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Identification of a complex peptidergic neuroendocrine network in the hard tick, *Rhipicephalus appendiculatus*

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Abstract

Neuropeptides are crucial regulators of development and various physiological functions but little is known about their identity, expression and function in vectors of pathogens causing serious diseases, such as ticks. Therefore, we have used antibodies against multiple insect and crustacean neuropeptides to reveal the presence of these bioactive molecules in peptidergic neurons and cells of the ixodid tick *Rhipicephalus appendiculatus*. These antibodies have detected 15 different immunoreactive compounds expressed in specific central and peripheral neurons associated with the synganglion. Most central neurons arborize in distinct areas of the neuropile or the putative neurohaemal periganglionic sheath of the synganglion. Several large identified neurons in the synganglion project multiple processes through peripheral nerves to form elaborate axonal arborizations on the surface of salivary glands or to terminate in the lateral segmental organs (LSO). Additional neuropeptide immunoreactivity has been observed in intrinsic secretory cells of the LSO. We have also identified two novel clusters of peripheral neurons embedded in the cheliceral and paraspiracular nerves. These neurons project branching axons into the synganglion and into the periphery. Our study has thus revealed a complex network of central and peripheral peptidergic neurons, putative neurohaemal and neuromodulatory structures and endocrine cells in the tick comparable with those found in insect and crustacean neuroendocrine systems. Strong specific staining with a large variety of antibodies also indicates that the tick nervous system and adjacent secretory organs are rich sources of diverse neuropeptides related to those identified in insects, crustaceans or even vertebrates.

Keywords

Neuropeptides; Synganglion; Salivary glands; Lateral segmental organs; Tick, *Rhipicephalus appendiculatus* (Arachnida, Acari, Ixodidae)

Introduction

The general anatomy and structure of the central nervous system (CNS; synganglion) and associated peripheral nervous system and secretory organs of ticks have been extensively studied (for reviews, see Chow et al. 1972; Coons et al. 1974; Sonenshine 1991; Coons and Alberti 1999; Rees 2004; Chang and Kaufman 2005; Lees and Bowman 2007). However, little is known about peptidergic neuroendocrine and neuromodulatory systems in ticks. Classical paraldehyde-fuchsin or other staining techniques have revealed 18 neurosecretory centres in the synganglia of representative ixodid and argasid ticks (Obenchain 1974; Obenchain and Oliver 1975; Pound and Oliver 1982; Binnington 1987; Prullage et al. 1992; Szlendak and Oliver 1992). Although these studies have described a complex network of neurosecretory cells presumably producing and releasing some bioactive molecules, their structures and functions remain to be identified.

Several neurohaemal organs and release sites for these neurosecretory cells have been proposed. These include two layers of neural sheath surrounding the synganglion and major nerve roots, viz. the proximal neurilemma (neural lamella) and the more distal periganglionic sheath. A narrow space called the periganglionic sinus lies between the neurilemma and periganglionic sheath; this space is continuous with the heart and supplies the synganglion with fresh haemolymph (Fig. 1; Prullage et al. 1992; Szlendak and Oliver 1992). Ultrastructural studies have shown that nerve fibres with neurosecretory vesicles are common in the neural lamella and may discharge their granules into the periganglionic blood sinus (Coons and Alberti 1999). Another layer, the periganglionic sheath, contains branching axons and axon terminals and is regarded as a putative neurohaemal release site for specific neurosecretory cells (Sonenshine 1991; Neupert et al. 2005).

The lateral segmental organs (LSO) are additional structures with a proposed neurohaemal role. They were first described in the ixodid tick *Boophilus microplus* (Binnington and Tatchell 1973) and later were observed in many other species (Chow and Wang 1974; Obenchain and Oliver 1975; Panfilova 1978; Binnington 1981; Marzouk et al. 1985; El Shoura 1989; Coons and Alberti 1999). Most species contain two to four pairs of the LSO interconnected by the lateral nerves along the synganglion (Sonenshine 1991). However, the true physiological function of the LSO remains unclear and no bioactive compounds confirming their secretory activity have been identified.

The retrocerebral organ complex (ROC) is also considered to be a neurohaemal organ in ticks (Sonenshine 1991; Coons and Alberti 1999). It is attached to the oesophagus close to the synganglion and some authors have proposed its analogous role to the corpora cardiaca/corpora allata complex of insects (Roshdy et al. 1973; Binnington 1981; Marzouk et al. 1985; Zhu and Oliver 1991). Although the ROC is a putative candidate for the production of juvenile hormone (JH) and/or neuropeptides in ticks, conclusive evidence for its neuroendocrine function(s) has not been reported (Chang and Kaufman 2005).

Numerous studies have provided evidence that tick salivary glands are innervated by several nerves from the synganglion (for a review, see Kaufman 1983). However, no neurons projecting their axons to the salivary gland surface have been identified. These observations indicate that the tick neuroendocrine system is composed of neurosecretory/modulatory

neurons in the synganglion and various neuronal projections extending into the neurilemma, periganglionic sheath, RCO, LSO and salivary glands. However, the true physiological roles of these putative neurohormonal and secretory organs await confirmation.

Neuropeptides are important active messengers in the neuroendocrine systems of most animals. Immunohistochemical staining with antibody to insulin suggested, for the first time, that the tick synganglion produced neuropeptides (Zhu and Oliver 1991; Davis et al. 1994), with insulin-like immunoreactivity (IR) being detected in the synganglion of *Ornithodoros parkeri* and *Dermacentor variabilis*. Subsequent immunohistochemical studies with antibodies to FMRFamide and cockroach allatostatin I indicated the presence of specific neuropeptides in various neurons and axonal arborizations in the synganglion of argasid and ixodid ticks (Zhu et al. 1995; Zhu and Oliver 2001). Whole-mount immunohistochemistry was used to detect insect kinin-like IR and periviscerokinin-like IR in specific neurons and axons of the ticks *Ixodes ricinus* and *B. microplus*. Periviscerokinin was the first neuropeptide identified in ticks by mass spectrometric analysis of single neurons (Neupert et al. 2005). Molecular cloning and functional analysis of the G-protein-coupled receptor for kinin-like peptide in the southern cattle tick *B. microplus* further indicated that ticks produced multiple neuropeptides and receptors (Holmes et al. 2000, 2003; Taneja-Bageshwar et al. 2006). Bioassays revealed the presence of ecdysteroidotropic neurohormone in the ixodid tick *Amblyomma hebraeum* (Lomas et al. 1997), and an opioid peptide sharing high sequence similarity with mammalian haemorphins was identified in synganglia extracts of *Amblyomma testudinarium* (Liang et al. 2005). However, the origin of this opioid peptide needs confirmation, because of possible contamination with the mammalian blood in the tick oesophagus, which cannot be separated from the synganglion (Neupert et al. 2005).

In this paper, we have used immunohistochemical techniques to detect the expression of multiple neuropeptide-like compounds in the tick synganglion and associated peripheral neuroendocrine organs. This is the first report describing the peptidergic nature of numerous previously unknown central and peripheral neurons, the elaborate innervation of salivary glands and the axon terminals and intrinsic cells in the LSO.

Materials and methods

Animals

A colony of *Rhipicephalus appendiculatus* was reared under the same laboratory conditions as those described for *Dermacentor reticulatus* (Slovák et al. 2002). The synganglia with attached peripheral nerves, LSO, RCO and salivary glands were investigated in 1- to 2-month-old unfed females. Representations were made from microphotographs of fixed ganglia by using Adobe Photoshop or were adapted from the published literature as indicated.

Whole-mount immunohistochemical staining

For the whole-mount immunohistochemical staining of tick neuroendocrine organs, we followed a procedure successfully used for the staining of insect endocrine cells (Žitnová et al. 2001). Ticks were dissected in icecold phosphate-buffered saline (PBS: 137 mM NaCl, 1.45 mM NaH₂PO₄·H₂O, 20.5 mM Na₂HPO₄, pH 7.2). The dorsal cuticle and gut were removed with sharp forceps and the synganglion with attached LSO were cleaned of remaining blood and fixed overnight in 4% paraformaldehyde in PBS (pH 7.2). Following washes in PBS containing 1% Triton X-100 (PBST), the periganglionic sheath was carefully removed from selected synganglia. The tissues were then pre-absorbed with 5% normal goat serum (Sigma, St. Louis, Mo., USA) in PBST for 10 min and incubated in monoclonal

antibody or polyclonal antiserum for 2 days. All the antibodies and the dilutions used in this study are listed in Table 1. After several washes with PBST, the tissues were incubated overnight with Alexa 488- or Alexa 555-labelled goat anti-rabbit IgG or goat anti-mouse IgG (Molecular Probes, Carlsbad, Calif., USA). Alternatively, we used horseradish peroxidase (HRP)-labelled secondary antibodies (Jackson ImmunoResearch Lab., West Grove, Pa., USA). The HRP was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.1 M TRIS-HCl buffer (pH 7.6) and 0.01% H₂O₂. The end of the reaction was determined visually to avoid excessive background staining. Stained tissues were washed in PBS and mounted in glycerol.

For whole-mount double-staining, we incubated fixed synganglia in a mixture of monoclonal antibodies and polyclonal rabbit antisera. After being washed with PBST, the tissues were incubated with a mixture of Alexa 488- and Alexa 555-labelled secondary antibodies, washed in PBST and mounted in glycerol.

Stained tissues were observed under a Nikon microscope (Eclipse 600) by using fluorescein and rhodamine filters for fluorescent microscopy, and Nomarski differential interference optics for light microscopy. Double-stained synganglia were analysed under a confocal microscope (Zeiss LSM510).

Negative controls included preabsorption of each antibody with its respective antigen. Each diluted antibody (see Table 1) was incubated with 1 μ M of the corresponding antigen for 24 h and used for immunohistochemistry as described above. The bombyxin antibody was pre-absorbed with 0.1 μ M synthetic N-terminal bombyxin. This treatment invariably abolished all immunohistochemical staining.

Nomenclature of peptidergic neurons

The synganglion of both argasid and ixodid ticks is composed of 18 neurosecretory centres containing a total of ~130–150 putative neurosecretory cells (Binnington 1987; Balashov 1998). Our antibodies reacted only with a limited number of these cells, whereas numerous previously unknown neurons were observed. To characterize these peptidergic neurons, we modified the established nomenclature of White et al. (1986) and named each neuron with letters. The first two letters refer to the position of each neuron in a specific lobe of the synganglion: cheliceral (Ch), palpal (Pa), stomodeal (St), prothocerebral (Pc), pedal 1–4 (Pd_{1–4}), opisthosomal (Os), preoesophageal (Pe) or postoesophageal (Po), whereas the following letters refer to its dorsal (D), ventral (V), anterior (A), posterior (P), medial (M) or lateral (L) location. The names of the peripheral neurons (PN) are indicated by their location in a specific nerve trunk: cheliceral (Ch) and paraspinal (Ps). Neurons projecting axons onto the surface of the putative neurohaemal periganglionic sheath or lateral segmental organs are considered to be neurosecretory cells (NC); interneurons (IN) arborize within the synganglion, whereas neurons innervating ducts and/or acini of salivary glands are denoted as SG.

Results

In this paper, we provide a comprehensive review of the peptidergic neurosecretory/modulatory systems by using neuropeptide antibodies and immunohistochemical staining of the synganglion and associated peripheral structures/organs of the tick *R. appendiculatus*. Since most tick neuropeptides have not been identified, we have tested the hypothesis that most arthropod neuropeptides are conserved and used antibodies generated against multiple insect and crustacean neuropeptides (Table 1).

Synganglion

The tick CNS contains a single synganglion that lies in the anterior-ventral part of the body and that is composed of several lobes or ganglia (Fig. 1; Obenchain 1974). Fifteen out of 18 antibodies showed strong specific reaction in various peptidergic cells and axonal projections in the synganglion, LSO, peripheral nerves and salivary glands. Only antibodies to *Manduca* allatostatin, eclosion hormone and adipokinetic hormones failed to react or showed inconsistent staining with any described cells or structures (Table 1). None of the antibodies used in this study showed positive reaction in the ROC, which was originally considered a major neurohaemal organ in ticks (Sonenshine 1991).

Bombyxin-like IR was distributed only on the dorsal side of the synganglion (Figs. 2, 3a). Three pairs of medium-sized neurons were observed in the anterior prothocerebrum (PcIN₁₋₃), whereas an additional three pairs of small neurons were stained in the posterior prothocerebrum (PcDM₁₋₃). A single pair of neurons was detected in the cheliceral lobe (ChD) and one pair of giant neurons was present in the opisthosomal ganglion (OsDM). Strong reaction was also detected in intrinsic cells of the LSO (Figs. 2, 3b).

Prothoracicotropic hormone (PTTH)-like IR (Figs. 2, 3c) was restricted to two pairs of small lateral neurons in the cheliceral lobes (ChD_{1,2}). As in insects, no other neurons were stained with this monoclonal antibody.

Ion transport peptide (ITP)-like immunostaining was found on the dorsal synganglion (Figs. 2, 3d); three pairs of medial neurons in the prothocerebrum (PcAM and PcDM_{1,2}), four neurons in each cheliceral lobe (ChD₁₋₄), one neuronal pair in the postoesophageal region (PoDM) and two pairs of neurons in the opisthosomal lobe (OsDM₁₋₂).

Pigment dispersing factor (PDF)-like IR was restricted to one pair of small neurons in the first pedal lobe (Pd₁DL) and two pairs of large cells in the opisthosomal lobe (Figs. 2, 3e). These large opisthosomal lobe neurons projected axons into lateral and opisthosomal nerves (Figs. 2, 3e,f). Each axon in the lateral nerve sent branches into salivary nerves II–IV to innervate the salivary glands (see below). These neurons were named OsSG_{1,2} to indicate their association with salivary glands. Immunoreactive lateral nerves were connected with two pairs of the LSO whose intrinsic cells also showed strong PDF-like IR (Figs. 2, 3f).

Corazonin-like IR was observed in four prothocerebral neurons (PcAM and PcIN), three pairs of small cells in the first pedal lobe (Pd₁DL₁₋₃) and clusters of five paired neurons in the opisthosomal lobe, viz. OsDM₁₋₅ (Figs. 2, 3g). PcIN neurons showed the colocalization of corazonin-like and MIP-like IR (Fig. 4a–c). Since PcIN project posteriorly a loop of paired axons that arborize in each pedal lobe, they are identified as interneurons (Figs. 2, 3g).

PRVamide-like IR was detected only in the dorsal region of the synganglion. We detected a pair of interneurons (PcIN) projecting short arborizing axons in the anterior prothocerebrum (Figs. 2, 3h). Posterior prothocerebrum contained three pairs of putative neurosecretory cells (PcNC₁₋₃) projecting contralateral axons to the periganglionic sheath where they branched into elaborate arborizations. A pair of neurons was stained in each pedal lobe 3, 4 (Pd_{3,4}DM) and two pairs of cells were observed in the opisthosomal lobe (OsDM_{1,2}).

RFamide-like IR was detected in a cluster of four paired small neurons in the anterior-medial prothocerebrum (PcAM) and seven other paired neurons were found more posteriorly in the medial prothocerebral lobe (PcDM₁₋₅ and PcVM_{1,2}; Figs. 2, 3i). A single neuron was stained in each cheliceral lobe (ChD). Another neuron in each palpal lobe projected axons into the palpal and salivary nerves I. Axonal projections of these palpal

neurons terminated along main ducts of salivary glands and were therefore named PaSG (Figs. 2, 3i, 4d,f; see below). Several neurons were detected in pedal lobes: two pairs of ventro-medial neurons (Pd₃DV₁₋₂), a pair of dorso-medial neurons (Pd₂DM), a pair of dorso-lateral neurons (Pd₃DL) and two paired neurons showing co-expression of RFamide-like and MIP-like peptides (Pd₃DM_{1,2}; Fig. 4d–f). A pair of small neurons was found in the opisthosomal lobe (OsDM). RFamide-like IR was also observed in axon terminals on the LSO surface (Fig. 3j). Therefore, the LSO may serve as neurohaemal organs for some neurosecretory cells of the synganglion, but their identity has not been determined.

Bombyx myosuppressin (BMS)-like IR was prominent in three clusters of anterior dorsal neurons. Three pairs of neurons in the prothocerebrum showed colocalization of BMS-like and CCAP-like IR (Fig. 4g–i). Double-stained axon terminals of these neurons were detected in the lateral neuropile of the pedal lobes indicating that they were interneurons (PcIN₁₋₃). Four pairs of neurons in the cheliceral lobes projected immunoreactive axons posteriorly to form elaborate arborizations in the periganglionic sheath and were therefore considered as neurosecretory cells (ChNC₁₋₄; Figs. 2, 3k). Two additional paired neurons were found in the palpal lobes (PaD_{1,2}). ChNC₁₋₄ and PaD_{1,2} showed double-labelling with antibodies against BMS and kinin (Fig. 4j–l). Five pairs of ventral segmental neurons were detected in the pedal lobes (Pd₁₋₄VM; Fig. 2).

Kinin-like IR was restricted to two pairs of small anterior prothocerebral neurons (PcAM), four pairs of anterior putative neurosecretory cells in the cheliceral lobes (ChNC₁₋₄) and two pairs of small neurons in the palpal lobes (PaD_{1,2}; Figs. 2, 3l, 4k,l). As described above, the two latter cell types produced kinin-like and BMS-like substances (Fig. 4j–l). Numerous immunoreactive axon terminals in the sheath apparently originating from ChNC₁₋₄ indicated their neurohaemal function.

Myoinhibitory peptide (MIP)-like IR was observed in numerous neurons of various sizes (Figs. 2, 5a). About eight pairs of small neurons were located in the anterior prothocerebrum (PcAM), whereas the central region of the prothocerebrum contained two pairs of neurons (PcDM and PcIN; Fig. 2). Double-staining revealed the colocalization of MIP-like and corazonin-like IR in the PcIN (Fig. 4a–c). The posterior prothocerebrum contained a pair of giant neurons that innervated specific acini of salivary glands (see below). To indicate that these prothocerebral neurons were associated with the salivary gland, we named them PcSG (Fig. 4b,c,e,f). A pair of small dorsal neurons was observed in each cheliceral lobe (ChD_{1,2}), whereas three small neurons were located in each palpal lobe (PaD and PaV_{1,2}). On the dorsal side of the pedal region, we detected a pair of small medial neurons (Pd₂DL), a pair of large medial neurons (PoDM) and two pairs of smaller medial neurons (Pd₃DM₁₋₂) that showed the co-expression of MIP-like and RFamide-like peptides (Figs. 2, 4d–f, 5a). The ventral side of pedal lobes 2–4 and the opisthosomal lobe contained 1–2 pairs of small medial neurons (Pd₂₋₄VM, OsVM, OsIN; Figs. 2, 5a).

Crustacean cardioactive peptide (CCAP)-like IR was distributed in a number of small neurons (Figs. 2, 5b). The most prominent cluster of interneurons in the anterior prothocerebrum (PcIN₁₋₃) showed the co-expression of CCAP-like and BMS-like peptides (Figs. 4g–i, 5b). Additional small neurons were observed in the posterior prothocerebrum (PcDM₁₋₃) and palpal lobes (PaD_{1,2} and PaV_{1,2}). Pedal lobes contain small paired neurons on the dorsal and ventral sides; two pairs were detected on the dorsal side of the pedal lobe 2 (Pd₂DM, Pd₂DL), whereas segmentally distributed neurons were observed on the ventral side of each pedal lobe 1–4 (Pd₁VM, Pd₁₋₄VL; Figs. 2, 5b).

Tachykinin-like IR was found in a cluster of about six small anterior neurons in the prothocerebrum (PcAM), a pair of dorsal larger cells in the cheliceral lobes (ChD) and a

prominent pair of large dorso-lateral neurons in the first pedal lobe (Pd₁SG; Figs. 2, 5c). Each Pd₁SG neuron projected a main axonal branch towards the oesophagus and then turned laterally. In the central part of the synganglion, each lateral axon branched in four directions; anteriorly into the palpal nerve innervating the salivary glands (see below), laterally into pedal nerve II, posteriolaterally into the pedal nerves III and IV, and posteriorly into the opisthosomal nerve. Axons from the pedal nerve IV terminate in the lateral nerves. These two large neurons therefore innervated most main central nerves, whereas their smaller axonal branches arborized on the lateral periganglionic sheath. Small paired neurons Pd₁₋₄VL and Pd₂VM were found in pedal lobes on the ventral side.

Diuretic hormone (DH)-like IR was observed in three pairs of dorso-medial neurons in the pedal region (Pd₂DM₁₋₃) and in a pair of larger neurons in the opisthosomal lobe (OsDM₁; Figs. 2, 4m). Double staining revealed the colocalization of DH-like and allatostatin-like IR in OsDM₁ neurons (Fig. 4m-o), which projected branching axons into the lateral, opisthosomal and pedal nerves IV.

Diptera allatostatin-like IR was detected in numerous small neurons distributed in clusters on the dorsal (~55 pairs) and ventral (~90 pairs) sides of the synganglion (Figs. 2, 5d). We observed ~25 pairs of dorso- and ventromedial prothocerebral neurons (PcDM, PcVM), two clusters of dorsal and ventral neurons (~3 and 6 pairs, respectively) in the cheliceral lobe (ChD₁₋₃, ChV₁₋₆) and ~6 pairs of cells in the palpal lobe (PaD₁₋₆). Each pedal lobe contained ~15–20 dorso-lateral (PdDL) or ventrolateral (PdVL) neurons, whereas 2–3 dorso-medial neurons were observed in the pedal lobes 2, 3 (Pd₂DM₁₋₃, Pd₃DM_{1,2}). About 25 pairs of cells were found in the opisthosomal lobe (Fig. 2, 5d). As described above, the largest pair of dorso-medial neurons in the opisthosomal lobe (OsDM₁) showed allatostatin-like and DH-like IR (Fig. 4m-o).

Allatotropin-like IR was found in a large number of neurons throughout the synganglion (Figs. 2, 5e,f). The prothocerebrum contained five pairs of dorso-medial (PcDM₁₋₅), two pairs of dorso-lateral (PcDL_{1,2}) and a pair of ventro-medial (PcVM) neurons (Fig. 5e,f). Paired neurons were found on the dorsal and ventral sides (PaD_{1,2}, PaV_{1,2}) of each palpal lobe (Fig. 5e). Most neurons were distributed in the pedal region; the first pedal lobe contained clusters of three dorso-lateral (Pd₁DL₁₋₃) and five ventro-lateral (Pd₁VL₁₋₅) neurons, whereas only one dorsal pair and three pairs of ventral neurons were found in more posterior pedal lobes 2–4 (Pd₂₋₄DL, Pd₂₋₄VL₁₋₃). Coupled neurons were also distributed in more medial regions of pedal lobes 2–4 (Pd₂₋₄VM_{1,2}). A group of six paired dorso-medial neurons was detected in the pedal lobe 3 (Pd₃DM₁₋₆) and two larger paired dorso-medial cells were located in the pedal lobe 4 (Pd₄DM_{1,2}). The opisthosomal lobe contained three pairs of dorsal neurons (OsDM₁₋₃) and two pairs of ventral neurons (OsVM_{1,2}).

Salivary gland innervation

We identified four types of neurons in the synganglion, each innervating different parts of salivary glands. The PDF antiserum revealed the entire anatomy of paired OsSG_{1,2} neurons located in the dorso-medial part of the opisthosomal lobe. All four cells (Figs. 2, 3e) projected axons through the peripheral nerves to innervate specific acini of the salivary glands (Figs. 6, 7a-c). A single axon from each OsSG neuron entered the lateral nerve and projected three branches into the salivary nerves II–IV. These three posterior immunoreactive axons exhibited anastomosis along the major salivary ducts and terminated exclusively on acini type II (Figs. 6, 7a-c). Axon terminals of OsSG were characterized by three to five small branches containing varicosities (Fig. 7a-c).

The antibody to MIP reacted with a prominent pair of giant neurons in the prothocerebrum PcSG (Figs. 2, 4b-f, 5a; see above). Each neuron projected a single thick axonal process

lacking varicosities in a loop, exited the synganglion through the palpal nerve and salivary nerve I and branched along the salivary ducts to terminate on salivary acini type II and III (Figs. 6, 7a–c). Each axonal branch showed characteristic bifurcating axon terminals at the base of these acini, which were clearly different from those stained with the PDF antibody (Figs. 6, 7a–c).

A single RFamide-like immunoreactive neuron in each palpal lobe (PaSG, Figs. 2, 3i, 4d,f) projected an axon through the palpal nerve and salivary nerve I and branches along the main ducts of salivary glands (Fig. 7d). The numerous varicosities along this axon indicated possible release sites of RFamide-like peptide(s) on the surface of salivary ducts (Fig. 7d). These axons never terminated on the secretory acini of salivary glands.

The anterior axonal branch of each tachykinin-like immunoreactive neuron (Pd₁SG; Figs. 2, 5c) innervated the ducts of the salivary glands. Fine axonal projections arborizing along salivary ducts contained numerous varicosities but did not innervate salivary acini (Fig. 7e).

Peripheral nervous system

Strong allatotropin-like IR was observed in three to six peripheral neurons contained in the cheliceral nerves. Since these cells had not been described before, we named them the cheliceral peripheral neurons (ChPN_{1–3}). The ChPN_{1–3} were elongated bipolar neurons, each projecting a single process into the periphery and another opposite bifurcating processes into the synganglion and a side branch of the cheliceral nerve (Fig. 8a).

We also immunostained five neurons attached to the paraspiracular nerves and named them the paraspiracular peripheral neurons (PsPN_{1–5}; Figs. 8a,b). Each PsPN had a round cell body projecting a single axon into the synganglion and another opposite process into a peripheral side branch of the paraspiracular nerve (Fig. 8b).

Discussion

Synganglion

Several tick species are medically important exoparasites and vectors of serious human pathogens. Thus, it is surprising that so little is known about the regulation of their growth, feeding and reproduction. Numerous studies of other invertebrate and vertebrate models indicate that neuropeptides control these physiological and behavioural processes essential for normal development and survival. Evidence for the presence of neuropeptides in ticks is limited to scarce immunohistochemical staining with antibodies to insect or vertebrate neuropeptides (Davis et al. 1994; Zhu et al. 1995; Zhu and Oliver 1991, 2001). So far, only one neuropeptide related to insect periviscerokinins has recently been identified by MALDI TOF/TOF mass spectrometry in specific central neurons of the ticks *Ixodes ricinus* and *Boophilus microplus* (Neupert et al. 2005). The primary structures, functions and cellular sources of other neuropeptides possibly produced by ticks are enigmatic. The genome project of the tick *Ixodes scapularis* (Hill and Wikel 2005) has recently accelerated progress in the identification of genes encoding putative bioactive peptides (Šimo and Park 2008). To provide more information about the presence of tick bioactive compounds and to associate them with appropriate physiological roles, we have determined the expression patterns of multiple neuropeptides in the CNS, putative neurohaemal/endocrine organs and salivary glands of the tick *R. appendiculatus*. For this purpose, we have used antibodies against identified and functionally characterized insect and crustacean neuropeptides. Our immunohistochemical study complements previously published observations but has also led to several interesting and novel findings.

Reaction of the bombyxin antibody in the synganglion of *R. appendiculatus* (this study) is strikingly similar to that revealed with the insulin antibody in the ixodid tick *D. variabilis* (Davis et al. 1994). Both antibodies immunostain similar prothocerebral, cheliceral and opisthosomal neurons. Insulin-like substance has also been detected in the posterior opisthosomal region of the argasid tick *O. parkeri* (Zhu and Oliver 1991). These data indicate that ticks produce neuropeptide(s) related to the insulin/bombyxin family in a conserved set of neurons. The possible role of these neuropeptide(s) in metabolic and/or reproductive processes is indicated by reduced insulin-like IR detected after blood feeding and mating in males and females of *D. variabilis* (Davis et al. 1994). Metabolic and growth regulating functions have been determined for bombyxin in the moth *Bombyx mori* (Iwami 2000) and insulin-related peptides in the fly *Drosophila melanogaster* (Colombani et al. 2005).

Cockroach allatostatin-like IR has been described in the synganglion of the American dog tick *D. variabilis* (Zhu and Oliver 2001); one to nine pairs of stained cells are distributed in each lobe, except for the pedal lobes, which lack immunoreactive neurons. Weaker allatostatin-like IR in freshly ecdysed females accumulate in unfed ticks one month later (Zhu and Oliver 2001). Using the same antibody, we have observed strong allatostatin-like IR in a considerably larger number of neurons in all regions of the synganglion of *R. appendiculatus*. At least 150 pairs of neurons have been detected in the synganglion of *R. appendiculatus* compared with ~30 pairs in *D. variabilis*. Accordingly, the position of allatostatin-like immunoreactive cells often differs in each species. These differences may indicate the functional diversification of allatostatin-like peptides in the different ticks. Indeed, various bioassays in insects indicate that allatostatins ending in FGLamide are multifunctional neuropeptides. They suppress JH biosynthesis only in some cockroaches and few other hemimetabolous insects, whereas the same or closely related peptides suppress gut and oviduct contractions or inhibit vitellogenin production in various cockroaches, locusts, moths or flies (Bendena et al. 1999; Gäde and Hoffmann 2005).

RFamides belong to a large family of neuropeptides widely distributed in the nervous and endocrine organs of all animals studied. Various neuropeptides sharing the same RFamide C-terminal are encoded by five to six different genes in insects (Roller et al. 2008). Immunohistochemical staining with the FMRFamide antibody indicates that these neuropeptides are also present in ticks. FMRFamide-like immunoreactive neurons are located in various regions of the synganglion of *D. variabilis* and *O. parkeri*. FMRFamide-like immunoreactive processes in coxal muscles suggest that these peptides control locomotory activity (Zhu et al. 1995). In this study, we have confirmed the expression of RFamide-related peptides in the synganglion and describe, for the first time, the presence of these peptides in axonal processes on the LSO surface and along salivary ducts. These data indicate that RFamides control a variety of central and peripheral functions, including the activity of secretory cells in the LSO and salivary ducts.

Kinin-like and periviscerokinin-like IR have been described in the synganglion of *I. ricinus* and *B. microplus*. The latter neuropeptide has been identified by using MALDI-TOF/TOF and named Ixori-PVK (PALIPFPRV-NH₂) since it shows high sequence homology with members of insect periviscerokinins (Neupert et al. 2005). Its C-terminal sequence (-PRVamide) is shared with preecdysis triggering hormone (PETH) and, thus, it is not surprising that our PETH antibody has revealed a similar staining pattern to that of the antibody to PVK. We conclude that the PETH antibody probably reacts with PVK in the synganglion of *R. appendiculatus*. Moreover, the distribution pattern of kinin-like IR is almost identical in *R. appendiculatus* (this study) and *I. ricinus* (Neupert et al. 2005) indicating their conserved function(s) in both species.

Neuropeptide expression patterns in the tick synganglion are, in some cases, comparable with those described in the insect CNS. For example, the restricted PTTH-like IR in only two pairs of lateral neurons of the tick resembles that in the paired PTTH-expressing neurosecretory cells (type III) in moths and *Drosophila* (Mizoguchi et al. 1990; Žit'an et al. 1993, 1995). Segmental neurons Pd₁₋₄VL showing CCAP-like IR in the tick pedal lobes are similar to interneurons 704 producing CCAP in the abdominal ganglia of many insects (Žit'an and Adams 2005), whereas posterior OsDM₁ neurons containing DH/allatostatin-like IR resemble the L₃ neurosecretory cells expressing the same neuropeptides in a moth (Davis et al. 1997). Likewise, large OsSG_{1,2} neurons showing PDF-like IR in the tick are similar to abdominal PDF neurons in a locust and a fly (Nässel et al. 1993; Park et al. 2000; Persson et al. 2001). These staining patterns indicate that some neurons and their active molecules have conserved functions.

The staining patterns with other neuropeptide antibodies appear to be unique for the tick synganglion and are difficult to associate with cellular neuropeptide expression in the CNS of other studied arthropods. This suggests the functional shift of the tick peptidergic network and its modification for the ectoparasitic life characterized by relatively short periods of specific feeding behaviour and prolonged quiescent periods in each developmental stage. Further studies are necessary to reveal the functions of the individual peptidergic neurons and their active messengers in ticks.

Salivary gland innervation

The neuroanatomy of the peripheral nervous system including the innervation of the salivary glands has been described by using classical histological, histochemical and ultrastructural techniques. The innervation of the salivary glands by a pair of nerves from the second pedal ganglion (lobe) was reported for the first time in *Haemaphysalis flava* (Saito 1960) and the innervation of nongranular and granular alveoli by individual axons was detailed in *D. variabilis* by Coons and Roshdy (1973). Later, Obenchain and Oliver (1976) reported the innervation of the salivary glands of *D. variabilis* and *Amblyomma tuberculatum* by palpal, lateral and paraspiracular nerves. The salivary glands of *B. microplus* are innervated by fine nerves branching from the palpal nerves and pedal nerves I–III (Binnington 1978). This innervation is involved in the direct control of salivary gland secretion, presumably through the release of catecholamines (e.g. dopamine) from the synganglion, although their cellular sources in the CNS are unknown (Megaw 1977). Quantitative radioenzymatic assay for catecholamines and immunohistochemical staining for tyrosine hydroxylase indicate that specific cells in salivary acini are a major source of dopamine, whereas only a minor component of dopamine is contained in the nerves of *Amblyomma hebraeum* (Kaufman et al. 1999).

Tick salivary glands are composed of three distinct types of acini (alveoli); nongranular (type I) and granular acini (types II and III) interconnected with salivary ducts. Nongranular acini I are attached to the main salivary duct in the anterior region of the salivary gland and each acinus is composed of a single central cell and several pyramidal, peritubular and constrictor cells. Granular acini II and III differ in their number and structure of granule-secreting cells and acini III are the most peripheral (Coons and Roshdy 1973; Binnington 1978; Megaw and Beadle 1979; Krolak et al. 1982). Salivary gland acini are innervated by axons containing neurosecretory vesicles but their origin has not been determined (Coons and Alberti 1999). Using antibodies to PDF, MIP, RFamide and tachykinin, we have identified several peptidergic neurons innervating salivary glands. Each type of peptidergic neuron produces distinct neuropeptide(s) and has a highly characteristic anatomy and, especially, axon terminals. For example, the OsSG neurons producing PDF-like peptide innervate only acini type II and exhibit anastomosis into three to five axon terminals containing varicosities, whereas the PcSG neurons expressing MIP-like substances show

characteristic bifurcating axon terminals for the innervation of acini II and III. MIP-like IR in two to three axon terminals has also been detected in acini II and III of *Ixodes scapularis* (L. Šimo and Y. Park, unpublished). The PaSG neurons showing RFamide-like IR and the Pd₁SG neurons producing tachykinin-like peptide(s) innervate only salivary ducts and never terminate on secretory salivary cells. A similar innervation of salivary acini and ducts has been observed with antibodies to serotonin, dopamine, octopamine and FMRFamide in several insect species. In vitro bioassays indicate that serotonin and dopamine control insect salivation through cyclic AMP, although the precise mechanisms of their action need further studies (Ali 1997). Certain FMRFamide-like peptides from the blowfly *Calliphora vomitoria* stimulate fluid secretion from isolated salivary glands, whereas other related peptides are inactive (Duve et al. 1992).

The complex innervation of salivary glands by neurons producing PDF-, MIP-, tachykinin- and RFamide-like peptides probably represents an adaptation for the specialized functions associated with the ectoparasitic life of ticks. Our preliminary data indicate a marked decrease of MIP-like and PDF-like IR in salivary gland innervation during the feeding of the adult *R. appendiculatus*. We speculate that each neuronal type producing distinct neuropeptides controls the activity of specific secretory cells or duct contractions of feeding ticks. Type II and III acini contain characteristic cuticular valvae, whereas type I acini lack these valvae (Binnington 1978). The central lumen of acini II and III opens via these valvae into salivary ducts. The type III acini of female ixodid ticks swell up as they accumulate fluid. Contractions of myoepithelial cells then force the fluid out into expanding salivary ducts (Coons et al. 1994; Lamoreaux et al. 1994, 2000). These observations indicate that MIP-like and PDF-like peptides modulate the opening of valvae and/or myoepithelial cell contractions during tick feeding, in order to release the content of specific secretory cells into the host's blood.

Additional tachykinin-like and RFamide-like IR has been observed in specific secretory cells of tick salivary glands (not shown in this paper). The active production of small bioactive compounds by salivary glands is not surprising, since tachykinin-related peptides (eleidosin and sialokinins) are produced by the salivary secretory cells of the curled octopus *Eledone cirrhosa* (Anastasi and Erspamer 1963) and the yellow fever mosquito *Aedes aegypti* (Champagne and Ribeiro 1994), respectively.

LSO as secretory glands

Staining with paraldehyde-fuchsin and vital dyes indicates that the LSO are secretory glands (Chow and Wang 1974; Obenchain and Oliver 1975; Panfilova 1978). They increase in size during feeding of females *Ixodes persulcatus*, as shown by Panfilova (1978). This author has proposed that the LSO are similar to neurohaemal perisymphatic organs of insects and that their size increase correlates with vitellogenesis in female oocytes. Ultrastructural studies suggest that LSO cells exhibit profiles characteristic for steroid-hormone-secreting tissues (Binnington 1981). These organs are likely candidates for endocrine functions but the nature of their secretion(s) has not been determined (Sonenshine 1991). Our immunohistochemical staining with antibodies against bombyxin and PDF indicate that the LSOs are composed of peptidergic endocrine cells and therefore probably do not produce ecdysteroids. RFamide-like IR in axons and their terminals on the LSO surface also indicates a neurohaemal function of these organs reminiscent of the corpora cardiaca in insects.

Peripheral nervous system

Our antibody to allatotropin has revealed clusters of novel peripheral neurons in the cheliceral and paraspiracular nerves (ChPN and PsPN). These bipolar neurons project axons into the synganglion and periphery. In insects, similar bipolar link neurons (L1) located in

the periphery close to spiracles innervate the heart and neurohaemal transverse nerves or perisymphatic organs. Immunohistochemical and in situ hybridization techniques have shown that L1 neurons are peptidergic and express CCAP, FLRFamide-related neuropeptide (F10 or myosuppressin) and non-amidated ITP-like peptide (ITPL; Davis et al. 1993; Dai et al. 2007; Lu et al. 2002). Crustacean hyperglycemic hormone (CHH)-like peptides related to ITPL are expressed in neurosecretory cells of the peripheral pericardial organs of crabs (Dircksen et al. 2001). These crab cells are probably homologous to insect L1 neurons (Dircksen and Heyn 1998). It will be interesting to determine whether peptidergic cells in the peripheral nervous systems of ticks, crabs and insects are of the same origin.

Concluding remarks

The results of our study indicate that ticks produce a large variety of neuropeptides by various types of central and peripheral neurons and by endocrine cells. The peptidergic nature and anatomy of various neurons, neurohaemal/neuromodulatory structures and endocrine cells has been described here for the first time in ticks. The physiological functions of these neuropeptides and cells are unknown and require further studies. Some of these peptidergic structures are probably adapted for the ectoparasitic life of ticks and the modulation of the host haemostatic and immune responses.

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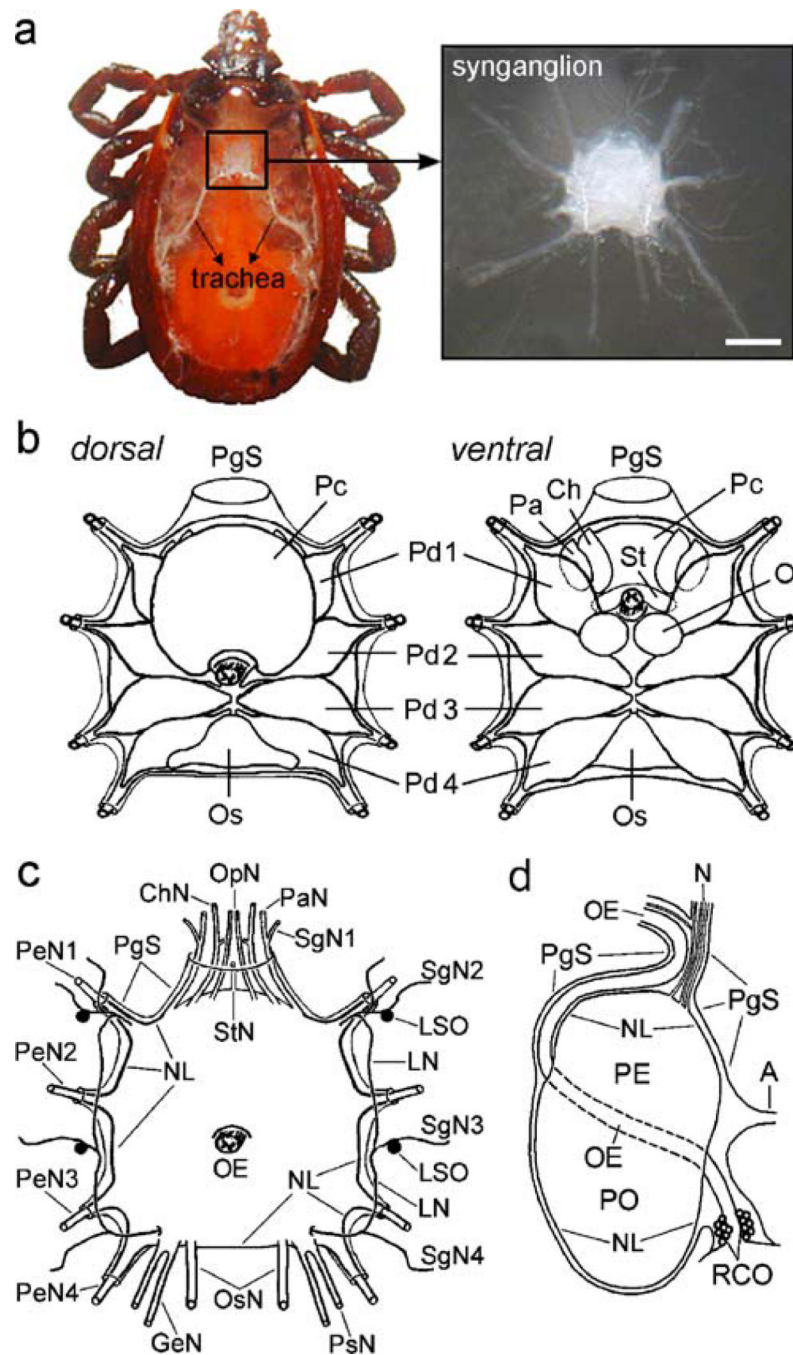


Fig. 1. Anatomy of the synganglion and associated peripheral nerves and organs. **a** *Left* Tick showing the position of the synganglion. *Right* Dorsal view of the tick synganglion and its general morphology. *Bar* 200 μ m. **b** Representations of various lobes in the synganglion (*Ch* cheliceral lobe, *Ol* olfactory lobe, *Os* opisthosomal lobe, *Pa* palpal lobe, *Pd1–4* pedal lobes 1–4, *Pc* prothocerebral lobe, *St* stomodeal lobe, *PgS* periganglionic sheath). **c** Representation of all peripheral nerves and lateral segmental organs (*LSO*); dorsal view (*ChN* cheliceral nerve, *GeN* genital nerve, *LN* lateral nerve, *OsN* opisthosomal nerve, *OpN* optical nerve, *PaN* palpal nerve, *PsN* paraspiracular nerve, *PeN1–4* pedal nerves 1–4, *StN* stomodeal nerve,

SgNI-4 salivary gland nerves 1–4, *E* oesophagus, *NL* neurilemma [neural lamella]. **d** Lateral view of the synganglion depicting positions of associated structures and organs (adapted from Obenchain 1974; *A* aorta, *OE*, *N* nerves, *NL* neurilemma, *PgS* periganglionic sheath, *RCO* retrocerebral organ, *PE* preoesophageal part of the synganglion, *PO* postoesophageal part of the synganglion)

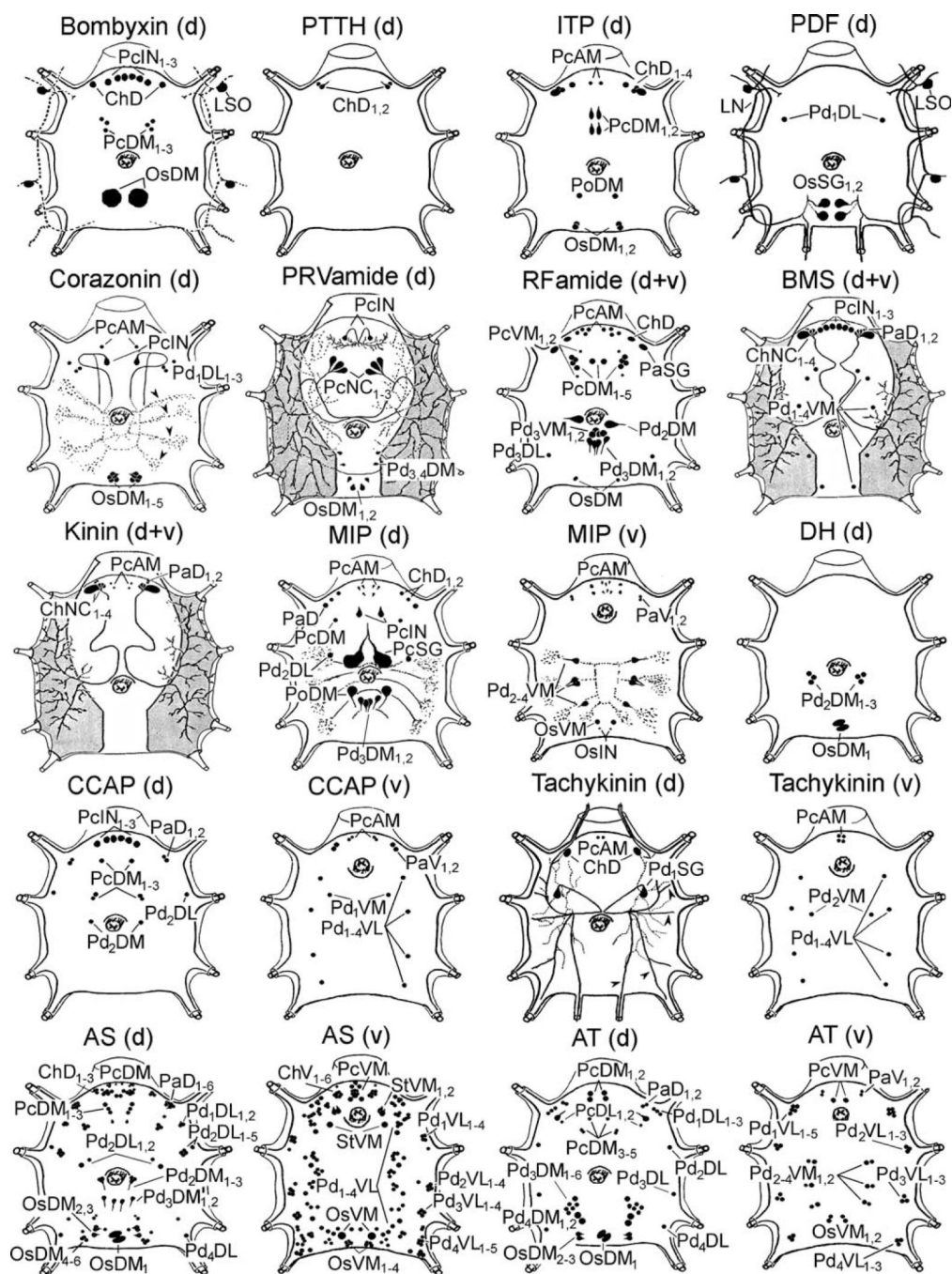


Fig. 2. Representations of all peptidergic neurons described in this study (an explanation of abbreviations of labelled peptidergic neurons is given in Nomenclature of peptidergic neurons; *d* dorsal, *v* ventral)

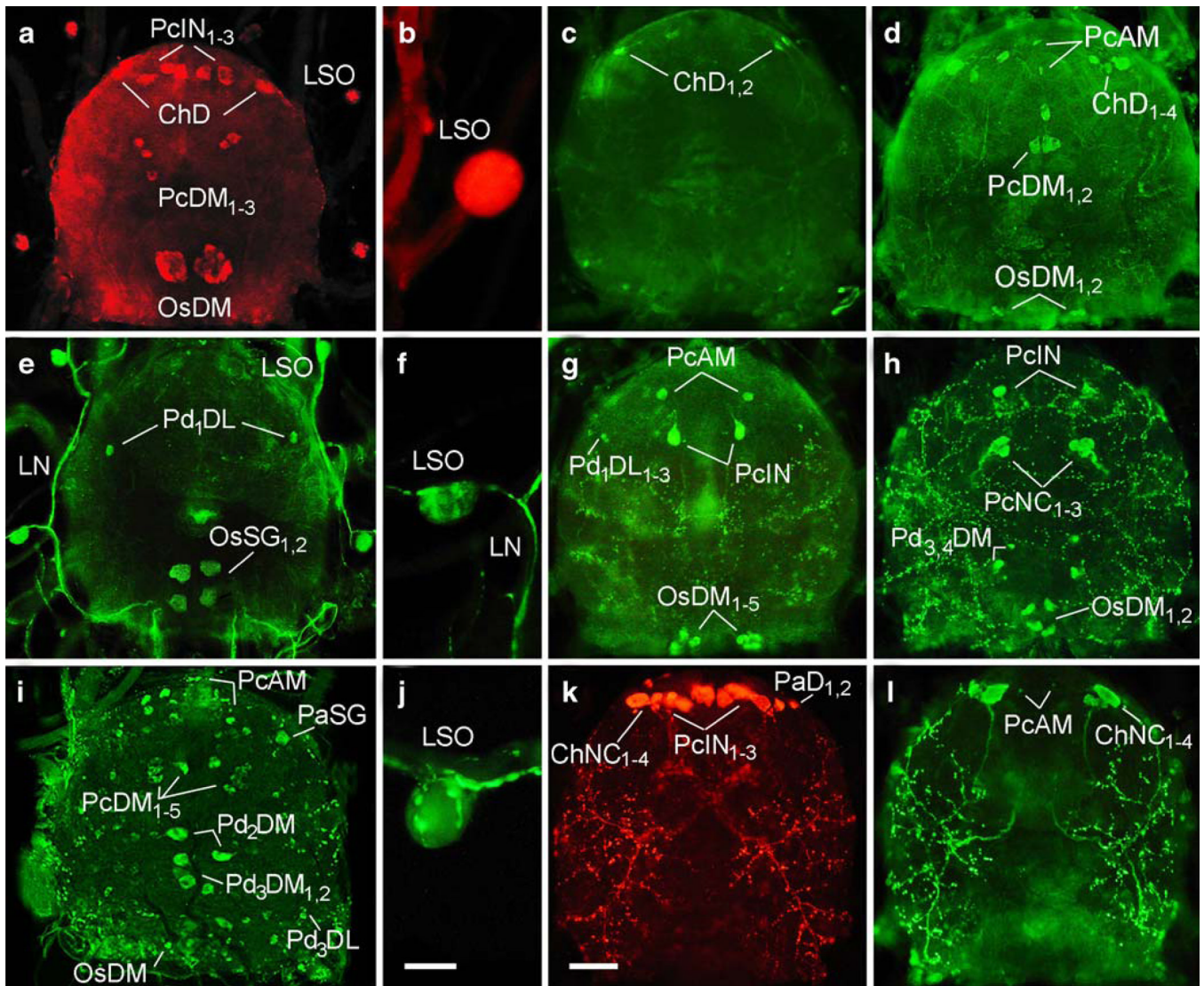


Fig. 3. Neuropeptide immunoreactivity in synganglia (dorsal side) and lateral segmental organs (abbreviations as in Nomenclature of peptidergic neurons). **a, b** Bombyxin-like IR in the synganglion and intrinsic cells of the LSO. **c** PTTH-like IR in the anterior synganglion. **d** ITP-like IR in the synganglion. **e, f** PDF-like IR in the synganglion, lateral nerves (*LN*) and intrinsic cells of the LSO. **g** Corazonin-like IR in the synganglion. **h** PRVamide-like IR in the synganglion. **i, j** RFamide-like IR in the synganglion and putative neurohaemal axon terminals containing varicosities in the LSO. **k** Myosuppressin-like IR in anterior neurons and putative neurohaemal areas in the dorsal periganglionic sheath of the synganglion. **l** Kinin-like IR in anterior synganglion neurons and the same neurohaemal areas as in **k**. Bars 50 μm (**a, c–e, g–i, k, l**), 20 μm (**b, f, j**)

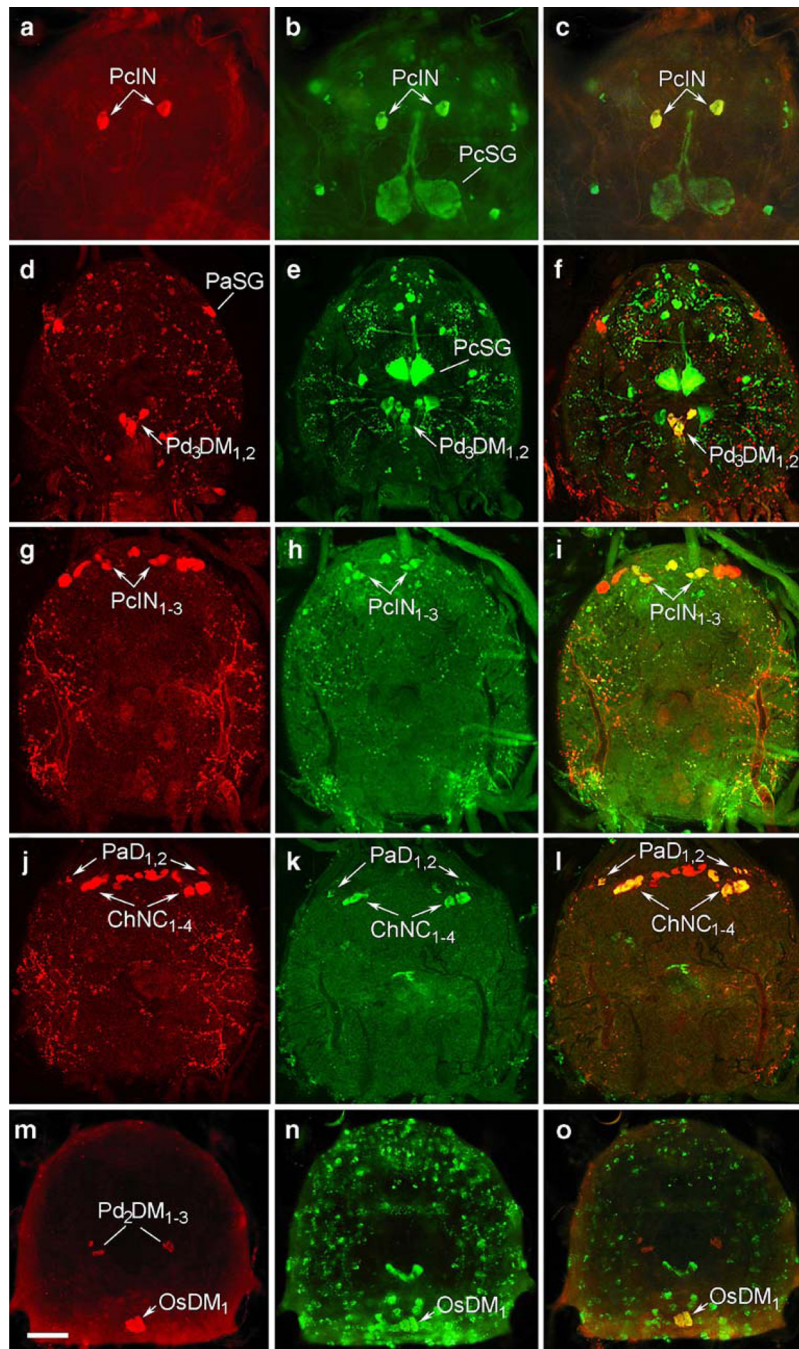


Fig. 4. Identification of central neurons in tick synganglia by using double-staining with neuropeptide antibodies. **a–c** Double-staining with antibodies to corazonin (**a**, red) and MIP (**b**, green) revealed the colocalization of these peptides in the prothocerebral interneurons (*PcIN*) as shown in **c** (yellow, arrows). **d–f** Coexpression of RFamide-like (**d**, red) and MIP-like (**e**, green) peptides was detected in medial pedal neurons (*Pd_{DM31,2}*) as presented in **f** (yellow, arrows). **g–i** Colocalization of BMS-like (**g**, red) and CCAP-like IR (**h**, green) in prothocerebral interneurons (*PcIN₁₋₃*) as seen in **i** (yellow, arrows). **j–l** BMS-like peptide (**j**, red) was also coexpressed with kinin-like peptides (**k**, green) in palpal neurons (*PaD_{1,2}*) and

cheliceral neurosecretory cells (*ChNC₁₋₄*) as revealed in **l** (*yellow, arrows*). **m–o**
Colocalization of DH-like (**m**, *red*) and allatostatin-like IR (**n**, *green*) was observed in
OsDM₁ neurons of the opisthosomal lobe (**o**, *yellow, arrows*). *Bar* 40 μm (**a–c**), 50 μm (**d–o**)

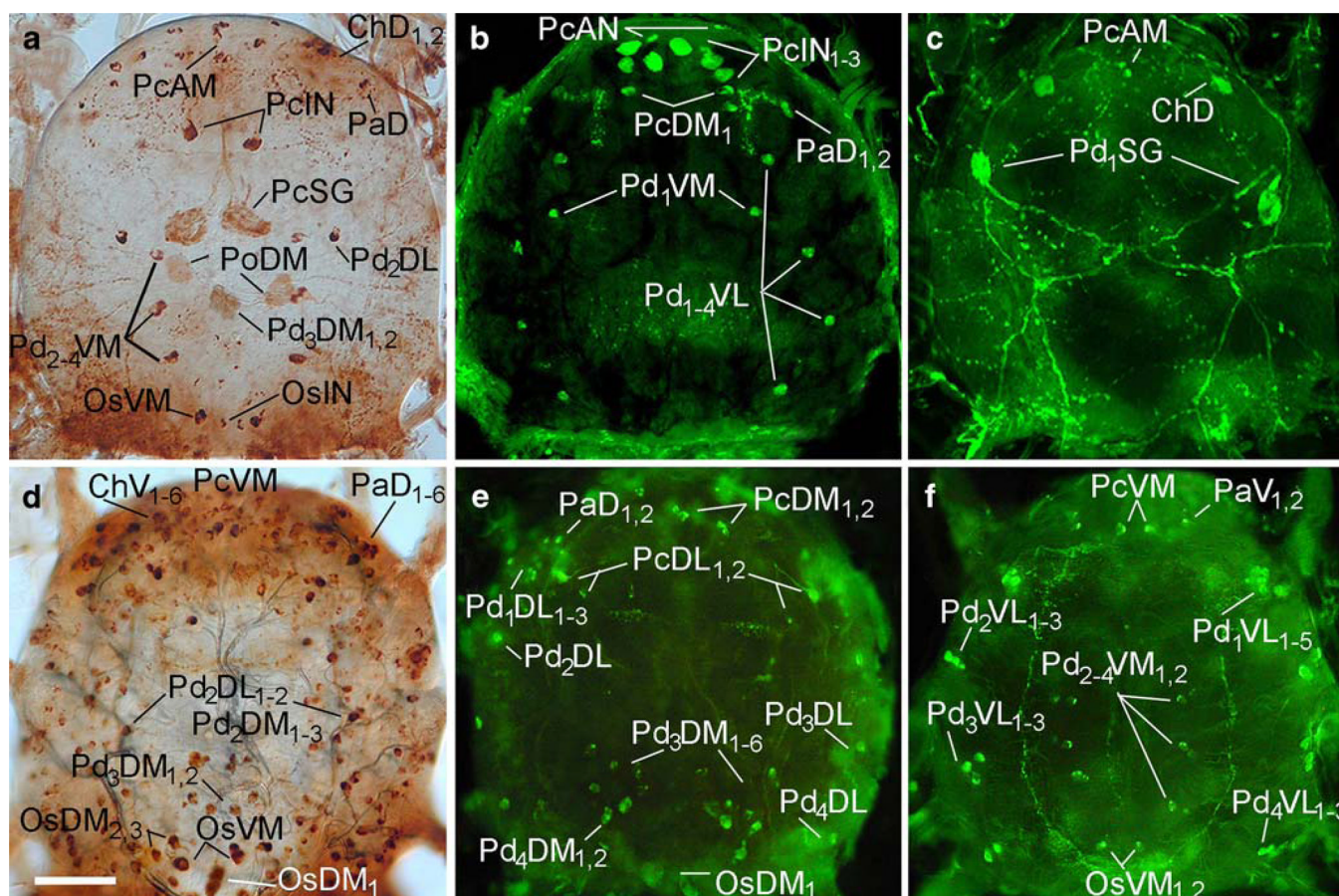
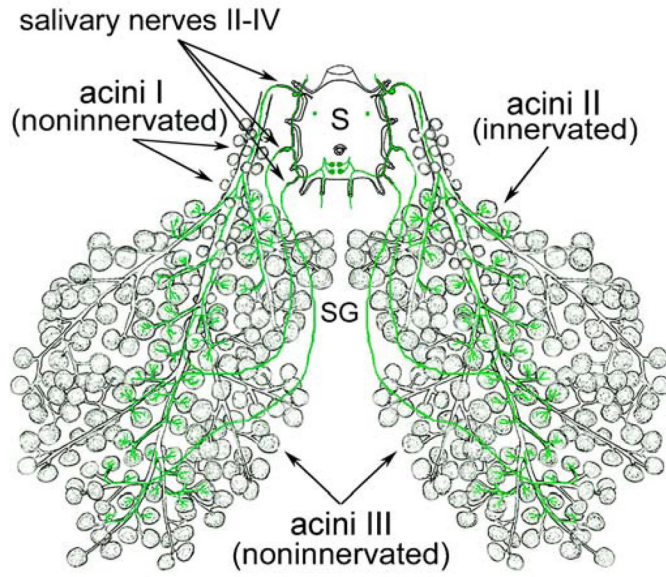


Fig. 5. Neuropeptide immunoreactivity in the tick synganglion (for an explanation of abbreviations, see Nomenclature of peptidergic neurons). **a** Composite view of MIP-like IR in most identified dorsal and ventral neurons. **b** Composite confocal sections of the synganglion showing CCAP-like IR in all described neurons. **c** Tachykininlike IR in dorsal cell bodies and elaborate axonal arborizations of Pd₁SG neurons. **d** Allatostatin-like IR in selected dorsal and ventral neurons. **e, f** Allatotropin-like IR in dorsal (**e**) and ventral (**f**) sides of the synganglion. Bar 50 μ m

PDF



MIP

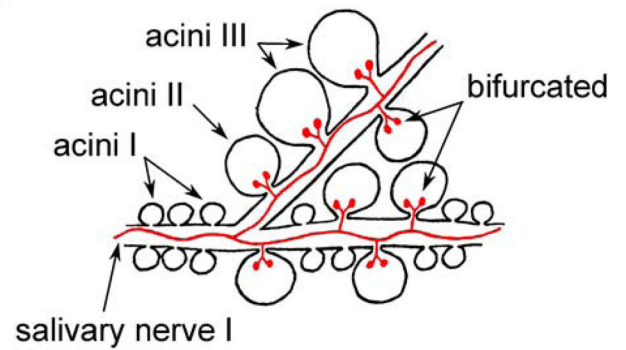
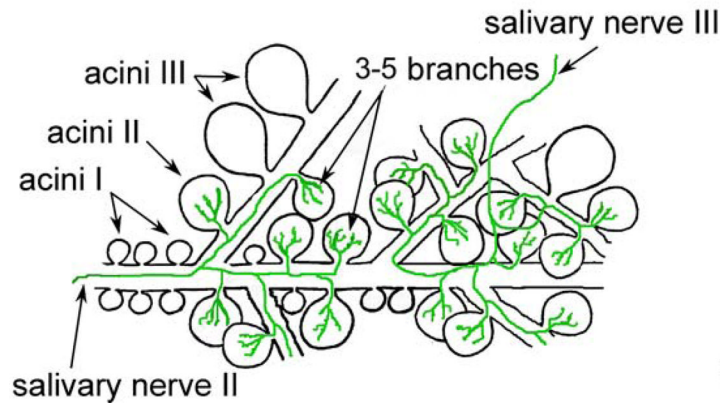
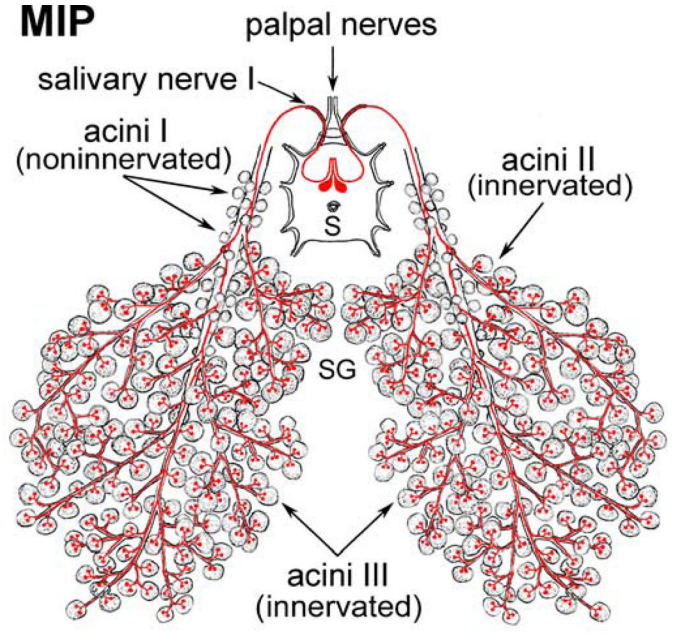


Fig. 6. Representations of identified central neurons innervating salivary glands (SG). PDF-like immunoreactive neurons (OsSG_{1,2}, green) exit the synganglion (S) through lateral and salivary nerves IIIIV to innervate specific acini type II. Branching axon terminals contain varicosities. MIP-like immunoreactive giant neurons (PcSG, red) exit through the salivary nerve I and innervate acini type II and III. Axons of these neurons lack varicosities and show typical bifurcating axon terminals

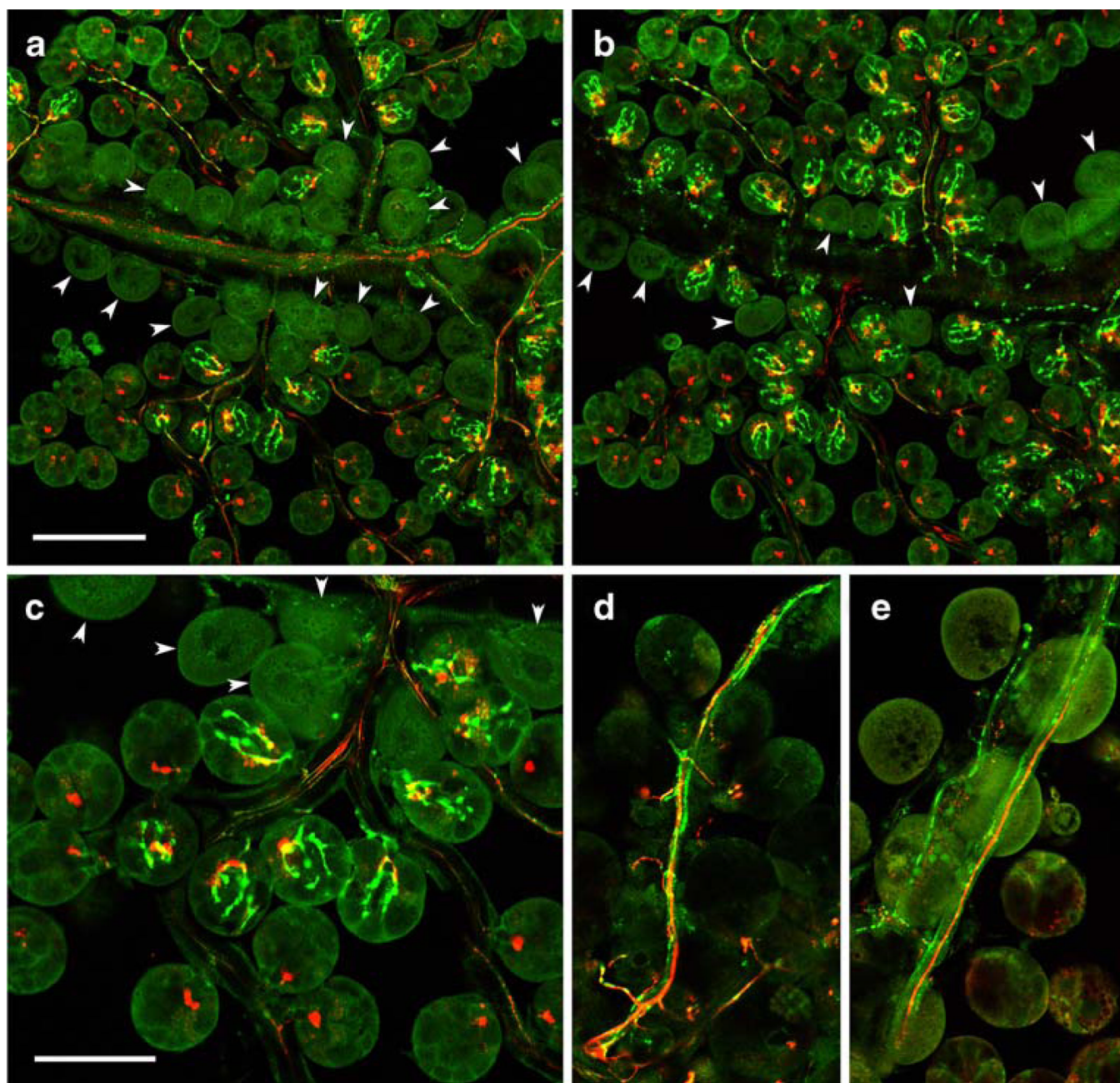


Fig. 7. Neuropeptide immunoreactivity in the salivary gland innervation. **a–c** Merged consecutive confocal sections of salivary glands showing double-staining with antibodies to PDF (*green*) and MIP (*red*). **a** Dorsal side. **b** Ventral side. **c** Higher magnification. Note the PDF-like IR in axons containing varicosities along salivary ducts and multiple axon terminals exclusively on acini type II, whereas axons containing MIP-like IR terminate on acini II and III. Acini I located along the main salivary duct are not innervated (*arrowheads*). **d** Double-staining with antibodies to RFamide (*green*) and MIP (*red*). Note that RFamide-like IR is restricted to axons running along salivary ducts, whereas adjacent MIP-like immunopositive axons terminate on salivary acini II and III. **e** Tachykinin-like IR (*green*) and MIP-like IR (*red*) in

branching axonal processes innervating ducts of salivary glands. *Bars* 100 μm (**a**, **b**), 50 μm (**c–e**)

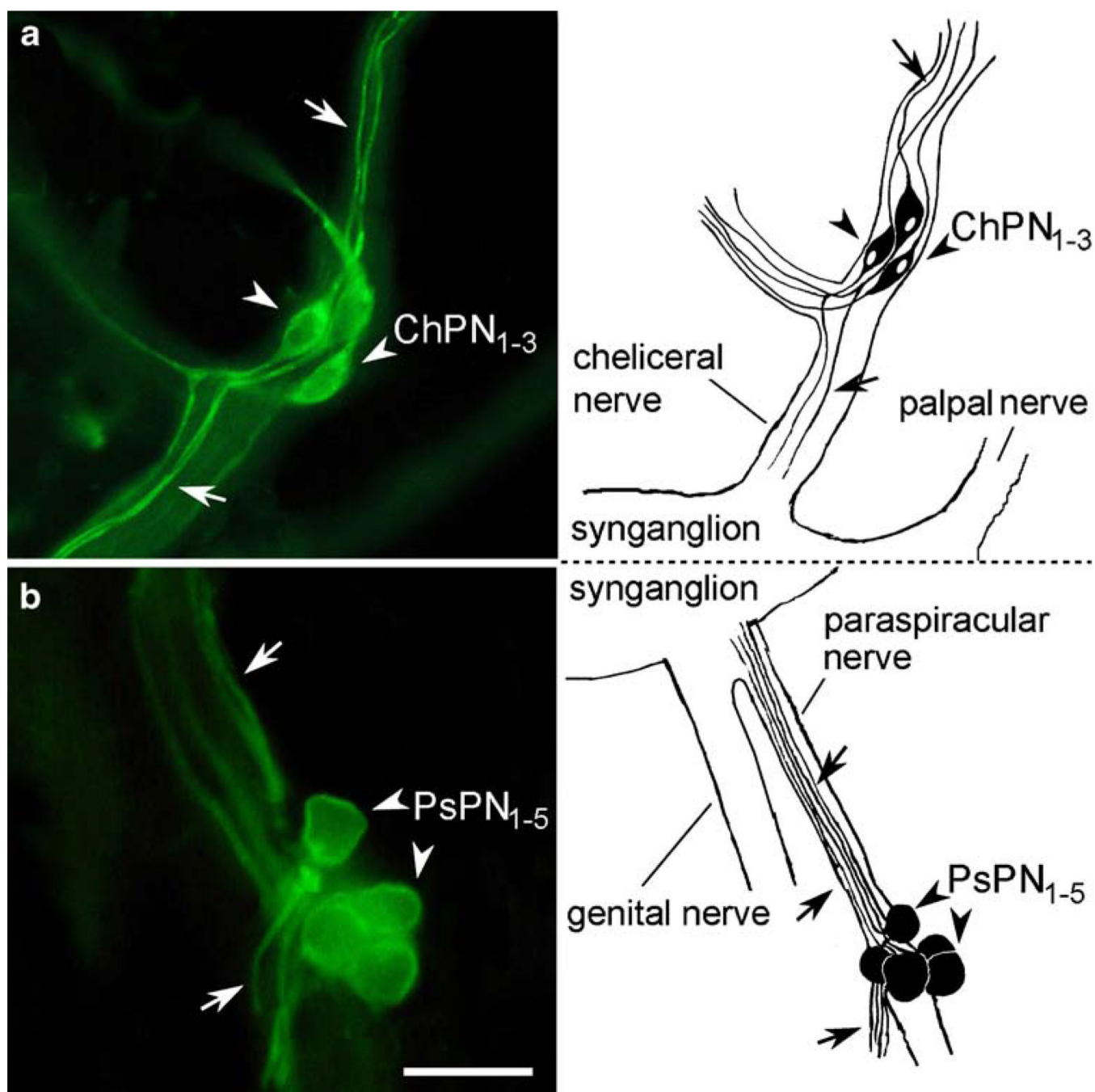


Fig. 8. Allatotropin-like IR in the peripheral nervous system. **a** Cluster of three cheliceral peripheral neurons ($ChPN_{1-3}$, arrowheads) in each cheliceral nerve. These bipolar neurons project axons into the periphery and into the synganglion (arrows). **b** Group of five paraspiracular peripheral neurons ($PsPN_{1-5}$, arrowheads) projecting axons into the synganglion and opposite processes into the periphery through a side branch of the paraspiracular nerve (arrows). Bar 5 μ m

Table 1Mouse monoclonal antibodies (*M*) and rabbit polyclonal antisera (*R*) used

Antibody to	Type	Dilution	Source/first characterization
Adipokinetic hormone (AKH)	R	1:1,000	D. Kodr�k, unpublished
Arg-Phe-NH ₂ (RFa)	R	1:1,000	Grimmelikhuijzen and Spencer 1984
Bommo-bombyxin (insulin-like)	M	1:300	Mizoguchi et al. 1987
Bommo-prothoracicotropic hormone (PTTH)	M	1:1,000	Mizoguchi et al. 1990
Bommo-myosuppressin (BMS)	M	1:1,000	Yamanaka et al. 2006
Crustacean cardioactive peptide (CCAP)	R	1:500	Stangier et al. 1988
Drome-pigment dispersing factor (PDF)	R	1:1,000	Persson et al. 2001
Dippu-allatostatin	M	1:100	Stay et al. 1992
Leuma-kinin	R	1:1,000	N�ssel et al. 1992
Locmi-tachykinin	R	1:2,000	N�ssel et al. 1993
Locmi-tachykinin	R	1:1,000	Schoofs et al. 1993
Manse-allatostatin	R	1:1,000	Žit an et al. 1995
Manse-allatotropin	R	1:1,000	Žit an et al. 1995
Manse-diuretic hormone (DH)	R	1:2,000	Žit an et al. 1995
Manse-eclosion hormone (EH)	R	1:1,000	Žit an et al. 2002
Manse-ion transport peptide (ITP)	R	1:10,000	Dai et al. 2007
Manse-myoinhibitory peptide I (MIP-I)	M	1:1,000	Kim et al. 2006
Manse-pre-ecdysis triggering hormone (PETH; PRVa)	R	1:1,000	Žit an et al. 1999
Peram-corazonin	R	1:5,000	Roller et al. 2003