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Immunostaining for allatotropin and allatostatin-A and -C in the mosquitoes *Aedes aegypti* and *Anopheles albimanus*

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Abstract

Confocal laser-scanning microscopy was used to carry out a comparative study of the immunostaining for three families of neuropeptides, viz., allatostatin-A (AS-A), allatostatin-C (AS-C) and allatotropin (AT), in adult female mosquitoes of *Aedes aegypti* and *Anopheles albimanus*. The specific patterns of immunostaining for each of the three peptides were similar in both species. The antisera raised against AT, AS-A, and AS-C revealed intense immunoreactivity in the cells of each protocerebral lobe of the brain and stained cells in each of the ventral ganglia and neuronal projections innervating various thoracic and abdominal tissues. Only the AS-A antiserum labeled immunoreactive endocrine cells in the midgut. The distribution of the peptides supports the concept that they play multiple regulatory roles in both species.

Keywords

Immunocytochemistry; Neuropeptides; Confocal laser-scanning microscopy; Mosquitoes *Aedes aegypti*; *Anopheles albimanus* (Insecta)

Introduction

Allatostatins (AS) and allatotropins (AT) are structurally diverse peptides that were first described as modulators of juvenile hormone (JH) biosynthesis in the corpora allata (CA) of a number of insect species (Kataoka et al. 1989; Woodhead et al. 1989; Kramer et al. 1991; Lorenz and Hoffmann 1995; Gilbert et al. 2000). AS and AT have subsequently been recognized as having multiple physiological effects, controlling processes such as heart rate and gut motility, nutrient absorption, migratory preparedness, and modulation of the circadian cycle (Bendena et al. 1999; Nässel 2002; Petri et al. 2002; Elekonich and Horodyski 2003).

Three families of AS have been identified in insects: YXFGL-amide-AS (cockroach or type-A; AS-A), W_2W_9-AS (cricket or type-B; AS-B), and PISCF-AS (*Manduca* or type-C; AS-C); (Stay et al. 1994; Tobe et al. 1995; Duve et al. 1998; Weaver et al. 1998; Bendena et al. 1999). In contrast, only one type of AT has been isolated and functionally characterized; this AT was originally identified from the heads of pharate adult *Manduca sexta* (Mas-AT), with analogs being isolated from brain of *Spodoptera frugiperda* (Kataoka et al. 1989; Oeh et al.

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2000), from the male accessory glands of *Locusta migratoria* (Paemen et al. 1991), and from the mosquito *Aedes aegypti* (Veenstra and Costes 1999; Li et al. 2003).

The ability of these peptides to modulate JH synthesis in *A. aegypti* has recently been evaluated. *Anopheles gambiae* AS-C (homolog to *M. sexta* AS-C) significantly inhibits JH synthesis, whereas *A. aegypti* AS-A (homolog to cockroach AS-A) does not affect CA activity (Li et al. 2004). *A. aegypti* AT (Ae-AT) stimulates JH synthesis in a strong and dose-dependent manner (Li et al. 2003).

The aim in this work was to compare immunoreactivity for AT, AS-A, and AS-C in adult females of two important species of disease vector mosquitoes, viz., *A. aegypti* and *Anopheles albimanus*, by using confocal laser-scanning microscopy. The specific patterns of immunostaining for each of the three peptides were similar in both mosquito species. The AT, AS-A and AS-C antibodies stained brain and ventral ganglia cells and projections innervating various thoracic and abdominal tissues. Only AS-A antiserum showed immunoreactivity in midgut endocrine cells.

Materials and methods

Mosquitoes

A colony of *A. albimanus* white-striped pupa phenotype was established with insects collected in the state of Chiapas, Mexico (Chan et al. 1994). A colony of *A. aegypti* was established with insects collected in the state of Morelos, Mexico. Adults of both species were reared under a photoperiod cycle of 12 h light: 12 h dark, at 28°C and 70%–80% relative humidity, and were fed ad libitum with 5% sugar solution. All mosquitoes used in this study were 2–3 day-old mated females.

Tissue preparation

Mosquitoes were immobilized by brief exposure to ice, washed with 70% ethanol, and air-dried. Tissues were dissected in a drop of phosphate-buffered saline (PBS: 140 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 20.4 mM Na_2HPO_4, pH 7.2) containing a cocktail of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone, 1 mM EDTA, and 0.1 mg/ml leupeptin; Sigma, St. Louis, Mo., USA). The head and thorax were separated from the abdomen by making a small tear on both lateral pleural membranes. The abdomen was pulled off, and the gut was removed. Abdomens without gut were cut along the pleural membrane by using a needle. The abdominal body wall containing the fat body, epidermal sub-tegumental cells (mainly in pleural membranes), the dorsal vessel, the tracheal system, and the ventral abdominal ganglia will be referred to here as “abdomens”. The brain and subesophageal ganglion were removed with the corpus cardiacum–corpus allatum complex attached (Br–CC–CA). The three pairs of thoracic ganglia (prothoracic, mesothoracic, and methathoracic) were obtained by removing the coxae and dorsal thoracic area. Excess protease inhibitors in all tissues was removed by washes in PBS.

Primary antibodies against neuropeptides

Rabbit polyclonal antisera against *A. gambiae* AS-C and *A. aegypti* AT were produced against synthetic peptides conjugated to keyhole limpet hemocyanin by Genemed Synthesis (San Francisco, Calif., USA). Antisera titers were established, by enzyme-linked immunosorbent assay, to be adequate at dilutions of 1/10,000. The specificity of the AT and AS-C antisera was tested by liquid-phase preabsorption with the parent antigen at a concentration of 5.5 nmol/ml diluted antiserum (1/500), overnight at 4°C. Immunostaining was abolished following this treatment. The rabbit polyclonal antiserum against AS-A was a gift of Dr. Rene Feyereisen (Reichwald et al. 1994).

Immunocytochemistry

Midguts, Br–CC–CA complexes, thoracic ganglia, and abdomens were fixed for 4 h at room temperature with 4% formaldehyde in PBS. After fixation, tissues were rinsed in PBS (5×10 min each), permeated with 1% Triton X-100 in PBS at 4°C overnight, washed in PBS (3×20 min each) at room temperature, incubated for 2 h at 37°C in a solution of 2% bovine serum albumin in PBS containing 0.1% sodium azide (PBS-A; blocking solution), incubated at 4°C overnight with the primary antisera (anti-AT, anti-AS-A, or anti-AS-C) diluted 1:500 in PBS-A, and washed (5×10 min each) with 0.1% Tween-20 in PBS (PBS-T). All subsequent procedures were carried out with the samples being kept in the dark. Tissues were incubated for 2 h at 37°C with the secondary antiserum, a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Oncogene Researchc Products, Boston, Mass., USA), diluted 1:100 in PBS-A. Tissues were washed in PBS-T (5×10 min each) and mounted on glass slides in a fluorescence-preserving medium (Vectashield, Vector, Burlinghame, Calif., USA). Twenty females were analyzed in each of the studies.

Confocal microscopy

Images of the immunostained preparations were obtained with an Ultra View laser-scanning confocal system (model CSU10, Perkin-Elmer, Wellesley, Mass., USA) with an air-cooled argon-krypton laser and a 488 single-filter block (excitation 488 nm, emission 510 nm). The confocal system was used on an epi-fluorescent microscope (Nikon E-600, Japan). Images were processed by using Imaging Suite (Spatial Module) version 3.0 from Perkin-Elmer and by Power Point programs. Three-dimensional (3D) reconstructions were obtained from Z-stack data sets. Results are presented as 3D views in a single projection.

Results

The patterns of immunostaining for each of the three peptides were similar in both mosquito species. No staining was observed in the controls in the absence of the three primary antisera.

AT immunoreactivity

Seven to ten neurons were intensively stained in each protocerebral lobe, whereas others were weakly stained (Figs. 1a, 2a). A small group of stained cells was always observed in a posterior location, projecting processes toward the frontal region of the brain (Figs. 1a, 2a, arrowheads). We did not observe labeled varicosities along the nerve extending to the CC–CA complexes or inside the CC–CA complex.

All the abdominal ganglia of the ventral nerve cord showed AT immunoreactivity. Three cells were labeled in each ganglion, two in the posterior region and one in the anterior region (Figs. 1b–d, 2b, c). Immunostained projections from these cells were prominent in the ventral nerve cord (Fig. 1d, arrowhead) and emerging laterally (Figs. 1b, 2b, c, arrowheads). These varicose processes were observed innervating abdominal tissues (hindgut, heart, and oviducts; Figs. 1b, c, 2b, c, arrowheads). Strong staining was observed in many processes projecting from the nerve cord directly to the dorsal vessel; in addition, labeled varicosities were seen along the wall of the dorsal vessel (Fig. 2d). Immunoreactivity was not found in neurons of the thoracic ganglia or in midgut endocrine cells of either of the two species.

AS-A immunoreactivity

Six neurons were recognized in each protocerebral lobe (Figs. 3a, 4a). These cells had long processes (Figs. 3a, 4a, arrowheads) projecting toward the stomatogastric nervous system and leaving the brain most probably via the nervi corporis cardiaci. Only *A. albimanus* exhibited labeled processes innervating the CC–CA complex (Fig. 4b, arrowhead).

In the abdominal nerve cord (Figs. 3b–d, 4c, d), only one cell was stained per ganglion, except in the last ganglion (neuromeres 7+8) in which eight cells were stained (Figs. 3d, 4d). Immunoreactive processes projecting from the labeled cells of the last abdominal ganglion (Figs. 3d, 4d, arrowheads) innervated oviducts, bursa, and spermatheca (data not shown). In the other ganglia, only one process from a single cell bifurcated within the ganglion and projected laterally to the abdomen (Figs. 3b, 4c, arrowheads).

Oenocytes in the fat body were stained by anti-AS-A only in *A. albimanus* (Fig. 4d). The antiserum to AS-A intensely labeled processes projecting toward the pericardial cells but showed no staining that reached the wall of the dorsal vessel (Fig. 4f). About 50 endocrine cells located in the posterior midgut were strongly stained (Figs. 3e, 4e). Two prominent processes were labeled that extended along the hindgut and ended in the rectal papilla (Figs. 3e, f, 4e, arrowheads). Immunoreactivity was not observed in thoracic ganglia cells of either of the two species (data not shown).

AS-C immunoreactivity

Four cells were labeled in each protocerebral lobe (Fig. 5a–c). They had large nuclei (Fig. 5a, 2; Fig. 5c, 1) and large labeled vacuoles in their cytoplasm (Fig. 5b, inset). These cells projected toward the stomatogastric nervous system (Fig. 5a, b, arrowheads). We did not observe immunostaining in the CC–CA of either species (data not shown).

In the ventral cord nerve, two cells were labeled in each of the first six abdominal ganglia (Fig. 5d, e), whereas in the last ganglion (neuromeres 7+8), four cells could be recognized (Fig. 5f). In general, we did not observe immunostaining in processes extending from the ganglia, although weakly labeled varicosities were seen on a few occasions (data not shown). Immunoreactivity was not observed in neurons of the thoracic ganglia or in midgut endocrine cells of either of the two species (data not shown).

Discussion

This study provides evidence of major similarities in the expression of three neuropeptides in *A. aegypti* and *A. albimanus*, representing the first comparative study of regulatory peptides in members of two mosquito subfamilies with more than 100 million years of separate evolution (Knudson et al. 2002). *A. aegypti* has been an excellent model for the study of mosquito physiology for more than 50 years, and ample information thus exists regarding the neuroendocrine regulation of its reproductive physiology. On the other hand, knowledge of these aspects of species of *Anopheles* is much more limited.

In the brains of *A. aegypti* and *A. albimanus*, each of the tested antisera reveals strong patterns of immunoreactivity exclusively in a small specific number of neurosecretory-like cells in the protocerebral lobes (summarized in Fig. 6a). Five paired groups of neurosecretory cells have been described in species of *Aedes*, *Culex*, and *Culiseta* (Clements 1992); these cells have processes extending from the brain in the nervi corporis cardiaci I and II and projecting into the stomatogastric nervous and the CC–CA complex. The labeled cells in our studies are difficult to categorize into the five groups described by Clements (1992). However, the three antisera recognize some of the medial neurosecretory cells located in the posterior pars intercerebralis (Fig. 6a, arrowheads). Differences are discernible in the labeled processes extending from the immunostained neurons. The processes from all the labeled neurons are well stained with anti-AS-A, but only processes projecting from the posterior neurosecretory cells are well stained with anti-AT. The processes are weakly stained with anti-AS-C.

Immunoreactivity of peptides from these three families has been described in the CC–CA complex of Lepidoptera (Zitnan et al. 1995; Duve and Thorpe 2003). In *Diploptera punctata*

and *Gryllus bimaculatus*, protocerebral neurosecretory cells show AS-A or AS-B immunoreactivity, respectively, and immunoreactivity in nerves projecting into the CA (Stay et al. 1992; Neuhäuser et al. 1994). AS-A and AS-C immunoreactivities have been demonstrated in a large number of neurons in the adult brain of *Drosophila melanogaster* (Zitnan et al. 1993; Yoon and Stay 1995), whereas nerves to the CA are not stained by the AS-A antiserum (Yoon and Stay 1995). In contrast, protocerebral neurosecretory cells in *D. melanogaster* larvae send AS-C-immunoreactive processes to the ring gland, reinforcing the idea that this peptide is an endocrine regulator in Diptera. Although an AT precursor has not been identified in the *Drosophila* genome, *M. sexta*-AT immunoreactivity has been detected in small median neurosecretory cells and numerous interneurons of the brain of adult *D. melanogaster* (Zitnan et al. 1993).

We have only found AS-A immunoreactive processes innervating the CC–CA complex of *A. albimanus*. In contrast, processes innervating the CC–CA complex in *A. aegypti* are not immunostained for AS-A. Moreover, the CC–CA complex is not stained with antisera against AT or AS-C in either mosquito. This absence of immunoreactivity may be the result of the presence of only small amounts of the peptides because of the minute size of mosquito CC–CA and the physiological state of the females evaluated.

The three antisera recognize neurons in the seven abdominal ganglia of the ventral nerve cord, with differences in the numbers and types of neurons and their projections (Fig. 6b). Anti-AT normally recognizes three cells per ganglion, whereas anti-AS-A stains only one neuron from the first to the sixth ganglion and eight cells in the last one (7+8). The AS-C antiserum recognizes two neurons from the first to the sixth ganglion and four in the last one (7+8). The locations of the stained neurons in the ganglia suggest that different cells are labeled by each antiserum (Fig. 6b). If immunoreactivity against AT and AS-A was detected in the perikarya of a particular neuron, it was also observed in the projecting processes; in contrast, AS-C antiserum stained the ganglion neurons but not their projections (Fig. 6b).

Immunoreactivity against AS-A has been previously described in a specific group of endocrine midgut cells in female *A. aegypti* (Veenstra et al. 1995). We have found a similar result in *A. albimanus*. In addition, in situ hybridization has shown the expression of AS-A mRNA in the same group of midgut cells in *A. aegypti* (F.G. Noriega et al., unpublished). In adult *D. melanogaster*, AS-A immunoreactivity has also been described in endocrine cells of the midgut and in the hindgut axons (Yoon and Stay 1995). Veenstra and Costes (1999) have described the absence of AT immunoreactivity in midgut endocrine cells of *A. aegypti*. We have confirmed this observation in *A. albimanus* and the absence of immunoreactivity to AS-C in the midguts of both mosquitoes.

What does the distribution of the three peptides tell us about their functions? AT was first isolated from *M. sexta* as a factor that stimulates the synthesis of JH in the CA (Kataoka et al. 1989). Previously, we have reported that the CA of a newly emerged *A. aegypti* female needs to be exposed to AT before it is capable of synthesizing JH (Li et al. 2003). We have also reported that only females that emerge with large amount of nutrients have CA capable of synthesizing enough JH to activate reproductive maturation (Caroci et al. 2004). Factors from the head are essential for CA activation and reproductive maturation in mosquitoes; decapitation of *A. aegypti* females within 1 h after adult ecdysis prevents normal development of the previtellogenic follicles (Gwadz and Spielman 1973). A similar effect has been described when the brain medial neurosecretory cells are removed (Lea 1967). Cells in the brain of some species of *Aedes* and *Anopheles* are immunostained by the anti-AT antiserum, and thus AT can be proposed as being one of these critical factors from the head playing a role in the nutritional activation of JH biosynthesis.

AT-immunoreactive cells in the first six abdominal ganglia of the nerve cord have processes that innervate the dorsal vessel, indicating that the dorsal vessel is a target organ of this peptide. AT is a cardioacceleratory peptide in *M. sexta* (Veenstra et al. 1994) and *Pseudaletia unipuncta* (Koladich et al. 2002). Rudwall et al. (2000) have reported that AT increases the heart rate in *Leucophaea maderae* and *Periplaneta americana* and have described AT immunostaining in processes projecting to the heart in these two species of cockroaches.

Intense labeled has also been observed with anti-AT in varicosities in processes innervating abdominal tissues, such as the hindgut, dorsal vessel, and oviducts. Veenstra and Costes (1999) have reported that *Aedes aegypti*-AT stimulates oviduct contractions in the blowfly *Phormia* sp. Mas-AT stimulates hindgut contractions in *L. maderae* (Rudwall et al. 2000) and gut motility in *Heliothis virescens* (Oeh et al. 2003).

The reason for the specific staining of oenocytes associated with the fat body by the anti-AT antibody remains to be explained. Oenocytes are large abdominal cells that are rich in smooth endoplasmic reticulum and mitochondria and have been implicated in hydrocarbon and lipid synthesis (Chapman 1998; Fan et al. 2002). Cuticular hydrocarbons and pheromones are produced by abdominal oenocytes and are shuttled through the hemolymph by high-density lipophorin (Gu et al. 1995; Schal et al. 1998; Sevala et al. 1999). In mosquitoes, oenocytes might produce AT or might be a target for the peptide. It should be noted however that oenocytes were stained in *A. albimanus* but not in *A. aegypti*.

AS-A was initially identified by its inhibitory activity on JH production in the cockroach *D. punctata* (Pratt et al. 1989; Woodhead et al. 1989). We have previously reported that *Aedes* AS-A have no effect on JH biosynthesis in female *A. aegypti* (Li et al. 2004).

AS-A has been described as an inhibitory regulator of visceral muscle contraction in several parts of the digestive tract in a number of insects (Stay 2000; Nässel 2002). Immunostaining of AS-A has been found in various tissues of several insects, including endocrine midgut cells of *A. aegypti* (Veenstra et al. 1995). These AS-A endocrine cells are “open type” cells. They have a bottle shape and extend from the basal lamina to the midgut lumen. The apical part of the cell is in contact with the lumen and might be able to sense the midgut contents (Brown et al. 1985; Stracker et al. 2002). AS-A-immunoreactive endocrine cells are located in the most posterior part of the midgut, close to the muscles of the pyloric sphincter; these cells might directly or indirectly affect the activity of midgut muscles. Caroci and Noriega (2003) have proposed that free amino acids in the midgut lumen might be sensed by these midgut endocrine cells and might play a key role as a signal used by the midgut to control the retention of its lumen contents.

The AS-A antiserum has not been seen to stain varicosities along the wall of the dorsal vessel, although stained processes seem to innervate the pericardial cells in both species. Putative roles for pericardial cells (nephrocytes) are the filtration, clearance, and regulation of the composition of hemolymph (Wigglesworth 1970; Dallai et al. 1994). Nephrocytes have both secretory and degradative functions; in *M. sexta*, pericardial cells remove JH esterase from the hemolymph (Bonning et al. 1997). In *A. gambiae*, scavenging nephrocytes, which are present alongside the dorsal vessel, harbor numerous peroxisomes and catalase-rich organelles that are active in the detoxification and neutralization of reactive oxygen species (Kumar et al. 2003). Yeast particles are aggregated by *A. albimanus* pericardial cells and induce a strong increase in lysosomal activity, indicating that this tissue could be important in cell-mediated immune responses (S. Hernández-Martinez, unpublished). The strongly AS-A-immunoreactive varicosities in processes innervating the nephrocytes suggest either that this peptide plays a role in the regulation of pericardial cell physiology or that these cells are involved in the clearance of the peptide.

AS-C was originally identified in *M. sexta* (Kramer et al. 1991). In this species and other moths, it inhibits JH biosynthesis (Kramer et al. 1991; McNeil and Tobe 2001). In *D. melanogaster*, an AS-C homolog slows heart contraction (Price et al. 2002). We have previously described that the biosynthetic activity of the *A. aegypti* CA in vitro is inhibited by factors present in the head and by physiological concentrations of synthetic *A. gambiae* AS-C (Li et al. 2004). The presence of AS-C immunostaining in the brain strengthens the hypothesis that AS-C is the brain factor involved in the regulation of JH biosynthesis in mosquitoes. Using a combination of high performance liquid chromatography and mass spectrometry, we have isolated and characterized a putative AS-C peptide from the brain of *A. aegypti* (Y. Li et al., unpublished).

In summary, we have performed a comparative study of the distribution of AT, AS-A, and AS-C and provided evidence of major similarities in the expression of these three neuropeptides in two species of mosquitoes. Our results support the idea that AT and AS-C are factors from the brain playing a role in the regulation of JH synthesis and oogenesis in mosquitoes, whereas AS-A is an important modulator of the contraction of visceral muscle.

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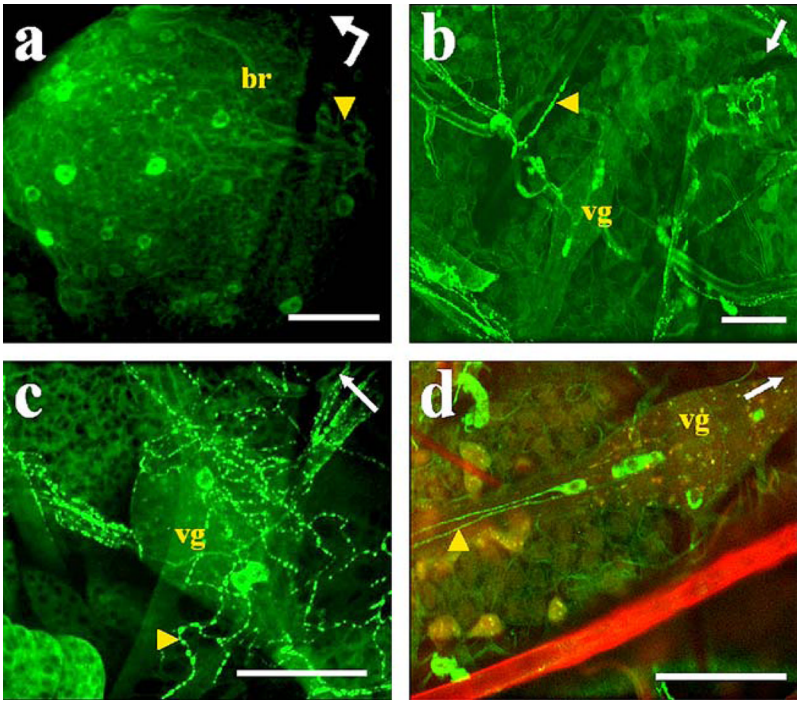

Fig. 1.

Immunostaining of AT in *A. aegypti* tissues. **a** Left protocerebral lobe of the brain (*br*) with a posterior group of labeled cells (*arrowhead*). *Bent arrow* Front (*arrowhead*) and left side (*arrow end*) of the brain. **b**–**d** Abdominal preparations with three cells labeled in ventral ganglia (*vg*) and varicosities labeled in ventral processes (*arrowhead*). *Arrows* Anterior part of the mosquito. **d** Combined images from confocal fluorescence (*green*) and light (*red*) microscopy. *Bars* 50 μm

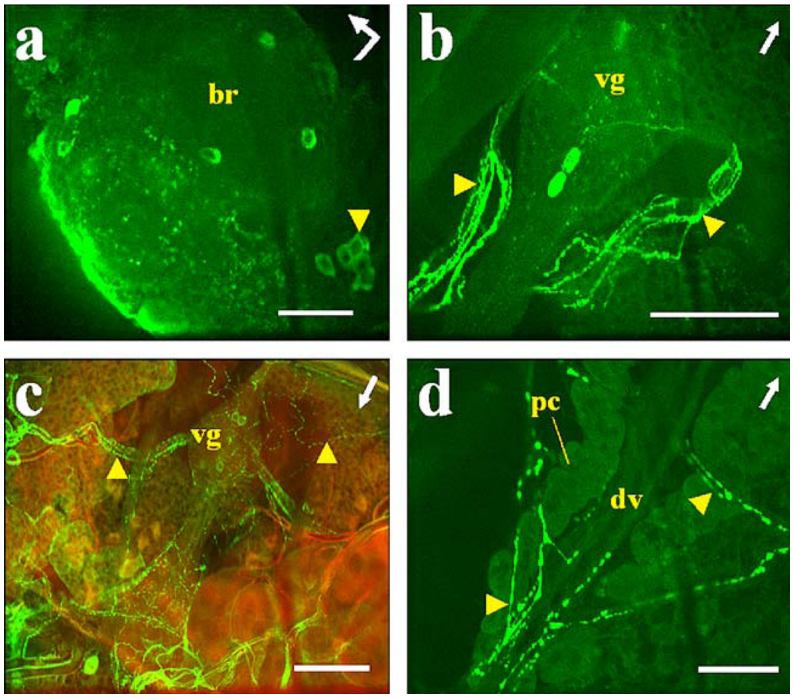

Fig. 2.

Immunostaining of AT in *A. albimanus* tissues. **a** Left protocerebral lobe of the brain (*br*) with a posterior group of labeled cells (*arrowhead*). *Bent arrow* Front (*arrowhead*) and left side (*arrow end*) of the brain. **b**, **c** Abdominal preparations with three cells labeled in ventral ganglia (*vg*) and varicosities labeled in ventral processes (*arrowheads*). **c** Combined images from confocal fluorescence (*green*) and light (*red*) microscopy. **d** Heart (*arrowheads* processes directly connected to the dorsal vessel containing immunostained varicosities, *dv* dorsal vessel of heart, *pc* pericardial cells). *Arrows* Anterior part of the mosquito. *Bars* 50 μm

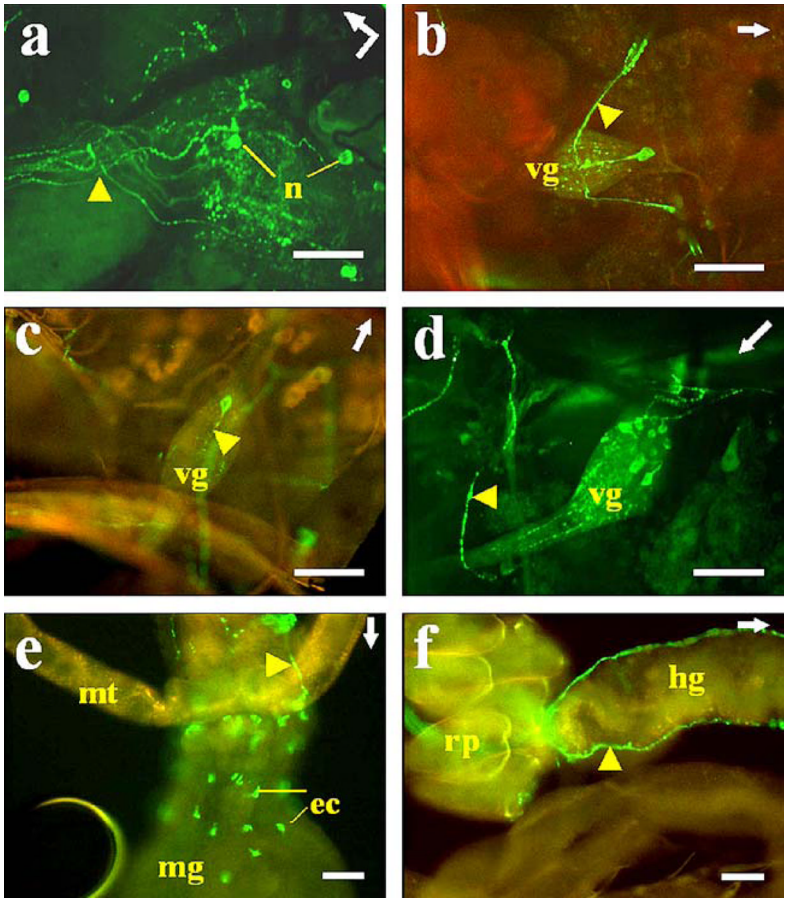

Fig. 3.

Immunostaining of AS-A in *A. aegypti* tissues. **a** Left protocerebral lobe of the brain. Neurons (*n*) with labeled processes (*arrowhead*) innervating the stomatogastric nervous system. *Bent arrow* indicates the front (*arrowhead*) and left side (*arrow end*) of the brain. **b**–**d** Abdominal ventral ganglia. Only one cell was stained in each ventral ganglion (*vg*), except in the last ganglion (**d**) in which eight cells were labeled. Note the immunostained varicosities in ventral processes (*arrowheads*). **e**, **f** Midgut. Endocrine cells (*ec*) showing immunoreactivity in the posterior midgut (*mg*) (*mt* Malpighian tubule). Two processes (*arrowhead*) can be seen running along the hindgut (*hg*) and ending in the rectal papillae (*rp*). *Arrows* Anterior part of the mosquito. **b**,**c**,**e**, **f** Combined images from confocal fluorescence (*green*) and light (*yellow-red*) microscopy. *Bars* 50 μm

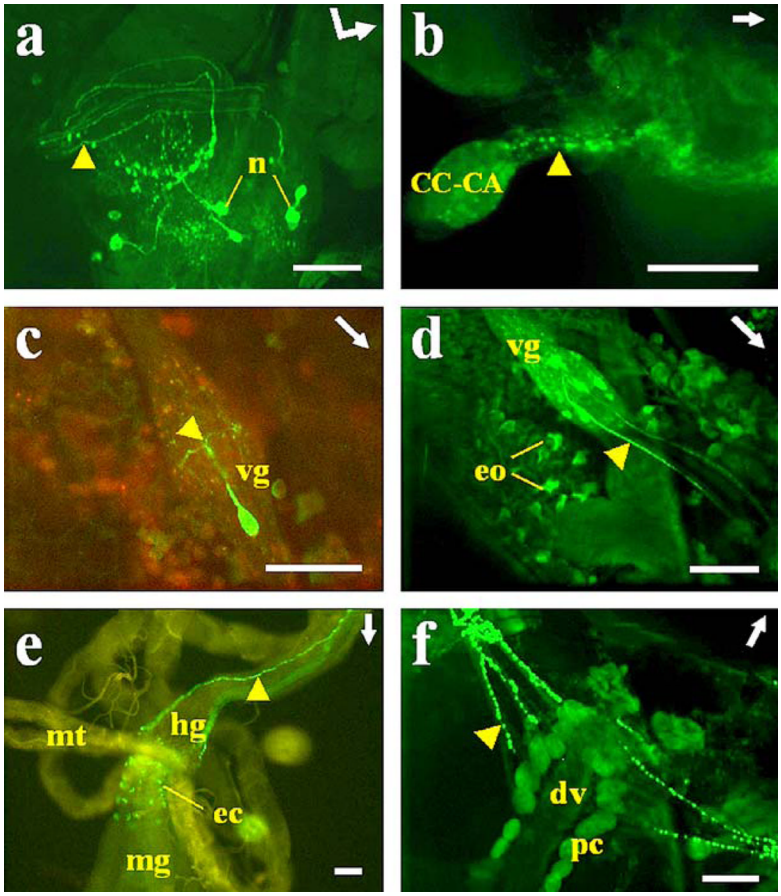

Fig. 4.

Immunostaining of AS-A in *A. albimanus* tissues. **a** Right protocerebral lobe of the brain. Neurons (*n*) with labeled processes (*arrowhead*) innervating the stomatogastric nervous system. *Bent arrow* Front (*arrowhead*) and left side (*arrow end*) of the brain. **b** Processes (*arrowhead*) with varicosities projecting into the corpus cardiacum–corpus allatum (*CC–CA*) complex. **c**, **d** Abdominal ventral ganglia: only one cell was stained in each ventral ganglion (*vg*), except in the last ganglion (**d**) in which eight cells were labeled. Note the immunostained varicosities in ventral processes (*arrowhead*). **d** Oenocytes (*eo*) ventrally located in abdominal fat body showing immunoreactivity. **e** Midgut (*mt* Malpighian tubule). Endocrine cells (*ec*) showing immunoreactivity in the posterior midgut (*mg*). Note the two processes (*arrowhead*) running along the hindgut (*hg*) and ending in the rectal papillae. **f** Heart. Inmmunolabeled varicosities in processes (*arrowhead*) ending in the proximity of pericardial cells (*pc*) around the dorsal vessel (*dv*). *Arrows* Anterior part of the mosquito. **c**, **e** Combined images from confocal fluorescence (*green*) and light (*yellow-red*) microscopy. *Bars* 50 μm

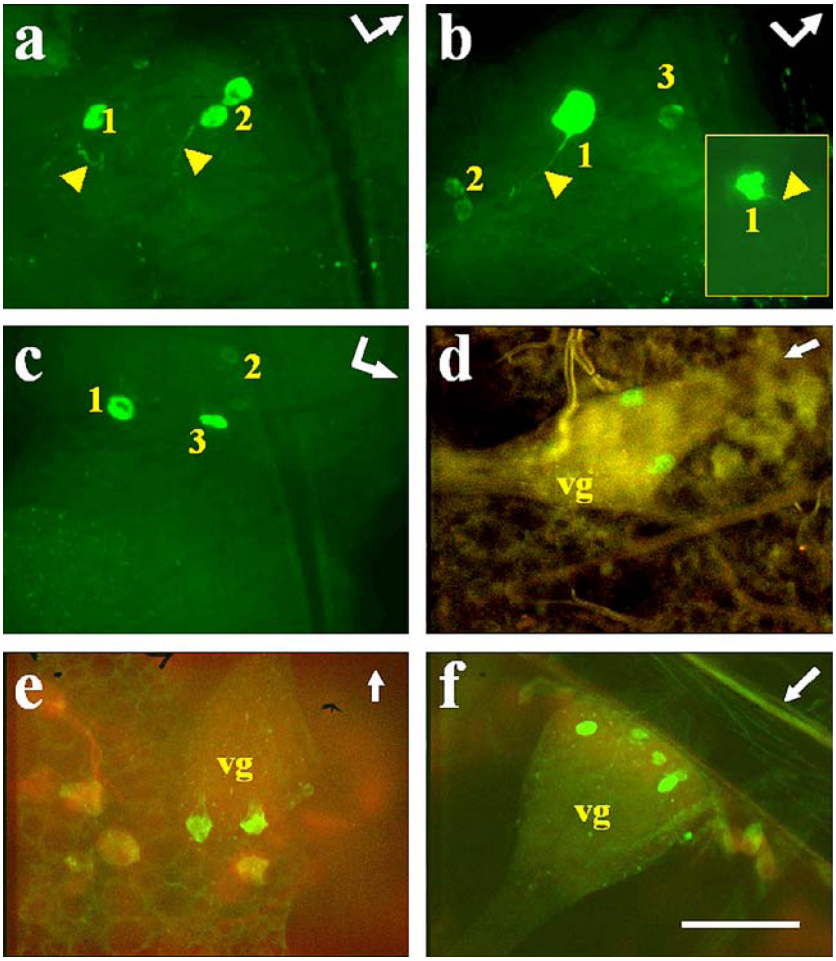

Fig. 5.

Immunostaining of AS-C in *A. aegypti* and *A. albimanus* tissues. Immunoreactivity in brain of *A. aegypti* (**a**, **b**) and *A. albimanus* (**c**). *Bent arrows* Front (*arrowhead*) and left side (*arrow end*) of the brain (*arrowheads* labeled processes, *numbers* location of the cells as depicted in Fig. 6a). *Inset*: Cell with large labeled vacuoles in the cytoplasm. **d**–**f** Abdominal ventral ganglia. Two cells were stained in each ventral ganglia (*vg*), except in the last ganglion (**f**) in which four cells were labeled. *Arrows* Anterior part of the mosquito. *Bar* 50 μm

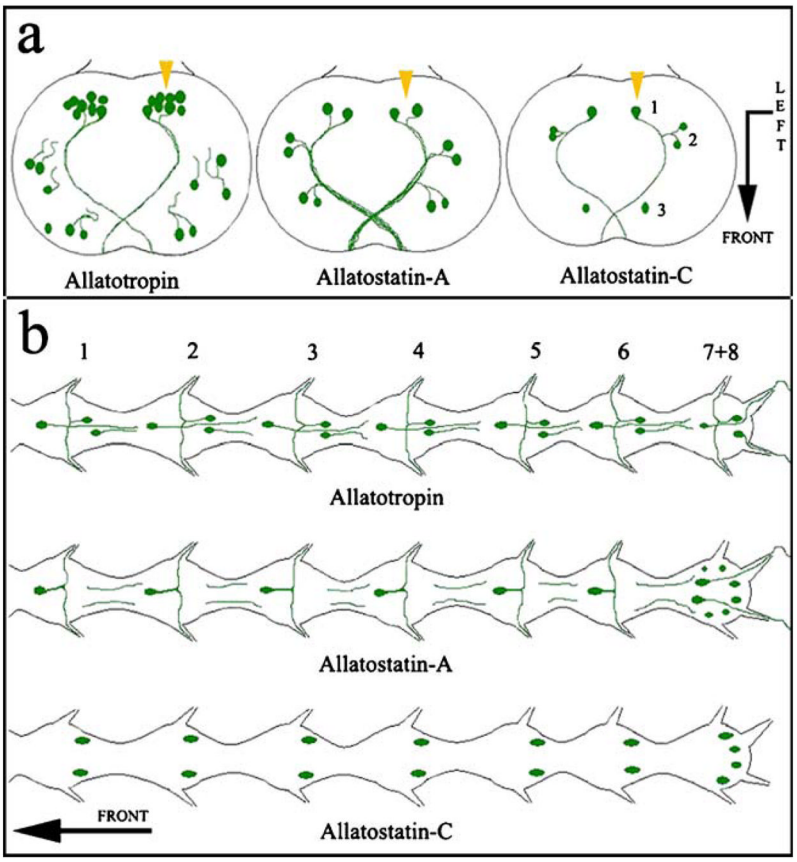

Fig. 6.

Representation of neuropeptide distribution in the central nervous system of *A. aegypti* and *A. albimanus*. The pattern of immunostaining for each of the three peptides is similar in both mosquito species. **a** Brains showing neurons and processes stained in the protocerebral lobes. *Bent arrow* Front (*arrowhead*) and left side (*arrow end*) of the brain (*numbers* relative location of cells). **b** Abdominal ganglia in the ventral nerve cord showing neurons and processes stained with the three different antibodies (*numbers* position of the ganglia). *Arrow* Anterior part of the mosquito