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Distribution of Ovary Ecdysteroidogenic Hormone I in the nervous system and gut of mosquitoes

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Abstract

Ovary ecdysteroidogenic hormone I (OEH I) is a gonadotropin in the female mosquito, *Aedes aegypti*. Whole-mount immunocytochemistry using OEH I antisera revealed an extensive distribution of immunostained cells in larvae and adults of this mosquito comparable to that observed in the African malaria mosquito, *Anopheles gambiae*. Medial neurosecretory cells were stained in brains of larvae and adult *Ae. aegypti*. In *An gambiae* the lateral neurosecretory cells were stained more often. In both species, immunostained axons from these cells extended out of the brain through the neurohemal organ associated with the aorta and branched extensively along the midgut. Immunostained endocrine cells were observed in larval and adult midguts of both species. In adults, abdominal metameric perivisceral organs were stained. Stained axons interconnected the perivisceral organs and neurosecretory cells in the abdominal ganglia. Episodic release of OEH I from these organs was evident in female *Ae. aegypti*, when staining disappeared at 12 hours after a blood meal and returned by 48 hours to levels observed before and up to 2 hours after the blood meal. Two sites were specifically stained only in *An. gambiae*: an axon net around the pyloric valve in the hindgut of larvae and adults and a ring of endocrine cells in the cardiac valve in the larval midgut. The markedly similar localizations of immunostained cells in larvae and adults of two distantly related species indicate that OEH I, or a homolog, is conserved within this group of Diptera and likely has stage- and sex-specific functions.

Keywords: insect, Diptera, *Anopheles gambiae*, *Aedes aegypti*, neurohormone, steroids, reproduction

Abbreviation:

CC	corpus cardiacum
LNC	lateral neurosecretory cells
MNC	medial neurosecretory cells
PVO	perivisceral organs
OEH I	ovary ecdysteroidogenic hormone I
VG	ventricular ganglia

Introduction

For invertebrates, the only steroidogenic gonadotropin identified to date is the ovary ecdysteroidogenic hormone (OEH I) from the yellow fever mosquito, *Aedes aegypti* (Brown et al., 1998). Ingestion of a blood meal by females of this species leads to the release of OEH I from neurosecretory cells, and it stimulates ovaries to secrete ecdysteroids, which modulate secretion of yolk proteins by the fat body (Clements, 1992). These proteins are taken up by oocytes during the first phase of egg maturation and utilized in the embryo. Native OEH I was isolated from female heads and partially sequenced. This sequence lead to the identification and cloning of a head-specific cDNA that encodes a prepropeptide that is processed into a bioactive peptide (Brown et al., 1998). Recombinant OEH I was purified from *Escherichia coli* transformed with modified *AeaOEH I* cDNA and shown to have the same bioactivity as the native peptide, as it stimulates yolk deposition *in vivo* when injected into blood-fed, decapitated *Ae. aegypti*, and ecdysteroidogenesis when incubated *in vitro* with ovaries from sugar-fed females (Matsumoto et al., 1989; Brown et al., 1998).

Throughout all life stages of insects, neurosecretory cells and midgut endocrine cells are known to be sources of an ever-increasing diversity of neuropeptides (Gäde et al., 1997). Initially, the source of OEH I in female *Ae. aegypti* was localized to medial neurosecretory cells in brains by microsurgery (Lea, 1967), and by immunocytochemistry on sectioned brains using an antiserum to the amino-terminus of OEH I (Brown et al., 1998). Other regions of the nervous system or midgut cells also may be a source of OEH, as suggested by the presence of OEH-like bioactive factors in headless bodies of female mosquitoes (Van Handel and Lea, 1984; Masler and Kelly, 1995). In addition, the existence of OEH I in *Ae. aegypti* larvae and males and other mosquito species has yet to be

determined. After chemical synthesis of the entire OEH I sequence, a polyclonal antiserum was produced to the peptide for use in an immunocytochemical study to address the above issues. As reported herein, cells containing OEH I, or homologs, were identified not only in brains but also in ventral nerve cords and guts of larvae and both sexes of *Ae. aegypti* and the African malaria mosquito, *Anopheles gambiae*. The tissue distribution of these cells is compared for the two species. Most notable was the immunostaining of an extensive perivisceral nervous system in adult mosquitoes that is described for the first time. Localization of OEH I, or a homologue, in different tissues, stages, and sexes suggests it that may regulate diverse processes during the development and reproduction of two mosquitoes that transmit devastating pathogens to humans and animals.

Materials and Methods

Mosquitoes

Larvae of *Ae. aegypti* and *An. gambiae* were reared at 27 °C on a mixture of yeast, lactalbumin hydrolysate and finely ground rat chow. Adults were maintained at 27 °C on 10% sucrose solution for the first two days, and thereafter, on water. Female *Ae. aegypti* were given access to anesthetized rats for blood feeding, and after 20 min, engorged females were separated and held for tissue dissections at different times after the blood meal.

Antiserum production

The entire sequence of OEH I, 86 amino acids including the pGlu amino terminus (8803 Da), was synthesized in the laboratory of Dr. Stephan Klauser (University of Zurich Hospital, Zurich, Switzerland), and the synthesis was confirmed by HPLC, amino terminus sequencing, and mass spectroscopy. After refolding and purification by HPLC, synthetic OEH I was shown to be bioactive in both the *in vivo* and *in vitro* bioassays (Brown et al., 1998; M. R. Brown, unpublished observations). The unpurified synthetic peptide was used as an antigen in rabbits (2 mg of peptide/animal in 0.5 ml of Freund's complete adjuvant and phosphate-buffered saline solution). Four antigen boosts (1 mg antigen/animal in same mix but with incomplete adjuvant) were made every four to five weeks. Two weeks after each immunization, sera were prepared and stored at –80 °C; only sera from the last two boosts (rabbit 303 C, D or rabbit 304 C, D) were used for immunocytochemistry.

Whole-mount immunocytochemistry

Whole tissues were dissected into 4% paraformaldehyde fixative solution (4% paraformaldehyde in 2.5 mM NaH_2PO_4, 8.5 mM Na_2PO_4, and 175 mM NaCl, pH 7.4, PBS) and then transferred into fresh fixative solution on ice for up to 2 h. After washing in PBS containing 0.5% Triton 100 (PBS-T) on ice for up to 30 min, tissues were permeabilized with chilled ethanol washes (30,50,70,50, and 30% ethanol in fixative solution; 5 min/step). Tissues next were washed in PBS-T on ice for 30 min, blocked with 5% goat serum in PBS-T for 2 h on ice, and incubated with diluted primary antiserum (1:1000 or 1:2000 in PBS-T-1% goat serum containing 0.05% sodium azide) at 4°C, overnight. Tissues then were washed in PBS-T-1% goat serum three times for 60 min on ice and then incubated overnight at 4 °C with fluorescent-labeled secondary antibodies (Alexa 488-goat anti-rabbit IgG (H+L); Molecular Probes, Inc; 1:2000 dilution in PBS-T) or peroxidase-conjugated secondary antibodies (Sigma; 1:50 dilution in PBS-T; stained with diaminobenzidine tetrahydrochloride). After washing in PBS-T three times for 60 min at 4 °C, tissues were mounted on slides in a 1:1 mixture of glycerol and PBS for observation. Tissues from five or more individuals treated or staged in the same way were examined or photographed with an Olympus BX60 microscope equipped with an epi-fluorescent light source.

To confirm staining specificity of the primary antiserum, tissues were treated as above with OEH I antisera (1:2000) preabsorbed with the antigenic peptide (40 µg/ml, overnight at 4 °C), preimmune sera, and fluorescent-labeled secondary antibodies alone.

Results

After treatment with antisera specific to synthetic OEH I and fluorescent-labeled secondary antibodies, the distribution of immunostained cells was observed and recorded for brains, midguts, and ventral nerve cords that had been dissected from larval and adult mosquitoes. Abdomens that had been split along one side and midguts removed were similarly treated, so that immunostaining in the nerve tracts connecting the PVO and ganglia could be observed *in situ*. These observations are summarized below in separate sections for the nervous system and gut of both larvae and adults of the two mosquito species. The localization of OEH I in cells was judged to be specific for tissues of *Ae. aegypti* based on the absence of immunostaining in experimental control tissues (results not shown). For these controls, tissues from larvae and adults were treated with antisera preabsorbed with synthetic OEH I or with preimmune serum, followed by labeled secondary antibodies, or with secondary antibodies alone. Tissues from *An. gambiae* subjected to the same experimental controls also were negative, thus the immunostained cells in the different tissues likely contain an OEH I homolog.

1. General description of the nervous system

The central nervous system of both mosquitoes is comprised of the brain and the ventral nerve cord, which includes the subesophageal ganglion, fused thoracic ganglia and abdominal ganglia (Clements, 1992). In larvae, the brain has two distinct hemispheres and is separate from the subesophageal ganglion. The ventral nerve cord includes eight abdominal ganglia (Fig. 1 A, B).

In adults, the subesophageal ganglion is fused to the brain, and only six abdominal ganglia are present due to the fusion of the first abdominal ganglion to the metathoracic ganglion and the seventh and eighth ganglia fusing into a terminal ganglion (Fig. 1 C, D).

The stomatogastric nervous system innervates the gut and associated organs in both larvae and adult mosquitoes (Fig. 2 A-D). It is comprised of the interconnected frontal ganglion, hypocerebral ganglion, corpus cardiacum, corpora allata, and ventricular ganglia (Clements, 1992). In adults, the corpus cardiacum (CC) is embedded in the cephalic aorta and connected by nerves to the brain, hypocerebral ganglion, and the corpora allata (Meola and Lea, 1972).

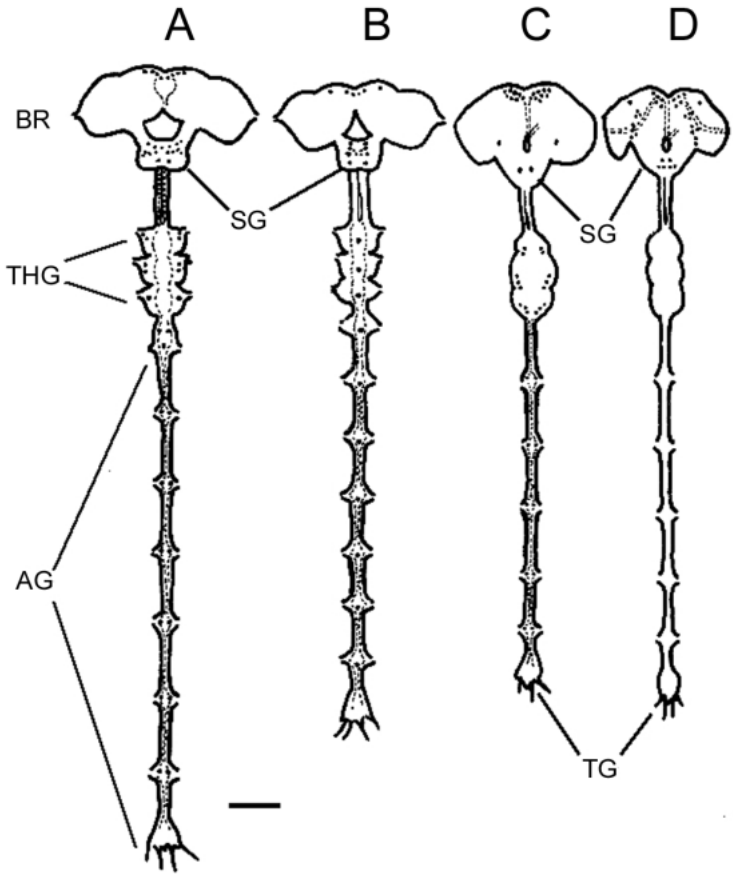

Figure 1. Distribution of OEH I immunostained cells and axons in the central nervous system of fourth instar larvae of *Aedes aegypti*, A, and *Anopheles gambiae*, B, and female *Ae. aegypti*, C, and *An. gambiae*, D. AG, abdominal ganglion; BR, brain; SG, subesophageal ganglion; THG, thoracic ganglion; TG, terminal ganglion. Filled circles: immunostained cells, dotted lines: immunostained axons. Scale bar = 200 μm.

Paired esophageal nerves connect the CC to the ventricular ganglia (VG) on each side of the cardiac valve. Nerve tracts extend from the VG to the salivary glands, midgut, and crops of adults (Clements, 1992).

Perivisceral organs (PVO, also known as perisympathetic organs) are distributed metamerically in the body of diverse insects and function as release sites for neurosecretory cells in the ganglia of the ventral nerve cord (Raabe, 1989; Nassel, 1996).

An unexpected result of this study was the immunostaining of organs in the abdomens of adult mosquitoes that resembled the PVO (see section 4 below).

2. Immunostaining in the nervous system of fourth instar larvae

Ae. aegypti—Figure 1A: Two clusters of medial neurosecretory cells (MNC), each with approximately ten cells, were stained in the dorsal protocerebrum of larval brains (Fig. 3A). Stained axons from the clusters formed a chiasma, exited the brain in discrete nerve tracts to the CC, and extended along the foregut to the midgut (see section 5 below). In thesubesophageal ganglion, three to five bilateral groups of two or more stained cells (Fig. 3A) were connected by stained axons. On occasion, up to four stained cells on each side of the thoracic ganglia were observed (Fig. 3A).

In the abdominal ganglia, a pair of lateral cells was stained, except for the first abdominal ganglion, which had one stained medial cell. Finely stained axons extended along the ventral nerve cord (Fig. 3A).

An. gambiae—Figure 1B: In larval brains, a pair of lateral neurosecretory cells (LNC) was stained consistently (Fig. 3B), and the MNC were weakly stained in a few brains. Stained axons, presumably from brain neurosecretory cells, were evident on the gut (see section 5 below), but were indiscernible in the brains or CC. The subesophageal ganglion contained three bilateral clusters of stained cells (Fig. 3C). An unpaired medial cell was weakly stained in the ventral region of the thoracic and abdominal ganglia (Fig. 3E). Occasionally, cells on each side of the abdominal ganglia were similarly stained (Fig. 3E). Stained axons were evident dorsally along the thoracic ganglia (Fig. 3D) and as a network in the abdominal ganglia (Fig. 3E).

3. Immunostaining in the adult nervous system

Ae. aegypti—Figure 1C: In female brains, up to twelve

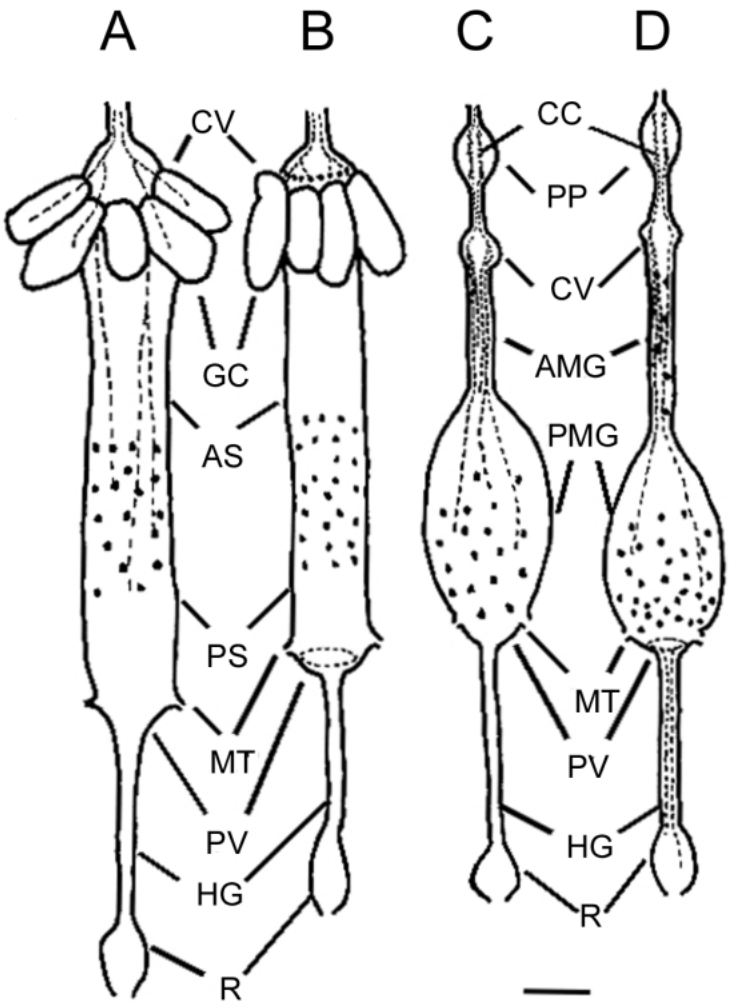

Figure 2. Distribution of OEH I immunostained cells and axons in the stomatogastric nervous system and gut of fourth instar larvae of *Aedes aegypti*, A, and *Anopheles gambiae*, B, and female *Ae. aegypti*, C, and *An. gambiae*, D. AMG, anterior midgut; AS, anterior stomach; CC, corpus cardiacum; CV, cardiac valve; GC, gastric caeca; HG, hindgut; MT, Malpighian tubules; PMG, posterior midgut; PS, posterior stomach; PV, pyloric valve; R, rectum. Filled circles: immunostained cells, dotted lines: immunostained axons. Scale bar = 400 μm.

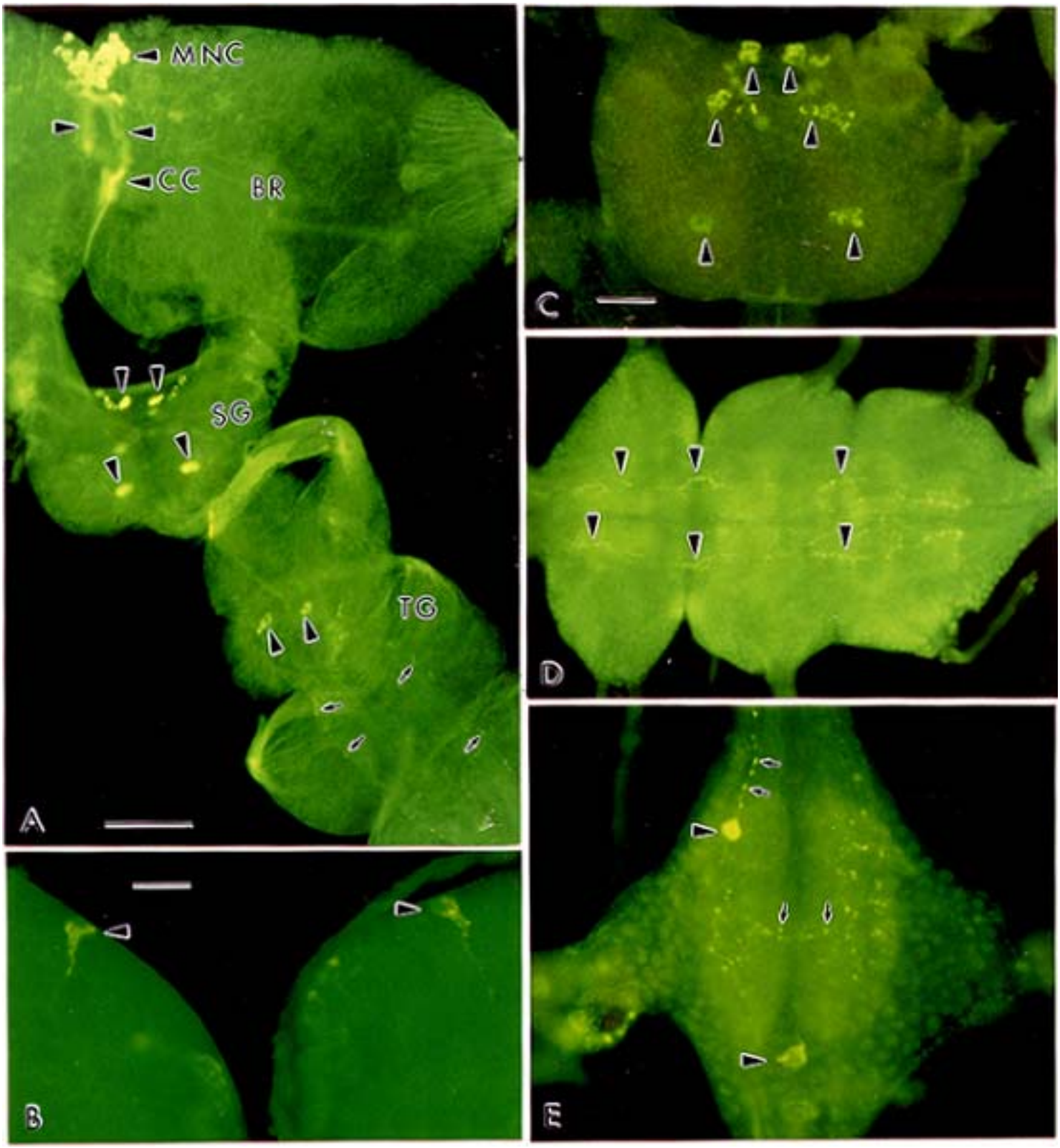

Figure 3. OEH I immunostaining in the nervous system of fourth instar larvae. A. Immunostained medial neurosecretory cells (MNC, arrowhead) with axon bundles (arrowheads) that exit the brain (BR) and enter the corpus cardiacum (CC) of an *Aedes aegypti* larva. Other immunostained cells (arrowheads) and axons (arrows) occur in the subesophageal ganglion (SG) and thoracic ganglia (TG). B. Pair of immunostained lateral neurosecretory cells (arrowheads) in the brain of an *Anopheles gambiae* larva. C. Groups of immunostained neurosecretory cells (arrowheads) in the subesophageal ganglion of an *An. gambiae* larva. D. Immunostained axons (arrowheads) in the dorsal region of the thoracic ganglia of an *An. gambiae* larva. E. Immunostained cells (arrowheads) and axons (arrows) in the seventh abdominal ganglion of an *An. gambiae* larva. Top or right of figure, anterior or dorsal. Scale bar = 67 µm for A; 40 µm for C and D; 20 µm for B and E.

pairs of MNC in bilateral clusters were densely stained in the dorsal protocerebrum (Fig. 4A). Stained axons extending from these clusters formed a chiasma and arched through the brain to emerge ventrally in the nervi corpris cadiaci (Fig. 4A). Thesenerve tracts continued over the pharyngeal pump to enter the CC (Fig. 4A). Within the CC, varicosities (Fig. 4A) were densely stained along axons that extended in the esophageal nerves to the anterior midgut as described in section 6 below. On occasion, stained axons were evident in the nerves connecting the CC to the CA. In seven day old and older females, two immunostained cells appeared between the optic lobes and protocerebrum.

Consistent staining of cells was observed in the ventral nerve cord: a pair of cells in the subesophageal ganglion (Fig. 4B), three or four pairs of cells in each thoracic ganglion (Fig. 4C), and two cells in each abdominal ganglion (Fig. 6D). Finely stained axons extended throughout ventral nerve cord. This same pattern of immunoreactivity was observed in the male brain and ventral nerve cord.

An. gambiae—Figure 1D: One or two pairs of weakly stained LNC were observed frequently in brains of both sexes (Fig. 4D, E), and in some males up to six pairs of MNC were stained similarly (Fig. 4D). In the ventral nerve cord of both sexes four pairs of cells were stained consistently in the subesophageal ganglion, but only a few cells and axons in the thoracic ganglia and abdominal ganglia were weakly stained.

In older *An. gambiae* females, the number of immunostained cells and the staining density of axons increased in the brain (Fig. 4E). Five or more immunostained cells first became evident in the optic lobes of seven-day-old females (Fig. 4E), and stained axons appeared to extend from these cells into the medulla (Fig. 4E). In addition, one or two pairs of cells were observed in the abdominal ganglia of some older females.

4. Immunostaining in the perivisceral nervous system in adults—Figure 5

Elongated sac-like organs resembling PVO were stained

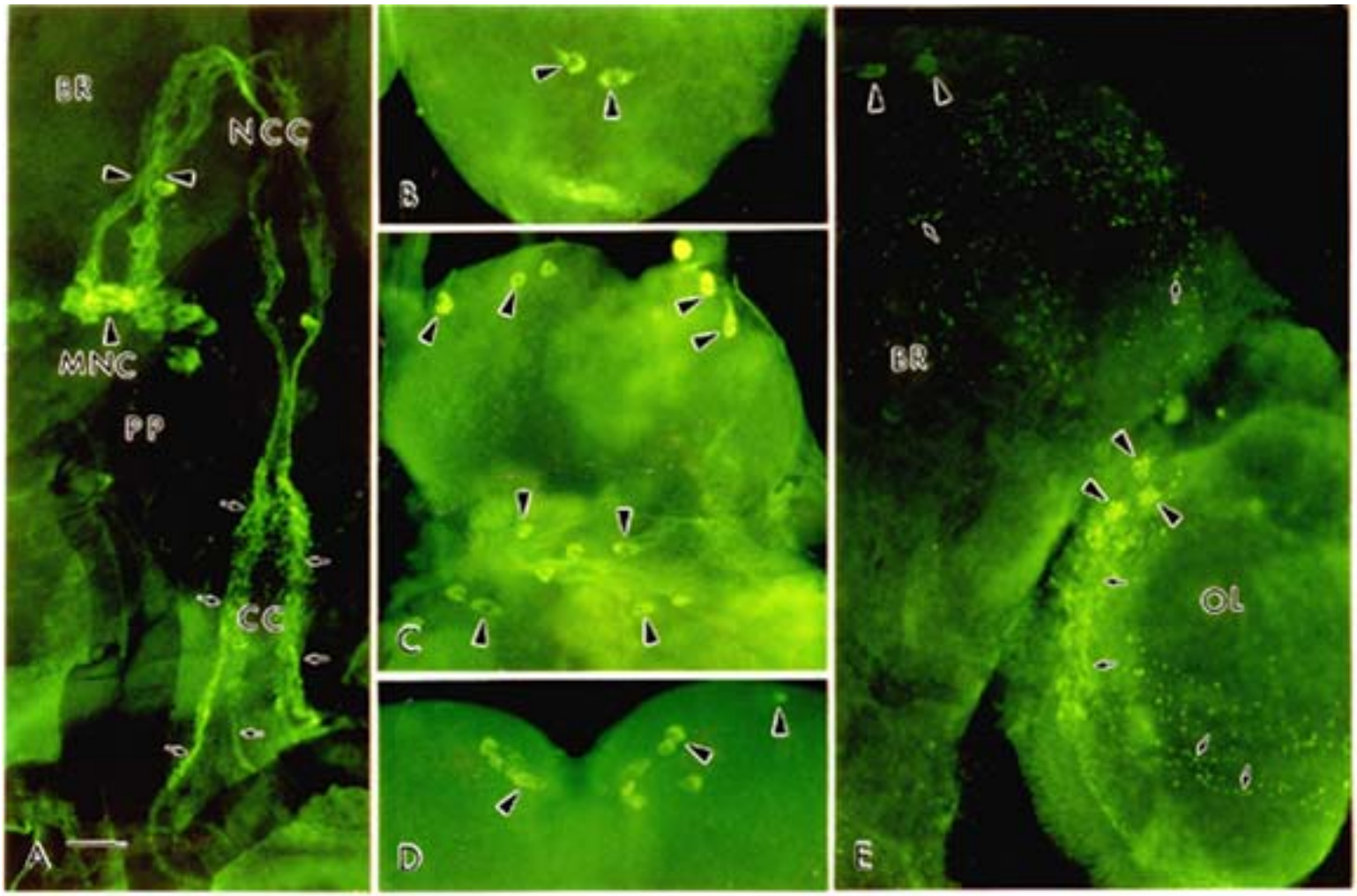

Figure 4. OEH I immunostaining in the nervous system of adults: A-C, 3 day old female *Aedes aegypti*. A. Clusters of immunostained MNCs (arrowhead) and their axons form a chiasma (arrowheads) and arch in the brain (BR), exit in the nervi corpris cadiaci (NCC), and pass over the pharyngeal pump (PP) to the corpus cardiacum (CC). In the CC, immunostained varicosities (arrows) occur along these axons, which exit in the esophageal nerves. Dorsal to ventral of whole brain; anterior, top. B. A pair of immunostained cells (arrowheads) in the subesophageal ganglion. C. Immunostained cells (arrowheads) in the fused thoracic ganglia. D. Immunostained MNCs (arrowheads) and a lateral cell (arrowhead) in the brain of a four day old male *Anopheles gambiae*. E. Immunostained cells (arrowheads) and axons (arrows) in the brain and optic lobes (OL) of a seven day old *An. gambiae* female. Top or right of figure, anterior or dorsal. Scale bar = 40 μm for A-E.

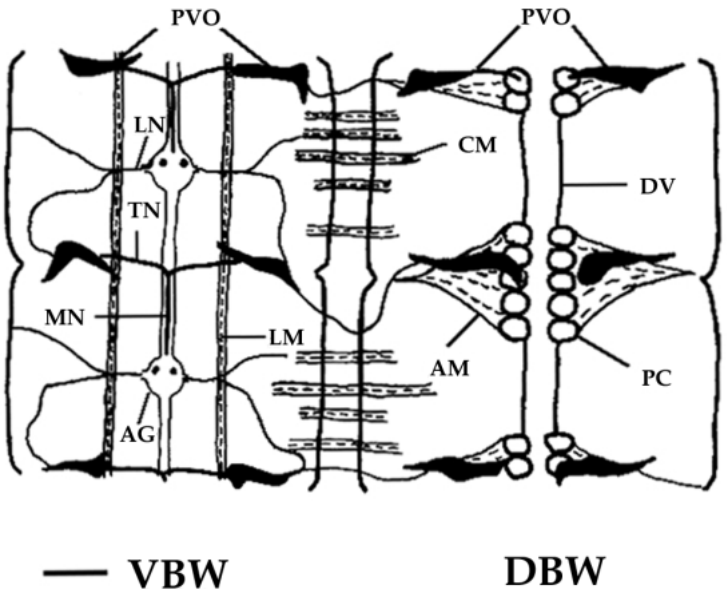

Figure 5. Distribution of OEH I immunostained cells and axons in the abdominal ganglia (AG) and perivisceral organs (PVO) associated with the dorsal (DBW) and ventral (VBW) abdominal body wall of female *Aedes aegypti*. AG, abdominal ganglia; AM, alary muscles; CM, circular muscles; DV, dorsal vessel; LM, longitudinal muscles; LN, lateral nerves; MN, median nerve; PC, pericardial cells; TN, transverse nerves. Filled circles: immunostained cells, dotted lines: immunostained axons. Scale bar = 400 μm.

in the abdomens of adults. In each of seven abdominal segments, a pair of PVO was associated with the ventral body wall and the other pair with the dorsal vessel (Fig.5)—a total of 28 PVO/abdomen. Shape, location, staining density, and number of the PVO in the abdomen were the same in female and male *Ae. aegypti* (Fig. 6A, B, D, E) and *An. gambiae* (Fig. 6C). Occasionally, one or two stained organs were found in tissues dissected from thoraces. No comparable organs were observed in *Ae. aegypti* or *An. gambiae* larvae treated with the OEH I antisera.

In adults, the dorsal and ventral pairs of PVO in each segment were positioned respectively in the sinus between the body wall and diaphragms at the anterior end of each cuticular plate (Fig. 5, 6A-C). The ends of the PVO were attached to the body wall by thin strands, with one end of the dorsal PVO in proximity to pericardial cells along the dorsal vessel (Fig. 6B). As observed with immunoperoxidase staining, the ventral PVO wrapped around the attachment of the diaphragm to the lateral body wall (Fig. 6E). In *Ae. aegypti* adults, weakly stained axons were observed in nerve tracts extending from the abdominal ganglion to the PVO in each segment.

These axons, presumably from a pair of stained ganglion cells, were first evident in the unpaired median nerve and transverse nerves that passed out of the ganglion over the ventral diaphragm to the ventral PVO (Fig. 6D), and continued along the body wall to the dorsal PVO. Lateral nerves extending from each side of the ganglion also contained stained axons. These nerves extended over the ventral longitudinal muscles, the circular muscles connecting the ventral and dorsal body walls, and to the PVO in the same and adjoining segment.

To discern whether or not the PVO function as neurohemal organs and release OEH I, the number of immunostained PVO was recorded for female *Ae. aegypti* before and at different times after a blood meal (Table 1). In non-blood fed females and ones up to 2 h after the blood meal, most PVO were stained densely (average of 22 or 23 per abdomen). At 6 h after the blood meal, the number of stained PVO began to decrease, and at 12 h and 24 h after the blood meal, the average number of stained PVO observed had decreased to eight and four per abdomen, respectively. The density of staining in the PVO at 6, 12, and 24 h after the blood meal was considerably less than that observed in females before or shortly after the blood meal. Together, these observations suggest that OEH I was released from these organs 6 to 12 h pbm and not replenished. Notably, by 48 h the average number of stained PVO per female was the same as that observed in sugar-fed females.

In contrast, there was no observable change in the immunostaining of cells or axons in the brain, CC, and esophageal nerves taken from the same and other females similarly staged after a blood meal.

5. Immunostaining in the stomatogastric nervous system and gut of larvae

For both mosquito species, the larval midgut consists of a cardiac valve surrounding the junction with the foregut, eight gastric caeca, and an anterior and posterior stomach extending to the Malpighian tubules and hindgut (Fig. 2A, B). Cone-shaped endocrine cells packed with secretory granules are dispersed throughout the midgut epithelium of larval and adult mosquitoes (Brown et al, 1985, 1986; Veenstra et al. 1995). Typically, these cells are positioned basally in the epithelium and have apical extensions to the lumen.

Ae. aegypti—Figure 2A: Stained axons originating from brain neurosecretory cells were evident in the esophageal nerves extending from the CC to the midgut. The nerves with stained varicosities branched over the cardiac valve, the caeca, and the anterior stomach (Fig. 7A). Approximately 140 endocrine cells were stained in each of the posterior stomachs examined.

An. gambiae—2B: Two aspects of the immunostaining in the gut of *An. gambiae* larvae differed from that of *Ae. aegypti*. A ring of endocrine cells in the cardiac valve (Fig. 7C) and an axon net in the pyloric valve were stained only in the gut of *An. gambiae*. In common were the stained axons in the esophageal nerves that

Table 1. Changes in the number of immunostained perivisceral organs (PVO, one dorsal and one ventral pair/segment—28 total) observed in abdomens of female *Aedes aegypti* before and after blood-feeding.

Time before or after a blood meal	Average number of immunostained PVO/female body wall (Number of female body walls observed)
Sugar-fed	22 (50)
1 h post blood meal (pbm)	23 (10)
2 h pbm	23 (10)
6 h pbm	17 (10)
12 h pbm	04 (10)
24 h pbm	08 (10)
48 h pbm	25 (10)

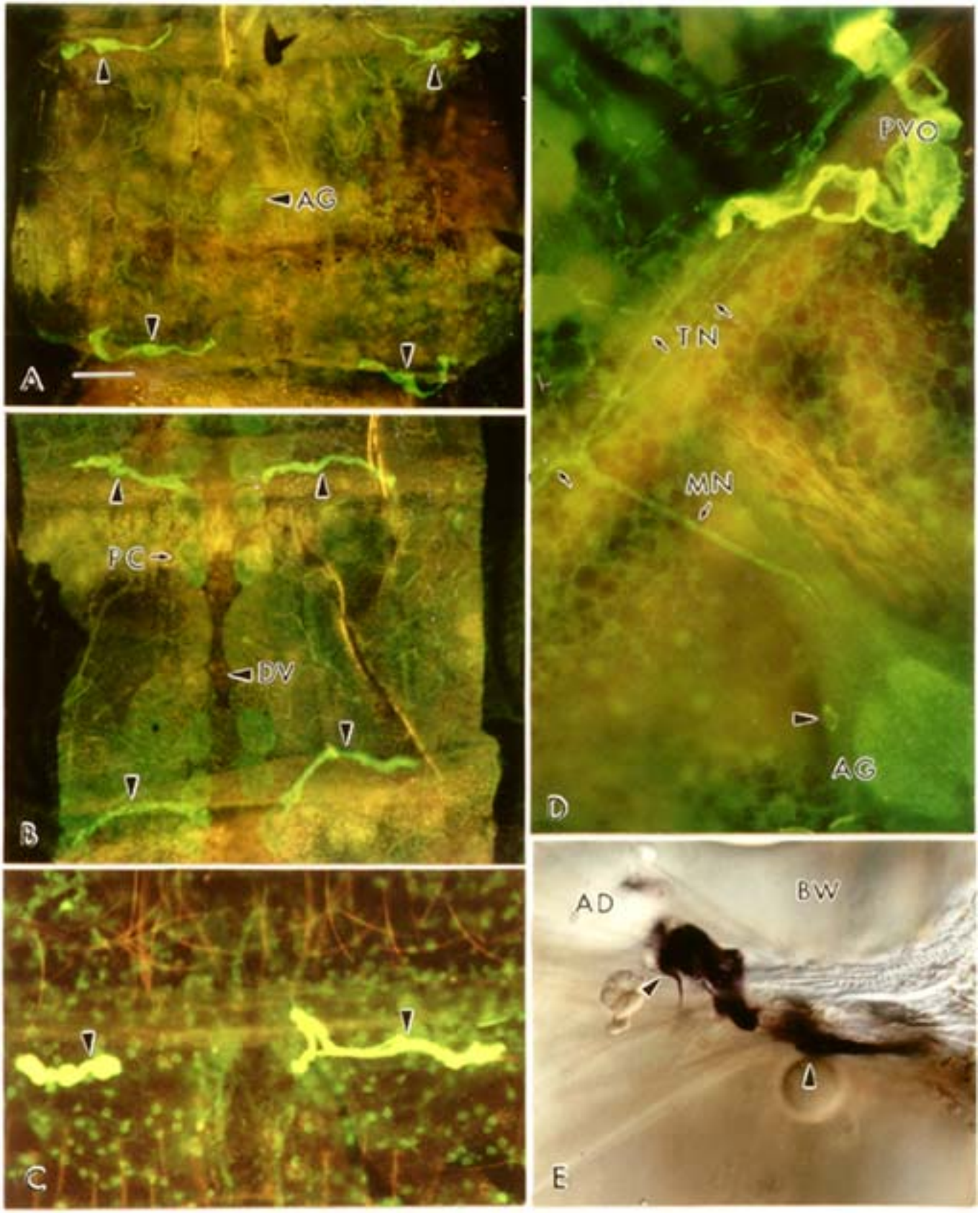

Figure 6. OEH I immunostaining in the perivisceral organs (PVO) and abdominal ganglia of adults. A. Pairs of immunostained PVO (arrowheads) at the anterior end of segments in the ventral body wall of a three day old *Aedes aegypti* female. AG, abdominal ganglion. B. Immunostained PVO (arrowheads) similarly positioned near the pericardial cells (PC) associated with the dorsal vessel (DV) in the dorsal body wall of the same female as in A. C. Immunostained PVO (arrowheads) in the dorsal body wall of an 11 day old *Anopheles gambiae* female. D. Detail of an abdominal ganglion and immunostained PVO in the ventral body wall of a five day old *Ae. aegypti* male. Immunostained axons (arrows) extend (out of the focal plane) from the immunostained cell (arrowhead) in the AG through the unpaired median nerve (MN) and transverse nerves (TN) to the PVO. E. Immunoperoxidase-stained PVO (arrowheads) wrapped around the attachment of the abdominal diaphragm (AD) to the ventral body wall (BW) of a six day old *Ae. aegypti* female. Top or right of figure, anterior or dorsal. Scale bar = 80 µm for A and B; 40 µm for C; 20 µm for D and E.

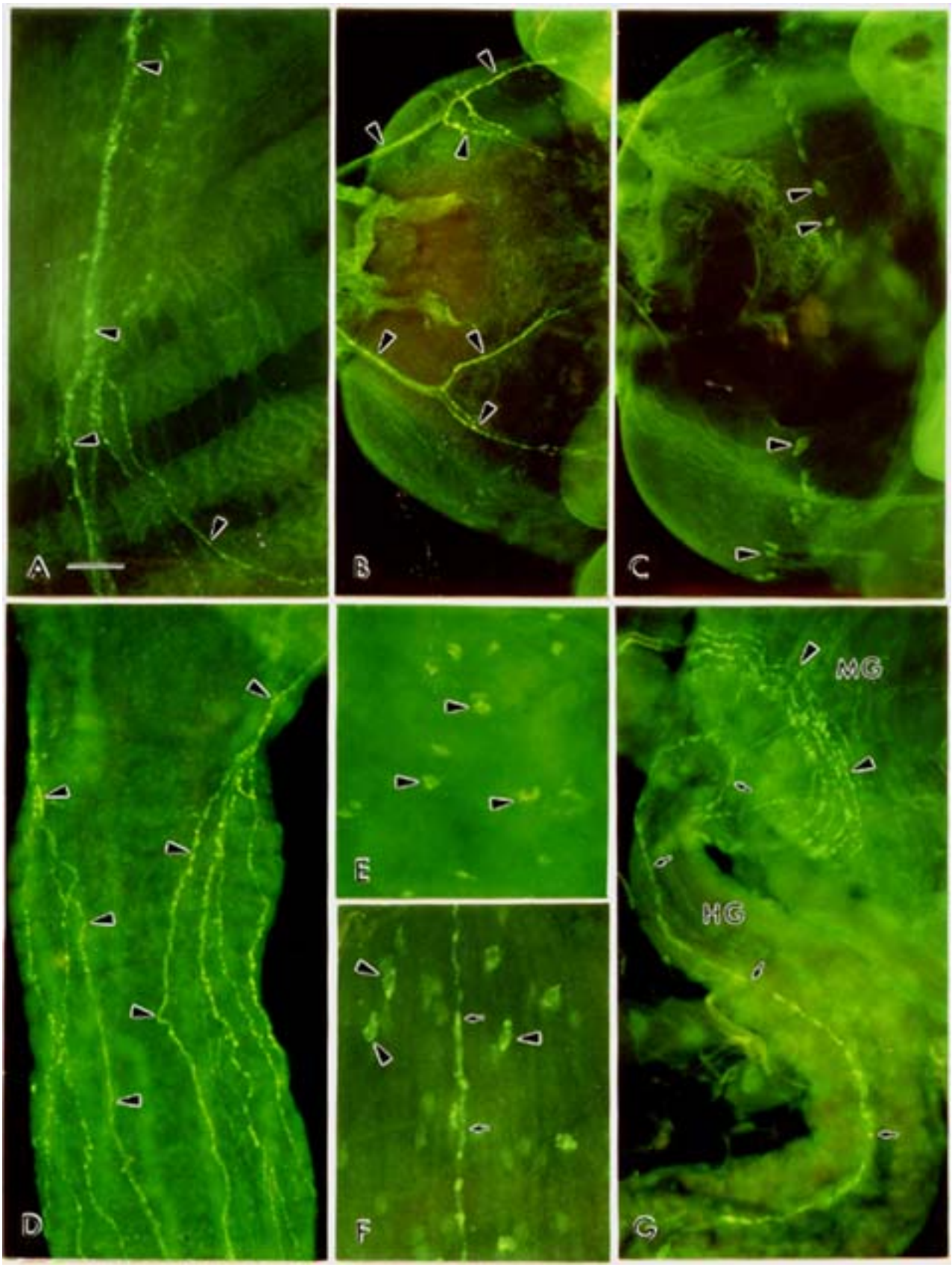

Figure 7. OEH I immunostaining in the stomatogastric nervous system and gut of fourth instar larvae and adults. A. Detail of immunostained axons (arrowheads) branching over the anterior stomach of an *Aedes aegypti* larva. B. Immunostained axons (arrowheads) in the esophageal nerves branching over the cardiac valve of an *Anopheles gambiae* larva. C. Ring of immunostained endocrine cells (arrowheads) in the cardiac valve of an *An. gambiae* larva. D. Immunostained axons (arrowheads) branching over the anterior midgut of a four day old *Ae. aegypti* female. E. Immunostained endocrine cells (arrowheads) in the posterior stomach of an *An. gambiae* larva. F. Immunostained endocrine cells (arrowheads) and axons (arrows) in the posterior midgut of a four day old *Ae. aegypti* female. G. Immunostained axon net (arrowheads) around the pyloric valve with a pair of axons (arrows) extending along the hindgut (HG) of a five day old *An. gambiae* female. MG, midgut. Top or right of figure, anterior or dorsal. Scale bar = 20 μm for A and F; 40 μm for B-E and G.

branched only over the cardiac valve (Fig. 7B) and the stained endocrine cells (ca. 70 cells) in the posterior stomach (Fig. 7E).

6. Immunostaining in the stomatogastric nervous system and gut of adults

During metamorphosis, the larval gut is remodeled into a sexually dimorphic organ in both species. In adults, two small dorsal crops and a large ventral crop develop from the foregut just anterior to the cardiac valve, and nectar meals are stored in these organs. The caeca become the tubular, anterior midgut of adults, and the larval midgut changes into an expandable posterior midgut for blood digestion in females (Fig. 2C, D) and is much reduced in males. The midgut ends at the junction of the Malpighian tubules and pyloric valve of the hindgut.

Ae. aegypti—Figure 2C: In both sexes, immunostained axons were evident in the esophageal nerves connecting the CC and the ventricular ganglia (VG) on the cardiac valve. Past the VG, the nerves branched extensively along two sides of the anterior midgut and were marked by densely immunostained varicosities (Fig. 7D). Some of these nerves extended onto the posterior midgut, where approximately 140 endocrine cells (Fig. 7F) were stained in females, and 70 such cells in males. No stained cell bodies were observed in proximity to the CC or in the VG, thus the immunostained axons are thought to originate from the MNC.

An. gambiae—Figure 2D: A similar degree of immunostaining was observed in the esophageal nerves extending from the CC along the foregut and branching over the anterior midgut of both sexes of *An. gambiae*. These axons are also thought to originate from neurosecretory cells in the brain because no other stained cell bodies were observed in the CC or VG. Endocrine cells in the posterior midgut were smaller and weakly stained in this species, but more were observed: ca. 280 cells/female midgut and 120 cells/male midgut. The most notable difference between the two species was the densely stained axon net encircling the pyloric valve (Fig. 7G). A pair of stained axons extended from this nerve net along the hindgut (Fig. 7G) to the rectum. Presumably, these axons originated from cells in the ventral nerve cord, but a direct connection was never observed.

Discussion

The neurohormone, OEH I, originally was isolated from heads of adult *Ae. aegypti*, but as detailed in this report, cells were immunostained by OEH I antisera not only in the brain, but also throughout the nervous system and gut of larvae and both sexes of *Ae. aegypti*. Parallel studies of *An. gambiae* revealed a similar distribution of such cells in larvae and adults, thus providing the first evidence for an OEH I homolog in this species. In both species, the cells in the different tissues generally persisted from larvae to adult, although the degree of immunostaining and the number of cells varied between stages. The presence of OEH I, or a homolog, in immunostained cells of both species was verified by the absence of immunostaining when tissues known to contain such cells were treated with antiserum preabsorbed with synthetic OEH I or with preimmune serum. The surprisingly similar and extensive expression of OEH I in larvae and both sexes of two distantly related species indicates that this peptide hormone, or its homologs, may be multifunctional and conserved among mosquitoes.

Neuroendocrine release sites for OEH I

Neurosecretory cells are distributed throughout the nervous system of insects in all post-embryonic stages, and neuroendocrine messengers are released from these cells at specific sites along their axons. In adults of both species, the densely immunostained varicosities in the CC and esophageal nerve along the anterior midgut are indicative of release sites for OEH I or a homolog synthesized in the MNC. Classic endocrine studies first demonstrated that brain neurosecretory cells and their axons extending to the CC were the source of the "egg development neurosecretory hormone" in female mosquitoes (Lea, 1972). This hormone was later renamed "ovary ecdysteroidogenic hormone" (Matsumoto et al., 1989). Histological and ultrastructural studies of sectioned mosquito heads provided additional details about this neuroendocrine axis (Meola and Lea, 1972; Clements et al., 1985). The MNC of *Ae. aegypti* females have also been shown by immunocytochemistry to be the source of leucokinin-like peptides (Chen et al., 1994) and FMRFamide-like peptides (Brown and Lea, 1988). At this time, it is not known whether these peptides are localized in the same MNC as OEH I. After a partial amino-terminal sequence of OEH I was obtained, a specific antiserum to this sequence was shown to stain the MNC in brains of female *Ae. aegypti* and axons in the CC (Brown et al., 1998). These observations were confirmed and expanded by the use of an antiserum made to the entire OEH I sequence and reacted against whole-mounts of the nervous system and midguts of larvae and adults in the present immunocytochemical study. These preparations favored the tracing of immunostained axons from the densely stained MNC in the brain of either a larva or an adult *Ae. aegypti* through the CC and onto the anterior region of the midgut. In contrast, the LNC and MNC were weakly immunostained in *An. gambiae* larvae and adults, but the axons extending from the CC to the midgut were stained to the same degree as observed in *Ae. aegypti*. This difference in staining may be due to the presence of unprocessed OEH I homolog in cells that is not as readily recognized by the OEH I antiserum as the processed form in axons. Nevertheless, the staining of axons on the midgut suggests that this part of the stomatogastric nervous system, as well as the CC, are the major release sites for brain neurosecretory cells in both species.

Other neurosecretory-type cells were stained with OEH I antisera to varying intensities throughout the ventral nerve cords of larvae and adult of both species. In both sexes of *Ae. aegypti*, immunostained axons extended from a pair of cells on each side of the abdominal ganglia through the unpaired median and paired transverse nerves to the immunostained dorsal and ventral PVO in each segment (Fig. 5). Comparably stained axons were not observed in the abdomens of adult *An. gambiae*, but the PVO were immunostained to the same density as in *Ae. aegypti.* The localization of OEH I in the PVO of adult *Ae. aegypti* was specific as determined with antisera to Arg-Phe-amide peptides used on similarly prepared abdomens. These antisera did not stain the PVO but did immunostain cells in the abdominal ganglia (results not shown).

This is the first description of PVO in mosquitoes. In other insects, cells in the ventral nerve cord similarly innervate these organs (Raabe, 1989; Nassel, 1996). Ultrastructural studies of the

PVO in a variety of insects have shown that they are swellings in the median or transverse nerves with sinuses and numerous neurosecretory terminals ensheathed by glial cells (Raabe, 1989). These studies provide a structural basis for the assertion that the PVO are release sites for neurosecretory cells in the ventral nerve cord. This function is implied from results of an experiment performed with blood-fed female *Ae. aegypti*. The release of OEH I from the PVO between 6 to 12 h after the blood meal was indicated by the absence of immunostaining in the PVO at 12 and 24 hours, and its subsequent return by 48 hours to levels observed before and up to 6 hours after the blood meal.

Gut release sites for OEH I

Several hundred endocrine cells are dispersed throughout the midgut of a female *Ae. aegypti* (Brown et al., 1985; 1986; Veenstra et al., 1995) and considerably fewer such cells exist in the midgut of a larva or a male. Similar cells presumably exist in *An. gambiae*. In both species, a specific population of these cells in the posterior stomach of larvae and the posterior midgut of adults was immunostained with the OEH I antisera and thus are likely endocrine or paracrine sources of OEH I, or a homolog. Immunostained endocrine cells were localized in another midgut area only in *An. gambiae*. These cells formed a ring in the cardiac valve of larvae and were dispersed in the anterior midgut of adults. Different peptides have been localized to specific populations of endocrine cells in the midgut of female *Ae. aegypti* (Brown et al, 1986; Veenstra et al, 1995), and at this time it is not known whether OEH I defines yet another population of endocrine cells or is colocalized with cells producing another peptide hormone in the midgut of this species.

Another notable difference between the two species was the immunostaining of an axon net around the pyloric valve in the hindgut of *An. gambiae* larvae and adults. From this net, immunostained axons extended along the hindgut and rectum, and these axons are thought to originate from cells in the nervous system. A comparable axon net in the hindgut of female *Ae. aegypti* was stained by antisera to Arg-Phe-amide peptides and several kinins (Veenstra et al., 1995), but not by the OEH I antisera. Localization of different peptides in this axon net in the same gut region suggests that it is a conserved release site used by neurosecretory cells in the ventral nerve cord and, possibly, the brain of mosquitoes, as demonstrated for eclosion hormone in the tobacco hornworm, *Manduca sexta* (Riddiford et al, 1994).

Functional significance of OEH I immunolocalization

For female *Ae. aegypti*, OEH I is known to directly activate steroidogenesis by isolated ovaries and, as a consequence of this action, indirectly stimulate egg maturation when injected into blood-fed, decapitated female *Ae. aegypti* (Matsumoto et al., 1989; Brown et al., 1998). Surgical manipulations of the MNC, parabiotic twining, and timed decapitations of females after a blood meal point to the MNC as the source of OEH I and its presence in hemolymph (Klowden, 1997). The hemolymph titer of OEH I *per se*, however, has yet to be determined for blood-fed females. As described above for *Ae. aegypti* females, there are two main neuroendocrine sites for the release of OEH I into the hemolymph: 1) the axonal region of the MNC extending from the CC and along the anterior midgut and 2) the abdominal PVO. There were no qualitative changes in the immunostaining of the MNC or their axons in female *Ae. aegypti* observed at different times after a blood meal, thus suggesting either that release of OEH I from these axons is rapidly replenished, or that release does not occur from this site. In these same females, OEH I released from the PVO by 12 h pbm, however, was not replenished until 48 h pbm. Release of OEH I from both sites presumably is regulated by the brain, which has been shown by timed decapitations to be required for up to 16 h pbm for egg maturation to occur at a normal rate (Greenplate et al., 1985). Distention of the abdomen of female mosquitoes by the blood meal also mediates egg maturation (Klowden, 1997) and may be another required signal for the release of OEH I from the PVOs several hours after the blood meal.

More than one hundred endocrine cells in the posterior midgut, where blood digestion occurs in females, are a putative source of OEH I that is independent of the nervous system. The presence of OEH I in such cells and in the ventral nerve cord and PVO likely accounts for the reported presence of OEH I-like factors in abdomens of female mosquitoes (Van Handel and Lea, 1984; Masler and Kelly, 1995). Interestingly, in females decapitated shortly after a blood meal, the putative OEH I in any one or all of these tissues is not released in the quantity or time period needed for egg development. Further studies of the immunoreactive peptides in these tissues are needed to determine whether or not they are structurally related to OEH I, especially since the unpurified synthetic OEH I was used to immunize rabbits. The resultant antisera likely recognize multiple epitopes of the peptide, and some of these epitopes may be displayed differently as OEH I-related peptides are processed in the cells of different tissues. One or more of the epitopes may be shared by other proteins or peptide hormones, yet to be identified. It is important to note that the cells immunostained by the OEH I antisera in the different tissues are likely sources of peptide hormones based on their morphology, and as no general immunostaining of tissues was observed, other more common proteins did not posses such epitopes.

Because OEH I immunostained cells were similarly distributed in the nervous system and gut of larvae and both sexes of the two mosquito species, OEH I cannot be regarded as a female specific peptide. The regulatory roles of OEH I or a homolog in larvae and males, and even female *An. gambiae*, have yet to be defined. Results from preliminary studies indicate that OEH I does not stimulate the production of ecdysteroids by the body walls of *Ae. aegypti* larvae and pupae, which are known to be steroidogenic (Jenkins et al., 1992), or by isolated testes from male *Ae. aegypt*i (M.R. Brown, unpublished results). Once the homolog is isolated from *An. gambiae* or synthesized from a nucleotide sequence similar to the *AaOEH I* cDNA, its steroidogenic activity in females can be ascertained.

An OEH I homolog also may regulate water balance or movement of food through the gut as indicated by immunostaining of the axon net on the hindgut of *An. gambiae* larvae and adults. Interestingly, neuroparsin A, which has antidiuretic activity in adult *Locusta migratoria* (Fournier and Girardie, 1988), is the only peptide hormone from other animals known to share significant sequence similarity with OEH I (Brown et al.,1998). The lack of sequence similarity to any other known insect peptides also provides additional support for our observations that OEH I, or a homolog, is produced

by conserved cell populations in the nervous system and midgut of different life stages of two distantly related mosquito species. More importantly, these localizations point to the conservation of an OEH I homolog among mosquito species that likely plays a key role in female reproduction. In time, its function in larvae and males may be discovered, and the extent of its conservation among other orders of Insecta may be divined with antisera and molecular probes specific to *Ae. aegypti* OEH I.

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