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Neural and endocrine regulation of osmoregulatory organs in tick: Recent discoveries and implications

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ABSTRACT

Ticks can survive in harsh and fluctuating vegetated environments for long durations between blood feedings with highly developed osmoregulatory mechanisms. Like the unique life history of hematophagous ticks, osmoregulatory organs and their regulatory mechanisms are significantly different from those in the closely related insect taxa. Over the last ten years, research has uncovered several neuropeptidergic innervations of the primary osmoregulatory organ, the salivary glands: myoinhibitory peptide (MIP), SIFamide, and elevenin. These neuropeptides are thought to be modulators of dopamine's autocrine or paracrine actions controlling the salivary glands, including the activation of fluid transport into the lumen of salivary acini and the pumping and gating action of salivary acini for expelling fluids out into salivary ducts. These actions are through two different dopamine receptors, D1 receptor and invertebrate D1-like dopamine receptor, respectively. Interestingly, MIP and SIFamide are also involved in the control of another important excretory/osmoregulatory organ, the hindgut, where SIFamide is myostimulatory, with MIP having antagonistic effects. FGLamide related allatostatin is also found to have axonal projections located on the surface of the rectum. Investigations of the osmoregulatory mechanisms of these critical vector species will potentially lead to the development of a measure to control tick species.

1. Introduction

Ticks are obligatory hematophagous ectoparasites of animals and transmit a wide variety of pathogens, including bacteria, protozoa, and viruses, in addition to causing direct physical damage to their hosts. Hard ticks blood feed on a given host for several days in each life stage (larva, nymph, and adult) to develop into next next life stages, but they are able to survive for much longer durations in between feedings. Most ixodid tick species are classified as three-host ticks, taking a blood meal during each of their major life stages, and must survive the vast majority of their lives off-host in the vegetated environment. As such, osmoregulation, the controlling of water and ion balance, is a challenging physiological task for ticks in harsh and fluctuating environments; maintaining water during the long off-host periods, while also being capable of the rapid removal of excessive water and ions during blood feeding. Most importantly, the osmoregulatory capacity in off-host ticks is likely one of the major factors determining the habitat range of these important vector species and geographical distribution of their associated pathogens. Like the unique life history of ticks, their osmoregulatory physiology is substantially different from other related arthropods, i.e., insects. The salivary glands and excretory organs, including the hindgut and Malpighian tubules, are the major organs for maintaining water and ion balance, while these organs are thought to be controlled by the synganglion (brain of tick) located in the middle of ventral side (Fig. 1A and B). The salivary glands are also recognized for their important function in the secretion of bioactive components to facilitate blood feeding (Francischetti et al., 2009; Ribeiro, 1987, 1995). While there have been extensive studies and excellent review articles describing tick saliva and its bioactive components (Ribeiro et al., 2006; Ribeiro, 1995, 1987), the mechanisms controlling the salivary glands and the hindgut have not been fully understood. In this paper, we review our recent findings that introduced a new direction of studies for

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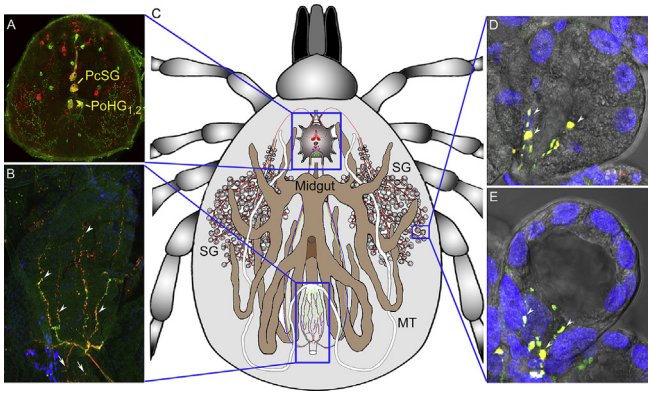

Fig. 1. Anatomy of hard ticks, showing the major internal organs. (A) Synganglion with cells showing positive immunoreactivity to antibodies against SIFamide (green) and MIP (red), where yellow indicates colocalization of both. PcSG, protocerebral salivary gland neuron; PoHG, post-oesophageal hindgut neurons. (B) Hindgut with projections immunoreactive to antibodies against SIFamide (green) and MIP (red), where yellow indicates colocalization. (C) Depicting the internal organization of organs. (D) Type II acinus and (E) Type III acinus with axon terminals showing positive immunoreactivity to antibodies against SIFamide (green) and MIP (red), where yellow indicates colocalization. Blue represents DAPI staining of nuclei. MT, Malphigian tubule; SG, salivary gland. The figure is reproduced and modified from Šimo et al. (2012, 2013) and Šimo and Park (2014).

understanding the neural and endocrine mechanisms of tick osmoregulation. This review including illustrations are from the studies with *Ixodes scapularis* the vecor of Lyme disease in U.S. unless specific species is mentioned.

2. Tick salivary gland and its regulation

2.1. Development and structure of tick salivary glands

The paired anterolateral salivary glands are the largest glands in the tick body, extending about two-thirds and one-seventh of the haemocoel length in ixodid and argasid ticks, respectively (Fig. 1C and D). In both families, the robust grape-like salivary glands are composed of different types of spherical acini, (also called alveoli) directly attached either to a main or branched salivary duct (Fig. 2A–C). In close proximity to the tick mouthparts, the main excretory/salivary ducts merge into a single salivarium (Binnington, 1978; Coons and Roshdy, 1973; Fawcett et al., 1986; Sonenshine, 1991). In ixodid ticks, three different types of acini (I, II and III) are present in females and four types (I-IV) are recognized in males, whereas only two types (I and II) of acini are present in the salivary glands of argasid ticks. For example, a single salivary gland of a *Rhipicephalus appendiculatus* female consists of about 1400 acini in total (Walker et al., 1985). The less abundant agranular type I acini occupy the anterior part of the main salivary duct, with occasional occurrence in the proximal portions of the thinner lobular ducts. The more abundant granular type II and III (most abundant) acini join the peripherally located secondary and tertiary branches, respectively (Binnington, 1978; Binnington and Stone, 1977; Binnington et al., 1983; Coons and Roshdy, 1973; Fawcett et al., 1981a,b; Megaw and Beadle, 1979; Sonenshine, 1991; Walker et al., 1985) (Fig. 2).

Although only the salivary duct has been discerned in early larvae of ixodid ticks (Till, 1961), low numbers of type I, II and III (not type IV) acini seem to appear in mature larval individuals (Chinery, 1965). As in all tick feeding stages, the salivary glands in larvae enlarge during the feeding process and degenerate once the larvae drop off of the host. Subsequently, the small salivary duct branches out, and small acini with undifferentiated cells form on their terminals. During metamorphosis to the nymphal stage, the number of acini increases and they differentiate into types I, II, and III acini in mature nymphs. As in larvae, a similar degeneration-differentiation process of the salivary glands occurs as recently fed nymphs metamorphose into molted adults, resulting in the development of all four types of acini I-IV (Binnington, 1978; Chinery, 1965; Till, 1961).

2.2. Excretory functions of type II and III acini

Granular type II acini are commonly found in both the argasid and ixodid ticks, with several complex cellular structures, including different types of cells containing dense secretory granules, epithelial, myoepithelial (or also named as adluminal cell or Cap), multiple abluminal interstitial and neck cells (Binnington, 1978; Coons and Roshdy, 1973; Fawcett et al., 1986; Sonenshine, 1991) (Fig. 1 and 2D). Type III acini are granular during the early feeding stage in basally located cells, but exhibit growth in the size of the expanded epithelial cell types in the apical region during feeding (Šimo et al., 2013, 2011). Both type II and type III acini commonly have a single myoepithelial cell located on the luminal surface of the epithelial cell junctions, in a web-like fashion. The myoepithelial cell also surrounds the valve-like cuticular structure at the junction between acini and salivary ducts (Fig. 2D–G).

An earlier study demonstrated that 75% of ingested water is excreted back into the host through the salivary glands during blood feeding (Kaufman and Phillips, 1973). This excretory function is achieved by water transport through the epithelial cells in types II and III acini. Thus far, the physiology involved in the salivary secretion can be simplified by the following steps.

1. Influx of water and electrolytes into the lumen of acini type II/III. This process is believed to be perpetrated through epithelial types of cells in type II/III acini using the major electrochemical gradient of sodium ions.
2. Pumping and gating in acini type III. After the lumen of an acinus is filled with primary saliva, expulsion of water into the salivary duct occurs. This process likely involves the actions of the myoepithelial cell and neck cells.
3. Resorption of ions and water in proximally located type I acini. Filtering of the primary saliva produced from the type II/III acini occurs through type I acini for resorption of potassium ions and water.

2.3. Neuropeptidergic innervations and the neuropeptide receptors in salivary gland acini types II and III

The complex neural and endocrine systems controlling tick salivary secretion have been extensively studied in a species of hard tick, *I. scapularis*. Among multiple neuropeptides newly identified in the genomic sequence (Gulia-Nuss et al., 2016), two neuropeptides, MIP and SIFamide, and their receptors were characterized for their roles in controlling the ticks' salivary glands. These neuropeptides were identified via immunohistochemistry and a matrix assisted laser desorption (MALDI) of samples enriched with either synganglion, hindgut, or salivary glands (Fig. 3). More specifically, MIP and SIFamide were commonly found in all three different organs and FGLamide related allatostatins were found in the synganglia and hindgut. The common peaks in different organs in this study implies the neuronal projections containing the neuropeptides from the synganglion reach to the specific organ for innervations.

In immunohistochemical studies, both neuropeptides, MIP and SIFamide, are co-localized in a pair of protocerebral salivary gland (PcSG) neurons, with their axonal projections reaching to the basal regions of the type II and III acini of the salivary gland (Fig. 2) (Šimo et al., 2009a). Additionally, the respective G protein-coupled receptors for MIP and SIFamide were identified in the tick salivary glands (Šimo et al., 2013). The SIFamide receptor exhibited immunoreactivity surrounding the acinar valve, extending to the luminal surface of basally

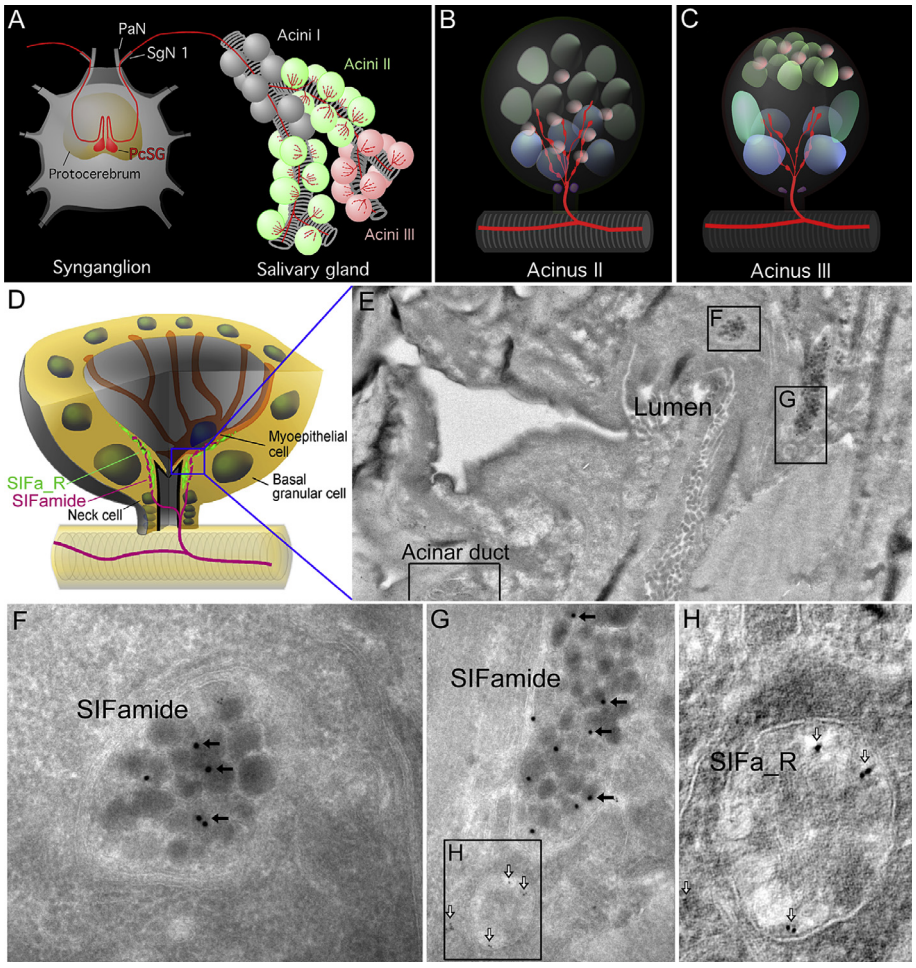

Fig. 2. Neural control of salivary gland acini. (A) Synganglion showing PcSG (protocerebral salivary gland neurons) with a neuronal projection via the salivary nerve 1 (SgN1) to salivary gland acini types II and III. (B) Type II and (C) type III acini with axon terminals at the basal regions. (D) Diagram showing the basal region of a type III acinus with immunolocalization of SIFamide and its receptor (SIFa_R), luminal myoepithelial cell, and basal granular cell in partially fed tick. (E) Transmission electron microscopy showing a SIFamide-positive axon with insets for higher magnifications in (F) and (G). (H) SIFamide receptor immunoreactivity via immuno-transmisison electron microscopy. Black arrows show the 15 nm gold nanoparticles for SIFamide and empty white arrows show 6 nm gold nanoparticles for SIFamide receptor. The figure include the figures reproduced and modified from Šimo et al. (2009a, 2013), while (E to H) are new data presented in this paper.

located acinar cells (Šimo et al., 2013), presumably the subcellular regions of the myoepithelial cell (Fig. 2D–H).

Based on the anatomy of the PcSG neuronal projections and the receptors in the salivary acini, the functions of these two neuropeptides are likely myoregulatory, possibly having antagonistic properties, which has been shown for the control of myoactivities in the tick hindgut (see section 3 for details). Differential expressions of MIP and SIF amide and their receptors throughout the different feeding stages are likely the mechanisms involved in the dynamic control of the salivary glands (Šimo et al., 2013). Although the anatomy suggests myoregulatory roles for MIP and SIFamide, we could not rule out the possible functional involvement of neuropeptides in the control of granular cells for the secretion of protein components.

An additional neuropeptide, elevenin, and its receptors have recently been identified in the salivary glands. Surprisingly, this neuropeptide was also found to be expressed/immunolocalized in the PcSG neuron and the axonal projections to the type II and III salivary gland acini. Based on these immunohistochemical studies, PcSG neurons express at least three different neuropeptides, SIFamide, MIP, and elevenin, and are likely the major controllers of salivary gland acini types II and III, although their functions need to be further investigated.

Axonal projections specific only to type II granular acini, originating from the two pairs of opistosomal neurons ($OsSG_{1,2}$), was identified in an immunohistochemistry using antibodies against PDF (pigment dispersing factor of insect) and orcokinin neuropeptides (Roller et al., 2015; Šimo et al., 2009b, 2012). However, as the PDF-like sequence has not been identified in the genomes of ticks and other arachnids, the PDF-immunoreactivity is likely the result of cross-reactivity with an unknown protein. The neural components that cross-reacted to PDF antibody is remaining to be identified yet. These observations provide another possible neuropeptidergic function for activating granular cells or controlling the myoepithelial cell in acini type II, leading to the secretion of salivary proteins.

2.4. Dopamine-mediated activation of salivary excretion

Earlier studies found that dopamine is the most potent endogenous compound that directly activates tick salivary secretion (Kaufman, 1976, 1977; McSwain et al., 1992; Schmidt et al., 1982). Another pharmacological stimulator, pilocarpine (PC, muscarinic acetylcholine receptor agonist), is considered to be acting through a cholinergic receptor, which activates the neural circuit for salivary secretion in the synganglion (central nervous system of the tick), because it exhibits activity only when the intact synganglion is attached to the salivary glands (Kaufman, 1978; Kaufman and Harris, 1983). Activation of the salivary glands through dopaminergic neurons has been a general assumption (Bowman and Sauer, 2004). However, presence of more than 1 ng of dopamine per pair of salivary glands in the *Amblyomma hebraeum* tick (Kaufman et al., 1999) and up to 100 pg in *Ixodes scapularis* (Koči et al., 2014), led to a hypothesis that autocrine or paracrine dopamine acts on the salivary glands. Additionally, the latter study showed that the dopamine level in the salivary glands increases upon feeding, peaking at day 5, and slowly declines afterward (white bar for dopamine in Fig. 4). High levels of conjugated dopamines in the salivary glands may have important roles in dopamine metabolism as either precursors, storage, or metabolically inactive forms of dopamine (Fig. 4).

The effects of dopamine on salivary secretion are dependent on both

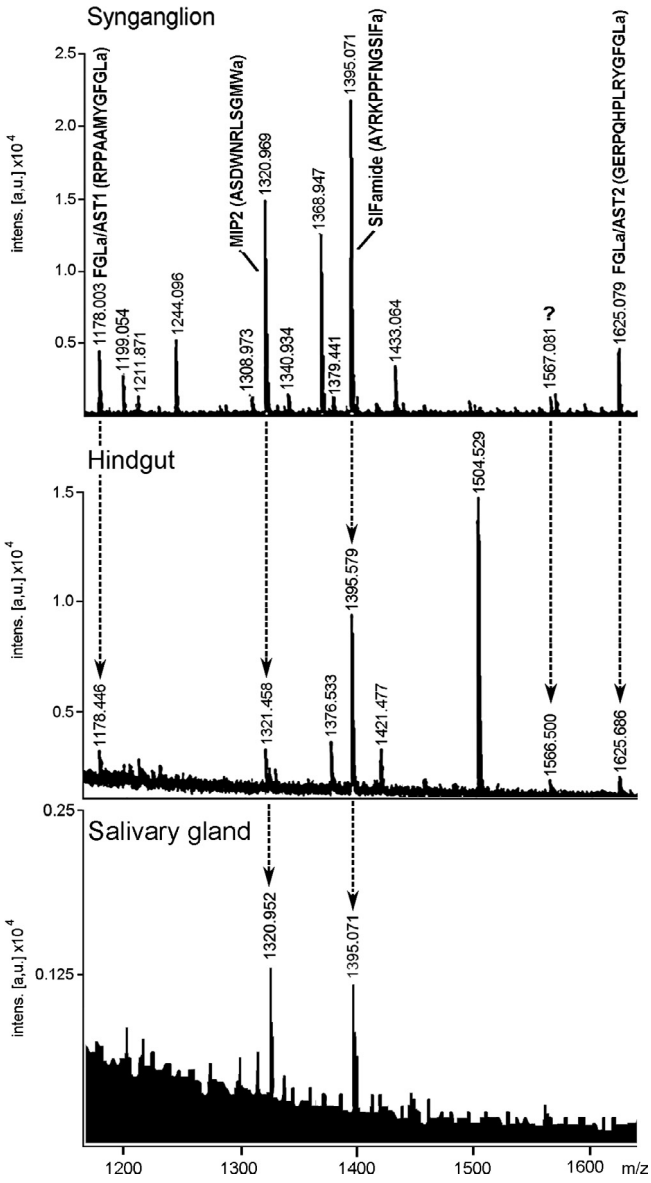

Fig. 3. Results from matrix assisted laser desorption (MALDI) of samples enriched with either synganglion, hindgut, or salivary gland. Note that there are a number of common peaks, implying the neural projections from the synganglion reaching to the target organs. The peaks are annotated based on the results of tandem mass spectrometry. The scales in Y-axis are different in different tissues. The figure is reproduced and modified from Šimo and Park (2014) and Šimo et al. (2009a).

extracellular Ca^{2+} (Kaufman, 1976; Needham and Sauer, 1979) and cAMP (Hume et al., 1984; Krolak et al., 1983; Schmidt et al., 1981). Two different dopamine receptors, dopamine receptor (D1) and invertebrate specific D1-like dopamine receptor (InvD1L), were characterized in both type II and III acini of the tick salivary glands (Šimo et al., 2014; Šimo et al., 2011). D1 immunoreactivity was located on the luminal surface of the epithelial cell types, while InvD1L was detected in the neural projections in close proximity to the acinar lumen.

Two dopamine receptors were further characterized for their localizations and activities in the heterologous expression system. These studies suggested that the downstream of the D1 receptor is likely for cAMP elevation in epithelial cells, and that of the InvD1L receptor is for Ca^{2+} mobilization in axonal processes innervating the luminal myoepithelial cell (Šimo et al., 2014, 2011). Therefore, two different dopamine actions orchestrate the first two steps of salivary secretion (steps 1 and 2 in section 2.2). The influx of fluids and pumping/gating actions occur through the D1 and InvD1L dopamine receptors, respectively, in specialized acini cell types (Kim et al., 2014).

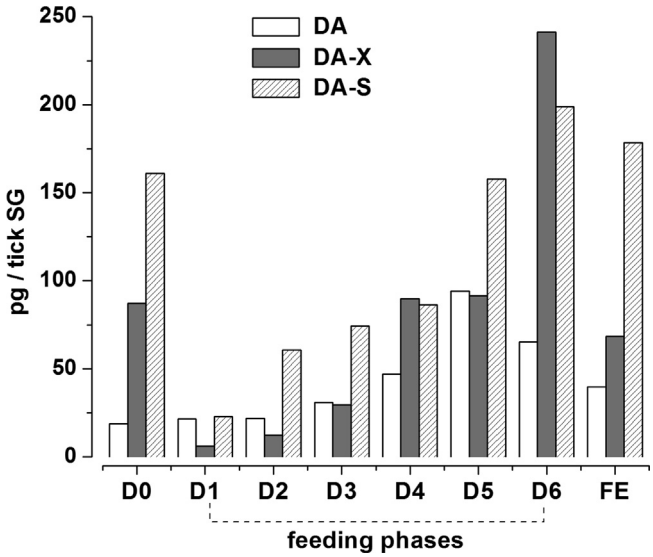

Fig. 4. Levels of dopamine (DA), dopamine sulfate (DS-S), and other conjugates (DA-X) in unfed (D0), 1 to 6 days fed, and fully engorged (FE) ticks' salivary glands. The figure is reproduced and modified from Kočí et al. (2014).

In vitro physiological experiments on isolated salivary glands treated with receptor-specific agonists and antagonists have discriminated the differential roles of dual dopamine receptors in the salivary glands. Salivary secretion assays (a modified Ramsay's assay, Fig. 5) showed the dose-dependent action of dopamine activation for salivary secretion. Observation of individual type III acini under a microscope, in real time, revealed the pumping actions (sudden reduction of the acini size) that are likely driven by contractions of myoepithelial cells, as well as the gating actions (slow release of luminal content through flapping valve located in the neck region, videos available (Kim et al., 2014) (Fig. 6). A series of experiments using a receptor-specific agonist and antagonist with the physiological assays supported the hypothesis that the D1 dopamine receptor activates cAMP elevation mediated fluid transport in epithelial cells for an increase in luminal volume. Meanwhile, InvD1L receptors trigger Ca^{2+} mediated contraction of the myoepithelial cell for expelling the fluid out to the salivary duct (Steps 1 and 2, respectively, in section 2.2).

2.5. *Absorptive function of type I acini*

For off-host ticks, surviving in vegetated environments for long durations between feedings requires physiological mechanisms for water uptake and conservation. Hard ticks can uptake water either by direct drinking or by capturing water vapor using hygroscopic (hyperosmolar) saliva, depending on the species and individuals (Kim et al., 2017; Rudolph and Knulle, 1978; Rudolph and Knülle, 1974). Earlier studies have suggested that type I acini are the site for the production of the hygroscopic saliva in the off-host stage. However, this hypothesis was contradicted by the ultrastructural observation of type I acini; the desiccated off-host tick contained resting phases of mitochondria, while the rehydrated tick has a highly active form of mitochondria in the lamellate cells of type I acini (Needham et al., 1990).

We found that type I acini are indeed the site of direct water absorption, which is consistent with the ultrastructural observation of low activity under desiccation and high activity in rehydration. Providing fluorescent water (1 mM rhodamine 123) to dehydrated off-host ticks demonstrated that the water immediately moved into type I acini through salivary duct upon drinking (Fig. 7). In addition, the type I

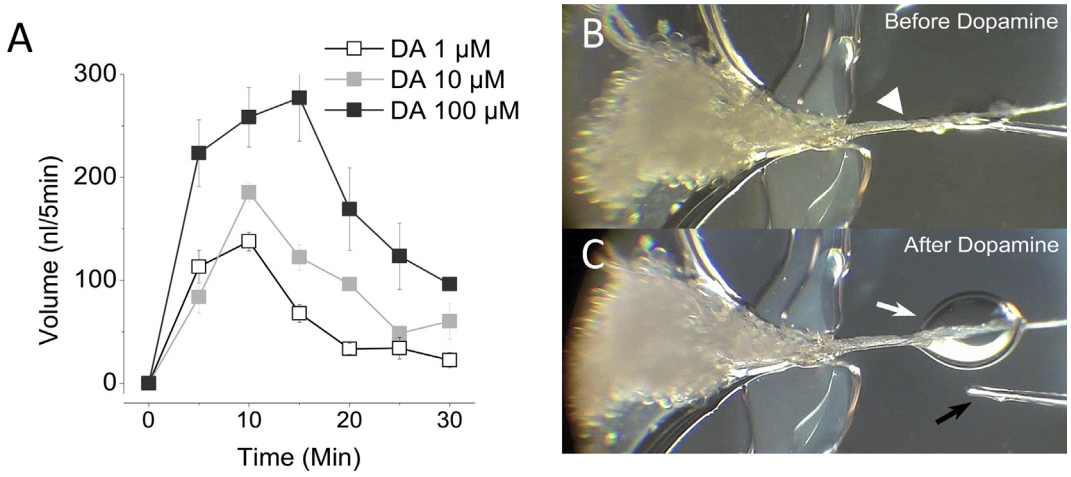

Fig. 5. Modified Ramsay's assay on tick salivary gland shows dopamine-induced secretory activities in a dose dependent manner. (A) Salivary secretions over time after different doses of dopamine treatments. (B and C) Experimental setup for the modified Ramsay's assay. The figure is reproduced and modified from Kim et al. (2014).

acinus is the site of water/ion resorption from the primary saliva produced from the type II and III acini in the feeding phase (the step 3 in Section 2.2. Fig. 8) (Kim et al., 2016). The absorptive function of type I acini was driven by the function of ouabain-sensitive Na/K-ATPase (Kim et al., 2016). This observation indicates the importance of the function of the type I acini in maintaining homeostasis of the water/ion balance in both unfed and feeding ticks. In addition, understanding the absorptive role of type I acini offers the means to deliver agents for use in the experimental manipulation of ticks and for development of the acricidal measures.

3. Regulation of tick hindgut

Tick excretion through the hindgut is mainly for the removal of metabolic wastes and potassium-rich water; 20–25 % of ingested water in the on-host tick (Kaufman and Phillips, 1973). The excretory organs in ticks are comprised of Malpighian tubules filled with the urea-rich white excretion, a rectal sac connected to a short anal canal, and the anus. In our observations, the heme-rich (red) and the urea-rich (guanine-rich, white) excretions are periodically defecated during feeding in different fecal pallets, indicating presence of a complex regulatory mechanism for separated excretory physiology.

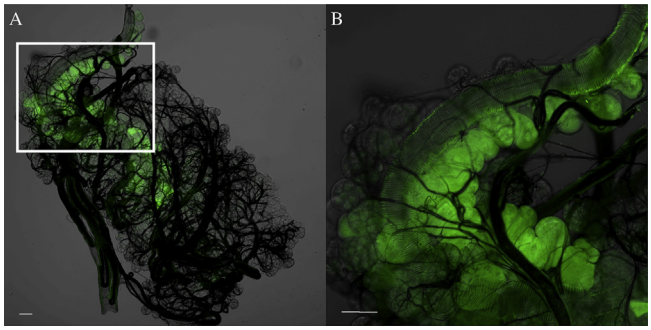

Fig. 7. Uptake of fluorescently labeled water by type I acini (green). (A) Entire salivary gland, including type I/II/III acini with tracheal branches. (B) A magnified view of the inset showing type I acini. The figure is reproduced and modified from Kim et al. (2016).

An experimental approach, similar to the one that was successful in identifying and characterizing salivary gland neuropeptides, has provided invaluable insight into tick hindgut physiology. Four immunoreactive neural projections were identified for the innervation of the hindgut, including FGLamide related allatostatin, MIP, SIFamide,

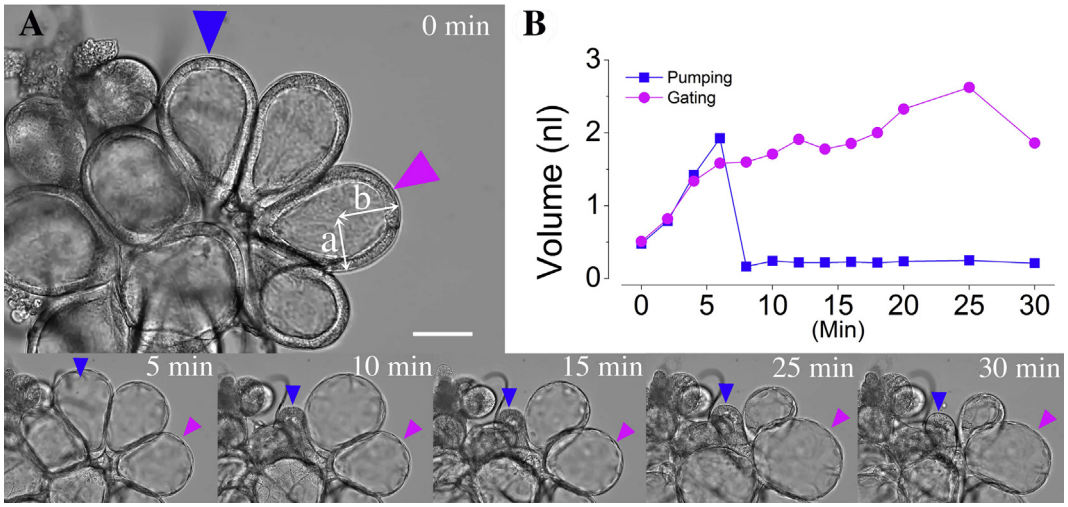

Fig. 6. Changes in acini size following dopamine treatment, showing pumping and gating actions of individual acini. (A) Type III acini under microscope, demonstrating changes in the acini size over time following dopamine treatment. (B) Changes in the acini volume after dopamine treatment. The figure is reproduced and modified from Kim et al. (2014).

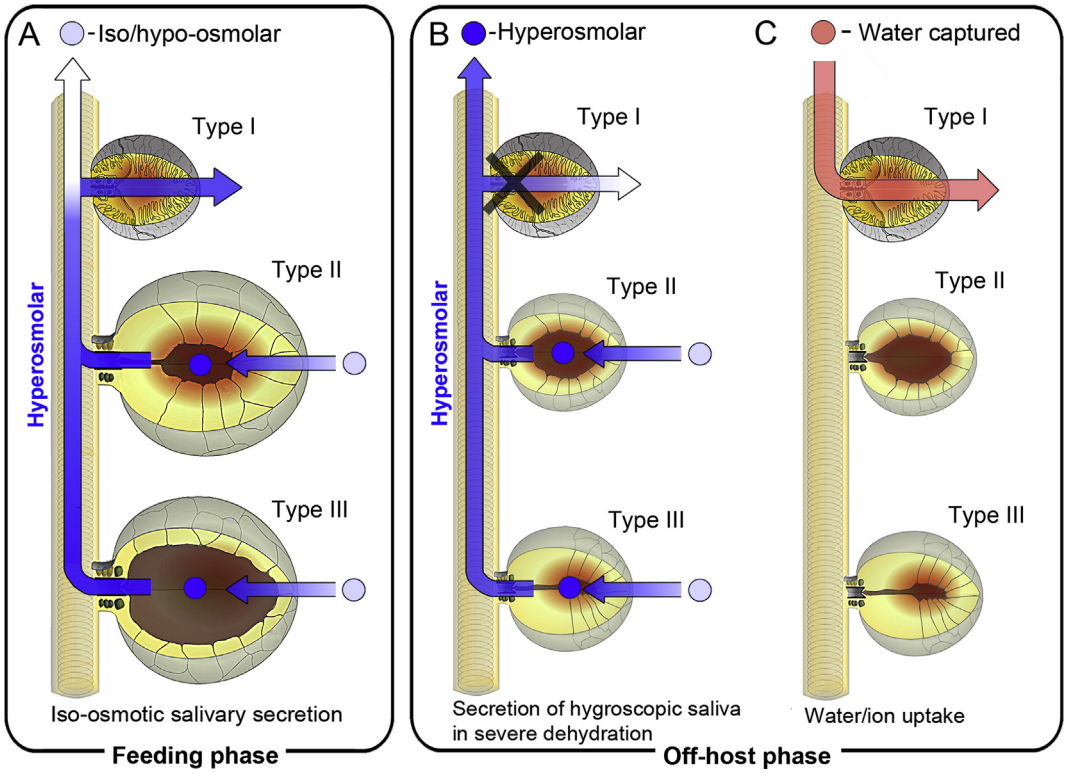

Fig. 8. Model explaining roles of specific acini types. (A) The process for iso/hypo osmolar salivary excretion in feeding tick. (B) Production of hyperosmolar saliva for capturing water molecules. (C) Uptake of water captured in the hygroscopic salivary secretion. The figure is reproduced and modified from Kim et al. (2017).

and orcokinin. Among the four peptides exhibiting positive immunoreactions, three were confirmed via MALDI analysis using a hindgut-enriched sample (Šimo and Park, 2014), (Fig. 3).

FGLamide related allatostatin originates from the pair of synganglion opisthosomal neurons (OsHG) and the projection reaches the surface of the hindgut. MIP/SIFamide neurons stem from two pairs of post-oesophageal hindgut neurons ($PoHG_{1\ and\ 2}$). Double-staining for FGLa/AST and MIP/SIFamide revealed that most of the immunoreactive axons run close to each other and contain multiple varicosities, but with clear separations (Šimo and Park, 2014). Similar orcokinin immunoreactive projections were also identified, but this immunoreactivity was not confirmed via MALDI for peptide identification, thus it remains as an unidentified neuropeptide (Šimo and Park, 2014; Roller et al., 2015).

The presence of four different FGLamide related allatostatin receptor transcripts were all confirmed through reverse transcription PCR (Šimo and Park, 2014). Based on the immunoreactivities, MIP receptors were found mainly along the cross and longitudinal visceral muscles on the surface of the hindgut, while SIFamide receptors were found both on visceral muscles and on hindgut epithelial cells.

Bioactivities of neuropeptides were tested on an isolated hindgut for the myoactivities. SIFamide showed strong myostimulatory activity with EC50 of 55 nM, while MIP was again found to have an antagonistic effect, suppressing SIFamide-mediated myostimulatory activity (Šimo and Park, 2014) (Fig. 9). Together, with the roles of MIP/SIFamide found in the control of salivary glands, it was proposed that concurring temporal needs of excretory functions in both the salivary glands and the hindgut during feeding stages are controlled by the antagonistic activities of these two peptides.

4. Implications of current knowledge, limitations, and future direction

The physiology and endocrinology of ticks, obligatory hematophagous arthropods, provide an excellent model system where dynamic developmental and physiological changes occur during blood feeding. The knowledge gained through these studies may be applicable to improve human and animal health by reducing tick populations, bite frequency, and/or disease transmission. Our studies using genomics, transcriptomics, and proteomics have been highly successful for identifying the components involved in the control of salivation and osmoregulation. Some of these components have been tested in the *semi-in vivo* physiological assays to understand their specific physiological activities. The actions of dopamine through two different dopamine receptors for the orchestration of salivary secretion has been well demonstrated by uncovering receptor subtype specific antagonists and agonists. Antagonistic actions of SIFamide and MIP are also shown in the hindgut motility assays.

Unfortunately, assays aimed at understanding neuropeptide actions on the salivary glands are impeded by the impermeability of peptides for activation of luminally located representative receptors. Additionally, we were unable to make tick RNA interference (RNAi) work in repeated trials, although we were able to achieve the goal by using pharmacological agonists and antagonists of specific target receptors (i.e., dopamine receptors, (Kim et al., 2014)). Difficulties in tick RNAi is the conclusion reached after failures in over 50 different trials testing possible variables that might be affecting the RNAi efficiencies, such as different concentrations of double stranded RNA, site and time of injections, different strains (Oklahoma and CDC strains), different target genes, including trials repeating experiments previously published by other groups. Therefore, development of a successful and robust RNAi method in tick will allow us to move forward in investigations of the neuropeptide functions in the salivary glands.

The current study has been focusing on the control of salivary glands for osmoregulation. In addition to water excretion, another important function of salivary glands is the secretion of bioactive compounds, including numerous proteins from granular cells. So far, only one type II-specific axonal projection has been identified by the

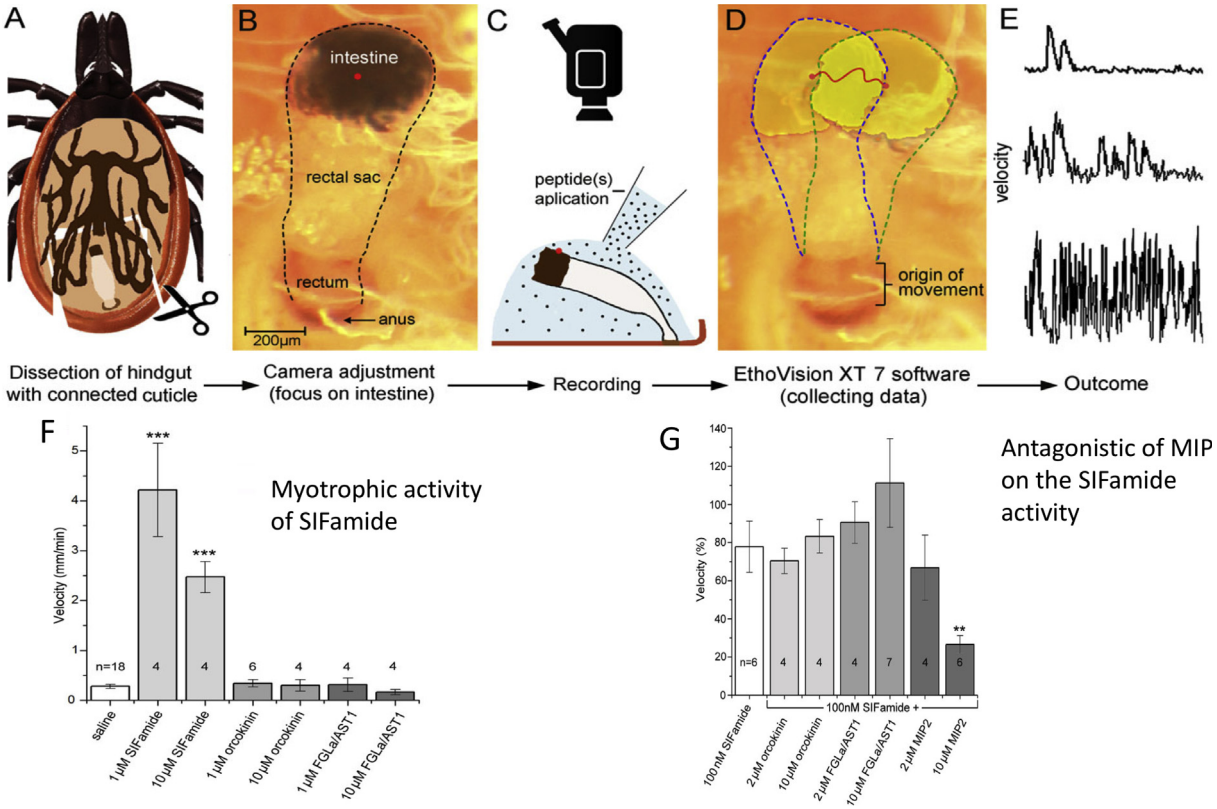

Fig. 9. Myoactivities of the tick hindgut, controlled by SIFamide and MIP neuropeptides. (A) Schematic of dissection and preparation of tick hindgut for *in vitro* assay. (B) The microscopic view of the hindgut. (C) The experimental setup for peptide treatments on the hindgut and the recording of treatment results. (D) An exemplary view of the hindgut movement. (E) An example of the data acquired and analyzed in EthoVision. Note that the origin of the recorded movement is in the posterior hindgut region called the anal canal. (F) Dose-dependent myotropic activity of SIFamide. (G) Antagonistic activity of MIP on the myotropic activity of SIFamide. The figure is reproduced and modified from Šimo and Park (2014).

colocalized immunoreactivity to PDF and orcokinin, although the nature of these components is not yet identified. Identifying the mechanisms controlling this apocrine type of secretion in the granular cells of acini will be an important breakthrough in this area of study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.08.004.

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