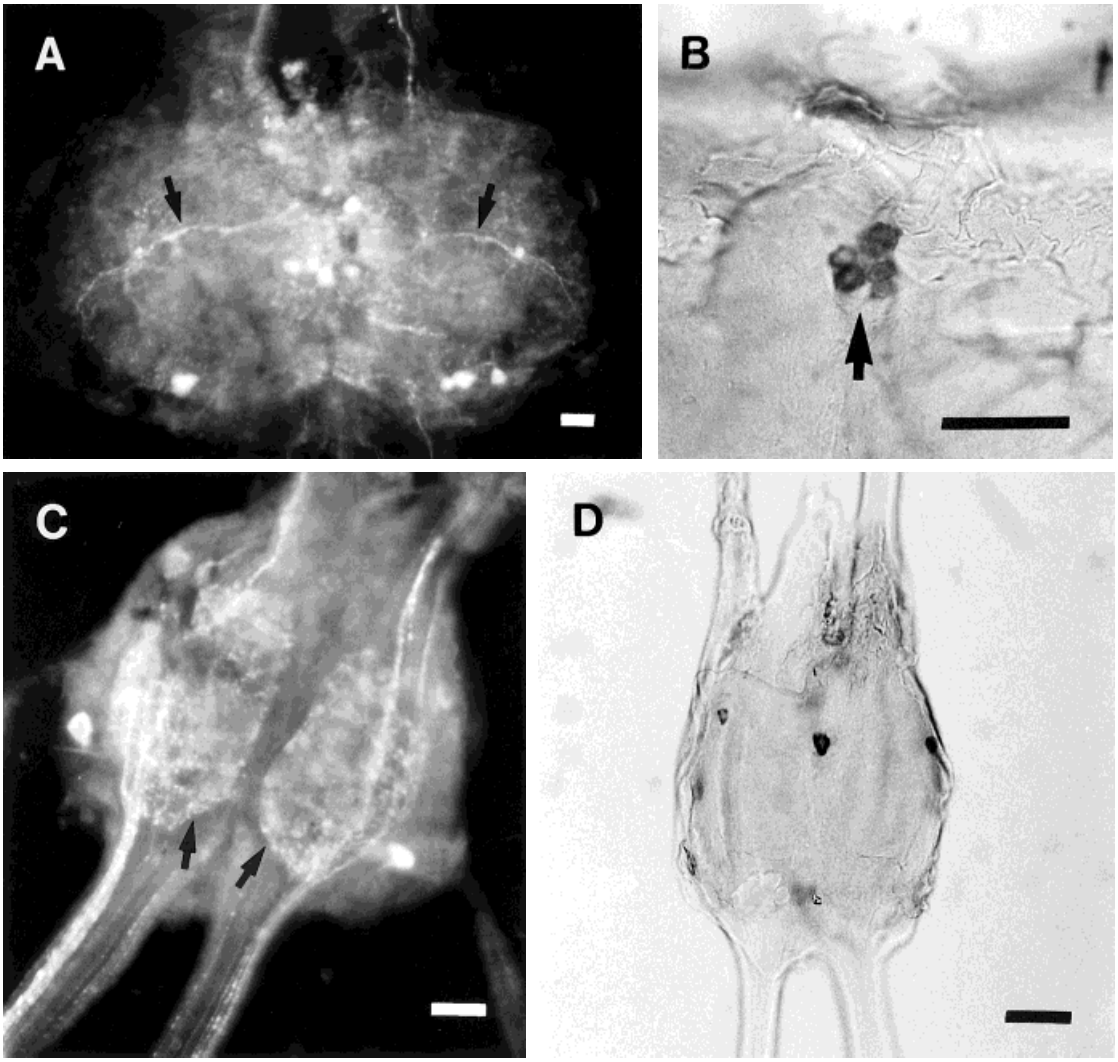

Fig. 5. Photomicrographs of wholemounts of *D. punctata* ganglia using immunohistochemistry and in situ hybridization. **A:** RFamide-like immunoreactivity of the mesothoracic ganglion. Arrows define the immunoreactive processes extending from two sets of highly immunoreactive mid-ventral neurons. **B:** Leucomyosuppressin mRNA-positive cells (arrow) of the mesothoracic ganglion. **C:** RFamide-like immunoreactivity of abdominal ganglion 5. Arrows define the immunoreactive neuropil. **D:** Leucomyosuppressin mRNA-positive cells of abdominal ganglion 5. Cellular debris in the posterior region of the ganglion was masked photographically to avoid confusion with positively stained cells. These cells are easily distinguishable from debris by color in the original preparation. Scale bars = 50 μm.

over the surface of the crop and appeared to connect to the salivary glands at the ducts. The salivary glands themselves were highly endowed with RFamide-like immunoreactive nerve processes following, and branching with, the salivary ducts and making contact with the acini. The salivary reservoirs had an intricate plexus of intense immunoreactivity coursing the anterior two-thirds of the sacs (Fig. 3C). No staining was seen in the posterior region of the sacs. No LMS mRNA staining was seen in these tissues (Fig. 6B). Immunoreactive processes were not observed to pass from the crop to the midgut, although they were observed traveling from the hindgut onto the midgut.

In the midgut, an abundance of endocrinelike cells and some nerve processes displayed intense RFamide-like immunoreactivity at the posterior end, with a declining gradient of cells anteriorly (Fig. 7A). At the point where RFamide-like immunoreactive cells were less abundant, immunoreactive nerve processes were more abundant. Just anterior to this point, the number of immunoreactive cells increased slightly, until, at a region just posterior to the gastric caecae/midgut junction, RFamide-like immunoreactive cells were no longer apparent. The gastric cecae contained a large number of immunoreactive endocrine-like cells. In contrast, LMS mRNA expression was most abundant in endocrinelike cells at the anterior region of the midgut near the crop/gastric cecae junction, in the area just anterior to the abundant immunoreactive processes, with a posterior declining gradient (Figs. 6B, 7B). No endocrinelike cells of the midgut were positive for LMS mRNA in the most posterior region, nor were any cells of the gastric cecae positive for LMS mRNA. The nerve processes that appeared to project up from the hindgut through the posterior region of the midgut stopped at the

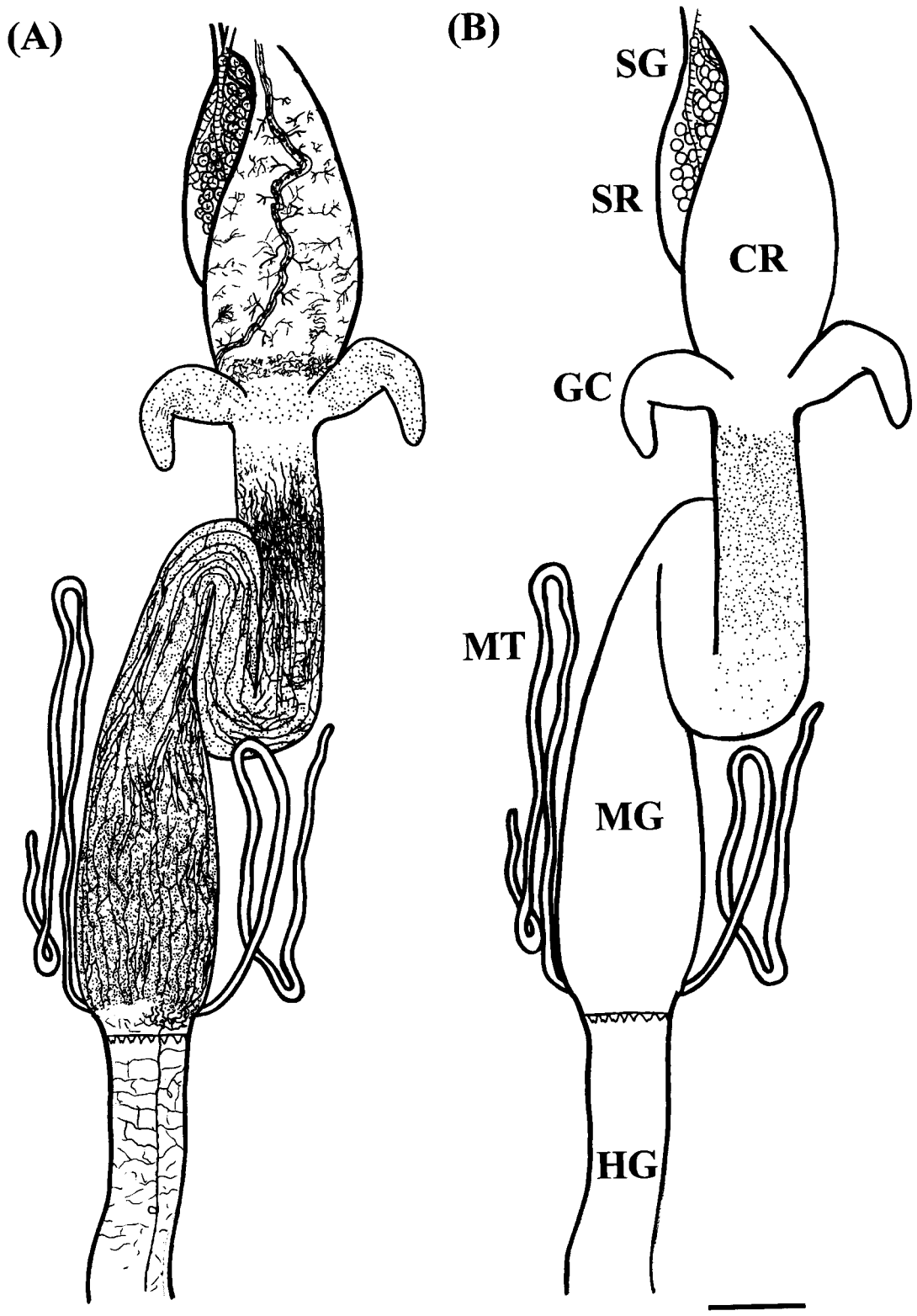

Fig. 6. Composite camera lucida representations of wholemounts of *D. punctata* alimentary canal detected by immunohistochemistry (**A**) and in situ hybridization (**B**). SG, salivary glands; SR, salivary reservoir; CR, crop; GC, gastric caecae; MT, Malpighian tubules; MG, midgut; HG, hindgut. Scale bars = 500 µm.

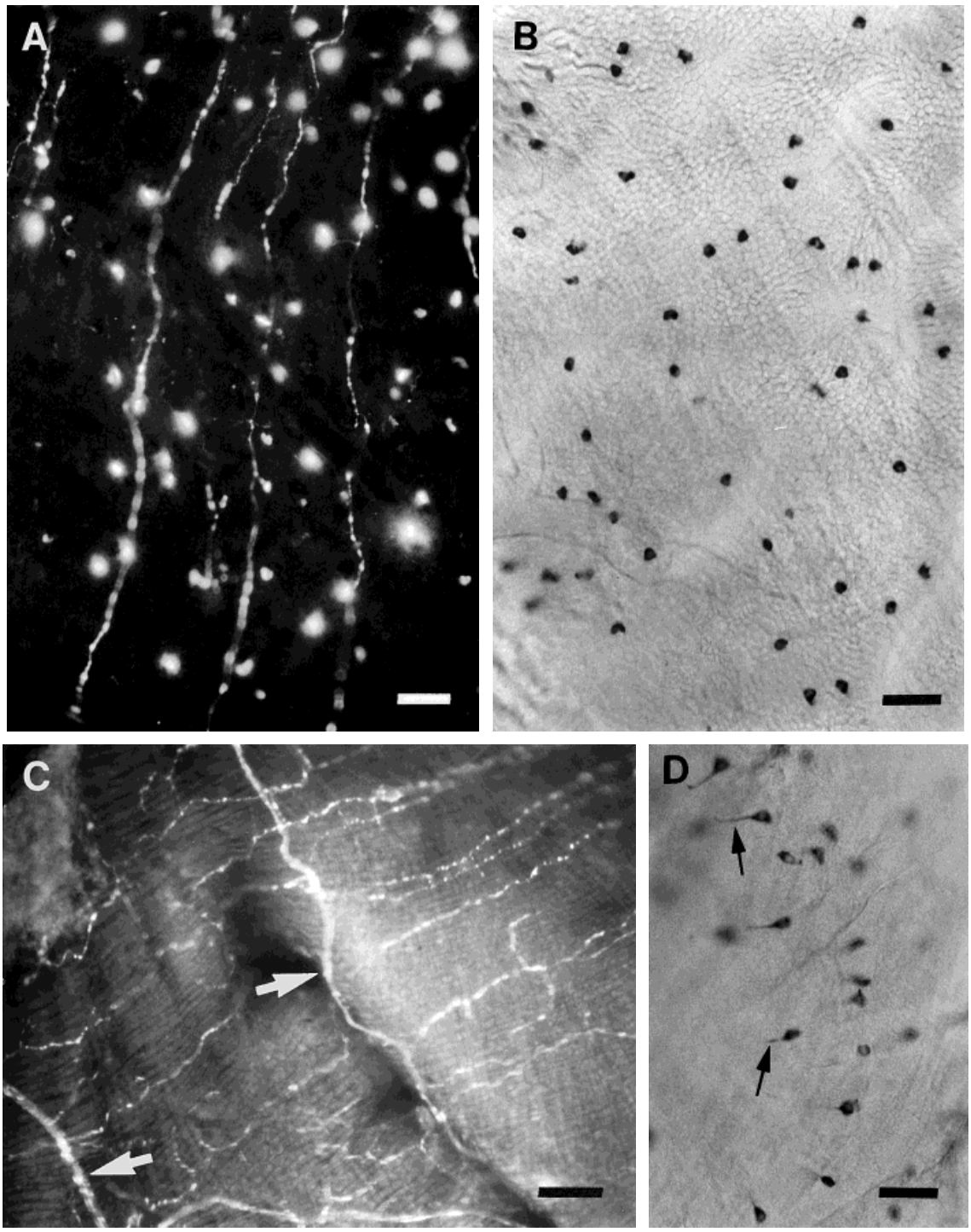

Fig. 7. Photomicrographs of wholemounts of *D. punctata* viscera using immunohistochemistry and in situ hybridization. **A:** RFamide-like immunoreactivity in endocrine cells and processes of the midgut. **B:** Leucomyosuppressin mRNA in endocrine cells of the midgut. **C:** RFamide-like immunoreactivity of nerves on the hindgut. Arrows point to longitudinal immunoreactive nerves that travel toward the midgut. **D:** Leucomyosuppressin mRNA in endocrine cells and their cytoplasmic extensions (arrows) in the midgut. Scale bars = 50 μm.

point just posterior to the region in which LMS mRNA cells were most abundant.

The cell bodies of the immunoreactive endocrinelike cells of the midgut appeared to be of the "open" variety (Nishiitsutsuji-Uwo and Endo, 1981). They were located against the serosal surface of the epithelium, with cytoplasmic extensions traveling to the lumen. Both cell bodies and cytoplasmic extensions possessed RFamide-like immunore-

activity. Extensions were long and meandering and easily visible by immunohistochemistry. LMS mRNA-positive staining was also observed in the cell bodies and along part of the length of the cytoplasmic extensions (Fig. 7D, arrows) but was never observed as far along the extensions as the RFamide-like immunoreactivity.

On the hindgut, three major nerves (Fig. 7C, arrows), which traveled longitudinally along its length, displayed RFamide-like immunoreactivity and had immunoreactive branches forming a grid radially across the entire hindgut surface. The large nerve processes traveled up to the midgut/Malpighian tubule junction and also appeared to project to the midgut. No LMS mRNA staining was detected.

DISCUSSION

Although the genes encoding sulfakinins (Nichols et al., 1988) and short FMRFamides (Taghert and Schneider, 1990) have been cloned in *Drosophila*, the first gene encoding a myosuppressin was described in the cockroach, *Diploptera punctata* (Donly et al., 1996). Unlike the multipeptide FaRP (and sulfakinin) precursors, this myosuppressin gene appears to encode a precursor containing only the single FaRP, LMS. Nevertheless, RP-HPLC analysis with RIA detection of FaRPs in brain extracts of *D. punctata* demonstrated at least six distinct peaks of RFamide-like-immunoreactive material (Fig. 1), only one of which co-eluted with authentic LMS. Multiple FaRPs were also isolated from midgut extracts, with one fraction co-eluting with LMS (unpublished observations). This result suggests that separate genes exist that express RFamides distinct from LMS, and therefore RFamide-like immunoreactivity in *D. punctata* represents FaRPs from multiple genes. These results also reflect the strength of using in situ hybridization as a tool for localizing LMS-producing cells in tissues of *Diploptera*.

By using a cDNA encoding the precursor for LMS, we created DIG-labeled RNA probes synthesized by in vitro transcription specific to the LMS coding region for use in in situ hybridization. In situ hybridization demonstrated abundant expression of LMS mRNA in the brain, optic lobes, frontal ganglion, subesophageal ganglion, ventral nerve cord, and endocrinelike cells of the midgut. Immunohistochemical analysis using a general anti-FMRFamide antiserum demonstrated the presence of FaRPs in the cell bodies of all tissues showing LMS mRNA, in other tissues, and in associated nerve processes. RFamide-like immunoreactivity was found in nervous and nonnervous tissues, including processes on the salivary glands and reservoirs, the ingluvial ganglion and ingluvial nerves on the crop, the processes of the hindgut, and both processes and endocrine-like cells of the midgut (Figs. 2–7).

Preabsorption of the antisera with either LMS or FMRFamide did not always fully block staining, suggesting that LMS may be colocalized with other FaRPs in some tissues. The ability of either LMS or FMRFamide to block staining of the SOG, salivary glands, and reservoir, however, also suggested that blocking of the RFamide moiety alone could be sufficient to eliminate staining of some RFamide peptides.

The distribution of RFamide-like immunoreactivity and LMS mRNA-positive cells in the brains of *D. punctata* has been described by Donly et al. (1996). Within the brain, FaRPs, and in particular LMS mRNA, are detected in the proto- and deutocerebra and in the optic lobes. RFamide-like immunoreactivity is also detected in various neuropil regions. On occasion, LMS mRNA-positive cells in *Diploptera* were not demonstrated by immunohistochemistry (compare Fig. 2A and 2B), and this was consistent for various periods in adult development (unpublished observations), suggesting that in situ hybridization is a very sensitive technique for detecting LMS-producing cells. However, these discrepancies may also represent cells that contain mRNA but have not processed peptide.

Most RFamide-like immunoreactivity and LMS mRNA expression in the brain is seen in the median neurosecretory cells of the protocerebrum. These cells may be the source of RFamide-like immunoreactivity localized in the CC, suggesting a neurohormonal role for FaRPs in *Diploptera*. Whether or not LMS is among the FaRPs present in the CC is under investigation.

Two LMS mRNA-positive lateral cells of the brain may be the lateral neurosecretory cells that project to the CC or may be the descending neurons described by Nässel et al. (1992) as leucokinin I-like immunoreactive, which send processes to the antennal lobes in *Leucophaea*. If this is the case, it will be interesting to determine whether kinins are colocalized with FaRPs in *Diploptera* because both can be myotropic on various tissues.

The presence of LMS mRNA in cells of the frontal ganglion and SOG and RFamide-like immunoreactivity in both the cells and processes of these ganglia and other parts of the stomatogastric system suggest that LMS plays a role in feeding-related functions in this species. The stomatogastric system innervates the foregut and midgut of most insects (reviewed by Huddart, 1985). For example, the frontal ganglion innervates the pharynx and proventriculus via the ingluvial nerves and may regulate the rate of crop emptying in *Periplaneta americana* (Davey and Treherne, 1963). RFamide-like immunoreactivity has been found in the ingluvial nerves in the cockroaches *Nauphoeta cinerea* and *Blabera craniifer* and on the crop surface (Zitnan et al., 1993). Although no LMS mRNA was detected in the ingluvial nerves or crop in *Diploptera*, there was abundant RFamide-like immunoreactivity in these tissues, and the frontal ganglion may provide a source of LMS to these areas. RFamide-like immunoreactivity has been found in the frontal ganglion of *Manduca sexta* (Copenhaver and Taghert, 1991), and Taylor et al. (1996) suggested that FaRPs may be colocalized there with other neuropeptides such as MAS-AT, the *Manduca sexta* allatotropin. In *L. maderae*, leucokinin VIII-like immunoreactivity has been found in six pairs of cells of the frontal ganglion (Meola et al., 1994). Whether the cells of the frontal ganglion of *Diploptera* also contain kinins or other neuropeptides needs to be addressed.

The SOG has been implicated in functions associated with feeding (Davis, 1987; Baines and Tyrer, 1989; Schachtner and Bräunig, 1993), and both LMS mRNA and RFamide-like immunoreactivity have been detected in cells of the SOG. HPLC analysis and double-label experiments in *Locusta migratoria*, however, have suggested that the LMS equivalent, SchistoFLRFamide, does not play a role in salivary gland function because this peptide is not present in the glands and is not localized in the cells of the SOG that innervate the salivary glands (Fusé et al., 1996). Instead, it has been suggested that FaRPs other than SchistoFLRFamide are present in the salivary glands, arising from the pro- and mesothoracic ganglia. Therefore,

the RFamide-like immunoreactive innervation seen in the salivary glands and reservoirs of *Diploptera* may reflect the presence of FaRPs other than LMS, originating from the ventral nerve cord. Interestingly, no LMS-like immunoreactivity was detected in the SOG or any abdominal ganglia of *L. maderae* when using an antiserum directed against LMS (Meola et al., 1991). This antiserum was preabsorbed with FMRFamide, which may have lowered its sensitivity to LMS. In contrast, in the locust *Schistocerca gregaria*, a pattern of cell staining using an antiserum directed against the N- terminal of SchistoFLRFamide (Swales and Evans, 1995) was similar, if not more abundant, to the pattern of LMS mRNA expression in the SOG of *Diploptera*. Thus, the roles of LMS and SchistoFLRFamide in the SOG need to be clarified.

All ganglia of the ventral nerve cord showed LMS mRNA-positive cells in *Diploptera*. Whereas the pattern of expression was dissimilar to that found in *L. maderae* (Meola et al., 1991), it was similar to the pattern observed in locusts when using the SchistoFLRFamide-specific antiserum (Swales and Evans, 1995). Even the terminal abdominal ganglion, which was originally suggested to lack FaRPs (Myers and Evans, 1985), has since been shown to contain SchistoFLRFamide-like peptides (Swales and Evans, 1995) and in the present study is shown to contain LMS mRNA and possibly other FaRPs (Fig. 4A,B). The terminal abdominal ganglion provides proctodeal nerves to the hindgut of many insects (reviewed by Huddart, 1985), and although there is no LMS mRNA observed in the hindgut of *Diploptera* (Fig. 6B), there is abundant RFamide-like immunoreactivity in nerve processes, many of which project to the midgut (Figs. 6B, 7C). It is likely that at least some of this immunoreactivity represents LMS expression because LMS mRNA is present in the terminal abdominal ganglion, and it was the hindgut that was first used as a bioassay for the isolation of LMS from cockroaches (Holman et al., 1986).

RFamide-like immunoreactivity in the thoracic ganglia demonstrates two clusters of ventral midline cells and other lateral cells that are located at the anterior and mid regions and that appear to send axons to the periphery (Fig. 5A). These cells are similar to the cells described in the ventral nerve cord of locusts (Ferber and Pflüger, 1992), which contain SchistoFLRFamide-like peptides (Swales and Evans, 1995). A single cluster of five cells in the same region also stain positively for LMS mRNA (Fig. 5B). A set of dorsal cells containing LMS mRNA are faintly visible in the prothoracic ganglion but are not detected by immunohistochemistry (compare Fig. 2A and 2B). These may be the DUM neurons described in locusts (Ferber and Pflüger, 1992; Stevenson and Pflüger, 1994) that innervate the heart muscles. Other DUM neurons have been described, which, although containing amines, affect both skeletal and visceral muscle (Orchard and Lange, 1985). Backfill experiments are necessary to ascertain specific origins of many of the cells in *Diploptera*.

Immunohistochemistry with the anti-FMRFamide antiserum and in situ hybridization of LMS mRNA in the midgut demonstrate positive signals in apparent intrinsic endocrine cells of the "open" variety (Zitnan et al., 1993), with staining in the cell bodies near the serosal membrane and in the cytoplasmic extensions leading to the lumen. RFamide-like immunoreactivity has been found in midgut endocrine cells of *N. cinerea* and *B. craniifer*, although not in the cytoplasmic extensions (Zitnan et al., 1993). The open cell types may monitor nutrient contents of the gut in *P. americana* (Fujita et al., 1981), possibly in a paracrine fashion (Yu et al., 1995). Leucomyosuppressin has been shown to induce increases in α-amylase secretion into the lumen of ligated weevil midguts (Nachman et al., 1996), suggesting a role in regulation of enzyme secretion into the midgut lumen. LMS acts similarly on the stomach–digestive gland complex of the scallop, *Pecten maximus* (Nachman et al., 1996). Alpha-amylase is the predominant carbohydrase in insects (reviewed by Terra and Ferreira, 1994) and is distributed unevenly in the midguts of locusts (Khan, 1961) and cockroaches (Day and Powning, 1949). This is discussed in greater detail below.

Allatostatins (first isolated as inhibitors of JH production in *Diploptera* and subsequently shown to inhibit hindgut contractions; Lange et al., 1995) and allatostatin mRNA are also found in open-type endocrine cells of the midgut of *Diploptera* (Reichwald et al., 1994), with a unique pattern of expression along the midgut length (Yu et al., 1995). The allatostatins have been shown to be released into the hemolymph in response to high $[K^+]$ and in response to starvation (Yu et al., 1995), supporting the notion that peptides in these endocrine cells could be involved in feeding-related processes.

LMS and SchistoFLRFamide are also inhibitors of contractions of midguts in *Locusta migratoria* (Orchard and Lange, 1997), as is ManducaFLRFamide in *Agrius convolvuli* (Fujisawa et al., 1993), indicating a possible role in movement of food along the length of the gut. Of particular interest in the present study is the fact that the distribution of RFamide-like immunoreactivity differs from that of LMS mRNA along the length of the midgut. The majority of RFamide-like immunoreactive endocrinelike cells are found in the posterior region of the midgut, with fewer cells anteriorly. Most LMS mRNA-containing cells are located in the anterior region, with none in the posterior section immediately adjacent to the midgut/Malpighian tubule junction. Blocking with LMS reduces the intensity of staining of the anterior region, whereas only a combination of FaRPs successfully abolishes all staining in the midgut. Because LMS is processed from a gene separate from other FaRPs, its expression can be independently regulated in different regions of the gut. HPLC analysis of brains (Fig. 1C) and midguts (unpublished observations) indicates that multiple FaRP fractions exist in *Diploptera*. Moreover, a unique FaRP (ANRSPSLRLRFamide) has been sequenced at the amino acid level from midguts of *P. americana*; this peptide or other FaRPs may exist in midgut axons and endocrine cells (Veenstra and Lambrou, 1995) with a staining profile very similar to that of *Diploptera*. Differential gene regulation therefore may be a means of controlling different regions of the midgut using different FaRPs. Regional differences in peptide expression have been shown in the midguts of *Diploptera* (allatostatin mRNA and peptides; Yu et al., 1995) and other insects (e.g., allatostatins, sulfakinins, tachykinins, and FaRP peptides; Agricola and Braunig, 1995; Muren et al., 1995; Veenstra et al., 1995). Carbonic anhydrase (Ridgway and Moffett, 1986) and pH (Dow, 1984) are also distributed unevenly along the length of the gut of *Manduca sexta* larvae, and there are functional differences in amino acid/K^+ symporters along the lengths of lepidopteran midguts (Giordana et al., 1994). Some of these differences may affect nutrient absorption in different parts of the gut (Dow, 1986). Trypsin in *Musca domesticus* larvae is local-

ized in posterior midgut cells (Jordao et al., 1996), supporting the notion that different peptides may regulate the activity of different digestive enzymes within the midgut. Whether the distribution of enzymes such as α-amylase and invertase match the distribution of LMS-containing endocrine cells is currently under investigation.

The diversity of ganglia and tissue types containing LMS mRNA suggests that LMS may play several roles in *D. punctata*. The strong association of LMS with the stomatogastric system and alimentary canal indicate a likely role in feeding-related processes and digestion. Its presence in the ventral nerve cord also suggests other central roles. These results give us clues for establishing appropriate bioassays for physiological studies. Furthermore, the present study describes a unique spatial separation of FaRP expression in the midgut that strongly suggests differential gene expression and differing roles for different FaRPs within the same tissue. An understanding of the interplay between the various peptides present will require the isolation and sequencing of the complete complement of FaRPs produced in the brain and midgut of this cockroach, and this work is now in progress.

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