Post-feeding physiology in *Rhodnius prolixus*: The possible role of FGLamide-related allatostatins

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A B S T R A C T

Allatostatins (ASTs) are neuropeptides that were first identified as inhibitors of juvenile hormone biosynthesis by the corpora allata of some insect species. The FGLamide-related ASTs (FGLa/ASTs) belong to one of three families of insect ASTs. Previously, we showed that *Rhodnius prolixus* FGLa/ASTs (Rhopr-FGLa/ASTs) are present throughout the *R. prolixus* central nervous system and are associated with 5 dorsal unpaired median (DUM) neurons in the mesothoracic ganglionic mass. A similar set of neurons contain serotonin which is a diuretic hormone in *R. prolixus*. Rhopr-FGLa/ASTs inhibit both spontaneous contractions of the anterior midgut and leucokinin-1-induced hindgut contractions. Since these tissues are involved with post-feeding diuresis, these data suggest a possible role for FGLa/ASTs in events associated with feeding, and a possible interaction with serotonin. To investigate this possibility, we have examined the DUM neurons in more detail with regard to their peptide content, examined the potential release of Rhopr-FGLa/ASTs into the haemolymph following feeding, and further investigated the effects of Rhopr-FGLa/ASTs on feeding-related tissues. There are 10 DUM neurons in the abdominal neuromeres, 5 of which express serotonin-like immunoreactivity and the other 5 express FGLa/AST-like immunoreactivity. FGLa/AST-like immunoreactivity is reduced in the 5 DUM neuron cell bodies and their neurohaemal sites on abdominal nerves at 3–5 h post feeding. Rhopr-FGLa/ASTs do not inhibit serotonin-stimulated anterior midgut absorption or Malpighian tubule secretion but do inhibit hindgut contractions induced by an endogenous kinin, suggesting that they may only indirectly affect post-feeding diuresis in *R. prolixus.*

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1. Introduction

The ability of certain neuropeptides (referred to as allatostatins, ASTs) to inhibit the biosynthesis of juvenile hormone (JH) by the corpora allata (CA) was first shown in *Diploptera punctata* (Pratt et al., 1991; Woodhead et al., 1989). Since that time a number of AST neuropeptides have been isolated that arise from three peptide families. These families are encoded by three separate genes and processed into neuropeptides with distinct sequences and pleiotropic activities across arthropods. The families are referred to as the cockroach type (FGLa/ASTs), cricket type (MIP/ASTs), and moth type (PISCF/ASTs), each based upon their C-terminal characteristic sequences (see Bendena et al., 1999; Tobe and Bendena, 2006). The FGLa/ASTs are present in numerous insect orders where they have

physiological roles distinct from controlling JH biosynthesis, such as inhibition of visceral muscle contraction (see Tobe and Bendena, 2006). These latter authors suggest that the FGLa/AST role as inhibitors of JH biosynthesis is a secondarily-evolved function, and that inhibition of visceral muscle contraction might be their ancestral function (see Tobe and Bendena, 2006). It is also interesting to observe that the cricket type MIP/ASTs were first described based upon their ability to inhibit visceral muscle contraction. FGLa/AST-like immunoreactivity is found in neurons within the central nervous system (CNS), but is also associated with processes over the foregut, midgut, and hindgut, as well as open-type midgut endocrine cells (see Robertson and Lange, 2010; Sarkar et al., 2003), which suggest that ASTs might play an important role in feeding-related physiological events. Myoinhibitory activity of FGLa/ASTs on the gut has been found in a variety of species. For example, FGLa/ASTs inhibit spontaneous contractions of the foregut in *Leucophaea maderae* (Duve et al., 1995), proctolin-induced muscle contractions of the *D. punctata* midgut (Fuse et al., 1999) and spontaneous and proctolin-induced hindgut muscle contractions of *D. punctata* (Lange et al., 1993).

Recently, the FGLa/AST gene transcript as well as FGLa/AST peptides were identified in the medically-important bug, *Rhodnius prolixus* (Ons et al., 2009, 2011; Zandawala et al., 2012). This is of some

importance because of the possible involvement of FGL/ASTs in insect feeding activities and the fact that this blood-gorging bug transmits the parasite *Trypanosoma cruzi* to humans in its urine, causing Chagas disease. Zandawala et al. (2012) used fluorescent *in situ* hybridization (FISH) to localize cell-specific expression of the Rhopr-FGLa/AST transcript within the CNS, and in particular identified 5 dorsal unpaired median (DUM) neurons in abdominal neuromeres of the mesothoracic ganglionic mass (MTGM). These same cells are positive for FGLa/AST-like immunoreactivity (Sarkar et al., 2003). This is of some interest since a similar set of 5 DUM neurons in the abdominal neuromeres of the MTGM contain serotonin (5-hydroxytryptamine, 5-HT) which is a diuretic hormone in *R. prolixus* (Maddrell et al., 1991; Orchard et al., 1989), colocalized with a neuropeptide – a calcitonin-like diuretic hormone (CT/DH) (Te Brugge et al., 2005). In light of their similar location, we were interested in further examining the DUM neurons of the MTGM, and examining their properties and possible interactions in post-feeding diuresis and other feeding-related physiological events.

2. Material and methods

2.1. Animals

Fifth-instar *R. prolixus* were obtained from a long standing colony at the University of Toronto Mississauga. Insects were reared in incubators in high relative humidity at 25 °C and routinely fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada).

2.2. Immunohistochemistry and time-course analysis

The insects were fed on defibrinated rabbit blood or saline containing 1 mM ATP and 150 mM NaCl at pH 7 (Friend and Smith, 1982). CNS, anterior midgut, posterior midgut and hindgut were used for the immunohistochemical analysis from the following insects: 3–4 week unfed fifth-instars, 1 h post-feeding on blood or saline, and 3–5 h post-feeding on blood or saline. The fixation and staining of the tissues for FGLa/AST-like immunoreactivity was done as described previously (Sarkar et al., 2003) with some minor modifications. Tissues were incubated for approximately 60 h at 4 °C in a 1:1000 polyclonal rabbit antiserum generated against Dippu-AST-7 (formerly 1) (Vitzthum et al., 1996) diluted in 0.4% Triton X-100 with 2% BSA and 10% NGS. The preparations were then washed with phosphate-buffered saline (PBS) for 4–6 h at room temperature. Next, the tissues were incubated overnight at 4 °C in affinity purified goat anti-rabbit antibody conjugated to Cy3 at a dilution of 1:600 in PBS containing 2% NGS. The tissues were then washed in PBS for 4–18 h and cleared with a glycerol series before mounting in 100% glycerol. Images were taken using a confocal microscope equipped with ZEN 2009 LE software (Zeiss, Jena, Germany). Staining intensity of the DUM neurons and the background was determined using the ImageJ Software. Ratios of cell intensity to background intensity were calculated for all 5 DUM neurons and their means plotted as a bar graph. An unpaired *t*-test was used for the statistical analyses ($P < 0.05$). Double-label immunohistochemistry was performed on CNS as described earlier (Sarkar et al., 2003), using anti-dippu-AST-7 antiserum and anti-serotonin antiserum (DiaSorin, Stillwater, MN, USA).

2.3. Neurophysiology

Intracellular recordings and Lucifer yellow injection of DUM neurons was performed as previously described, as was immunohistochemistry following such treatments (Orchard et al., 1989). Briefly, for intracellular recordings, the dorsal thoracic and abdominal cuticle as well as the gut were removed to expose the MTGM.

The MTGM was stabilized with a metal spoon and kept moist with physiological saline. Electrodes were filled with 5% (w/v) Lucifer yellow CH in distilled water. The electrode resistances varied between 60 and 200 MΩ. The electrodes were connected to a World Precision Instruments S-7071A electrometer (Sarasota, Florida, USA) and oscilloscope, which allowed for recording or current injection via a bridge circuit. Lucifer yellow was injected into the cells by passing hyperpolarising direct current (4–7 nA) for up to 20 min. The ganglia were then fixed in 2% paraformaldehyde and processed for immunohistochemistry.

2.4. Peptides

Rhopr-FGLa/AST-2 (LPVYNFGLamide), Rhopr-FGLa/AST-3 (AHNEGRLYSFGLamide) and Rhopr-kinin-2 (AKFSSWGamide) were custom synthesised by GenScript (Piscataway, NJ, USA) at >95% purity.

2.5. Anterior midgut absorption assay

Fluid transport across the anterior midgut was measured using the absorption assay described earlier (Te Brugge et al., 2009). Briefly, the anterior midgut was exposed by removing the dorsal cuticle and diaphragm. The anterior end of the anterior midgut (foregut and anterior midgut juncture) was ligated using a silk thread. Next, the anterior midgut was nicked at the juncture with posterior midgut and 30–50 μl physiological saline (150 mM NaCl, 8.6 mM KCl, 2 mM CaCl$_2$, 4 mM NaHCO$_3$, 34 mM glucose, 8.5 mM MgCl$_2$, 5 mM HEPES [pH 7.2]) containing 0.01% methylene blue was injected into the anterior midgut before ligating its posterior end. Methylene blue was used to check for leakage. The anterior midgut was gently blotted and weighed on a Mettler AE 240 balance. Once weighed, the tissues were placed in a micro-centrifuge tube with 1 mL saline, saline containing only serotonin (10^{-7} M) or both serotonin (10^{-7} M) and peptide solution (10^{-6} and 10^{-7} M). Following 30 min incubation, the tissues were blotted and weighed again. The difference in weight (initial–final) was used to calculate the absorption rate (nL/min), assuming a specific gravity of 1. The results are expressed as mean ± s.e.m. One-way ANOVA and Tukey post-test were used for the statistical analyses ($P < 0.05$).

2.6. Upper Malpighian tubule (MT) secretion assay

The upper MT fluid secretion assay was performed using the methods described earlier, with few modifications (Donini et al., 2008; Paluzzi and Orchard, 2006). Briefly, upper MTs were dissected using a fine glass probe under saline that contained (mmol l^{-1}): 129 NaCl, 8.6 KCl, 4.0 NaHCO$_3$, 4.3 NaH$_2$PO$_4$, 8.5 MgCl$_2$, 2 CaCl$_2$, 8.6 HEPES and 20 glucose at pH 7. Upper tubule segments were transferred to a Sylgard-lined dish containing 90 μl drops of saline covered with water-saturated paraffin oil. The open end of the tubules were pulled out of the saline and wrapped around a nearby minuten pin. Next, 10 μl of saline (control) or saline containing 10^{-6} M peptide (Rhopr-FGLa/AST-2 or Rhopr-FGLa/AST-3) were added to the bathing saline droplet (final concentration of the peptide was 10^{-7} M). The tubules were then incubated for 15 min. Following this incubation, 10 μl of saline was removed from the bathing droplet and replaced with 10 μl of saline containing 10^{-6} M serotonin alone (control) or combined with 10^{-7} M peptide (Rhopr-FGLa/AST-2 or Rhopr-FGLa/AST-3). Droplets of secreted fluid that formed at the pin after 30 min were collected using fine glass probes. The diameter (d) of the droplet was measured using an ocular micrometer and used to calculate the volume of secreted fluid using the equation $V = (\pi/6)d^3$. The volume was divided by the time over which the droplets formed to obtain the

rate of secretion. One-way ANOVA and Tukey post-test were used for the statistical analyses ($P < 0.05$).

2.7. Hindgut contraction assay

The contraction assay using hindgut from unfed fifth-instar *R. prolixus* was performed as described previously (Te Brugge et al., 2002; Zandawala et al., 2012), except that contractions were monitored and recorded using PicoLog recorder (Pico Technology, St. Neots, Cambridgeshire, UK). Two-tailed unpaired *t* test was used for the statistical analysis ($P < 0.05$). Briefly, hindgut assays were conducted on isolated fifth-instar hindguts maintained under physiological saline. The preparation consisted of a small piece of ventral cuticle surrounding the anus to secure the hindgut to a Sylgard (Dow Corning, Midland, Michigan, USA) coated dish, while the anterior end of the hindgut and a small portion of the posterior midgut was tied by a fine thread to a miniature force transducer (AksjeselskapetMikro-elektronikk, Horten, Norway). Longitudinal contractions were recorded on the PicoLog. Hindguts from unfed *R. prolixus* rarely contract spontaneously *in vitro*, and so Rhopr-kinin-2 (Bhatt et al., 2013) was used to stimulate contraction before testing the inhibitory effects of Rhopr-FGLa/AST-2 or -3).

3. Results

3.1. Serotonin-like and FGLa/AST-like immunoreactivity in DUM neurons

Double-label immunohistochemistry indicates that the 5 serotonin-like DUM neurons in the MTGM are distinct from the 5 FGLa/AST-like DUM neurons (Fig. 1A). Each of these DUM neurons produces extensive neurohaemal sites on their corresponding abdominal nerve (see Orchard, 2009), and there is no co-localization of FGLa/AST-like and serotonin-like immunoreactivity in these neurohaemal sites (Fig. 1B). There are, however, other cells in the CNS which are immunoreactive for both serotonin and FGLa/ASTs (Fig. 1A).

3.2. Neurophysiology

Intracellular recordings from the FGLa/AST-like immunoreactive DUM neurons (confirmed by Lucifer yellow injection and immunohistochemistry) revealed that they have smaller cell bodies than the serotoninergic DUM neurons (30 μm diameter versus 40 μm diameter). They are capable of supporting overshooting action potentials of between 40 and 60 mV amplitude, with large after hyperpolarisations (Fig. 2). There are likely multiple spike initiation sites resulting in various spike types recorded from the cell body (Fig. 2). Lucifer yellow did not pass between the DUM neurons and so it is unlikely that they are electrically-coupled.

3.3. FGLa/AST-like immunoreactivity pre- and post-feeding

The FGLa/AST-like staining intensity of the DUM neuron cell bodies is significantly reduced at 3–5 h post-feeding (Fig. 3A–C). The bilateral axons were more visible in neurons 1 h after feeding. In addition, the number and intensity of neurohemal sites on the abdominal nerves decreased at 3–5 h post-feeding (Fig. 4A–C). FGLa/AST-like immunoreactive open-type endocrine cells are present in the anterior and posterior midguts (Fig. 5A and C) whereas the hindgut only contains immunoreactive nerve processes (Fig. 5E). The FGLa/AST-like immunoreactive endocrine cells remained brightly stained in anterior and posterior midgut from 1 h post-fed (not shown) to 3–5 h post-fed (Fig. 5B and D). Similarly, there was no difference in the staining-intensity of immunoreactive processes on the hindgut from unfed and fed insects at the times tested (Fig. 5F). Similar results were obtained from blood fed or saline fed insects.

3.4. Anterior midgut absorption assay

The anterior midgut absorption assay is a simple yet sensitive assay used to determine the rate of water transport across the midgut epithelium. The fluid transport rate across the anterior midgut incubated in saline is negligible but 10^{-7} M serotonin increased the absorption rate to 53.9 ± 4.1 nL/min (Fig. 6). This rate was not significantly altered when midguts were incubated in either 10^{-7} M or 10^{-6} M Rhopr-FGLa/AST-2 or 3 (Fig. 6).

3.5. MT secretion assay

We also tested the effects of Rhopr-FGLa/ASTs on serotonin-stimulated MT secretion. Tubules incubated in 10^{-7} M Rhopr-FGLa/AST-2 or 3 along with 10^{-7} M serotonin, showed no significant decrease compared to those incubated in 10^{-7} M serotonin alone (Fig. 7).
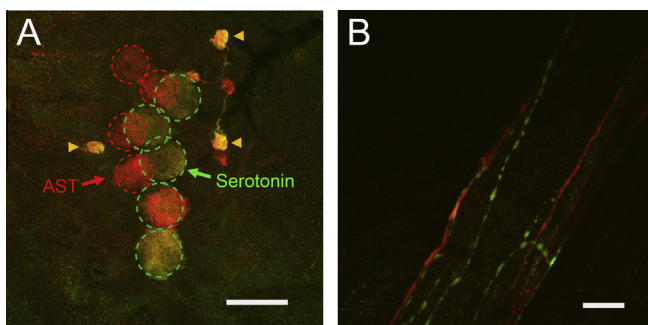

Fig. 1. FGLa/AST-like (red) and serotonin-like (green) double-label immunohistochemistry. (A) Dorsal view of MTGM. The 5 FGLa/AST-like immunoreactive DUM neurons are distinct from the 5 serotonin-like DUM neurons. The DUM neurons lie dorsal/ventral to each other and so overlap in the image. Each neuron has been circled to add clarity. Note the larger diameter of the serotonergic DUM cell bodies. Other neurons which are double-labeled (in yellow) for both serotonin-like and FGLa/AST-like immunoreactivity are indicated with an arrow head. Scale bar: 50 μm. (B) Abdominal nerves: note the absence of FGLa/AST-like and serotonin-like immunoreactivity co-localization in the neurohemal sites on the abdominal nerves. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
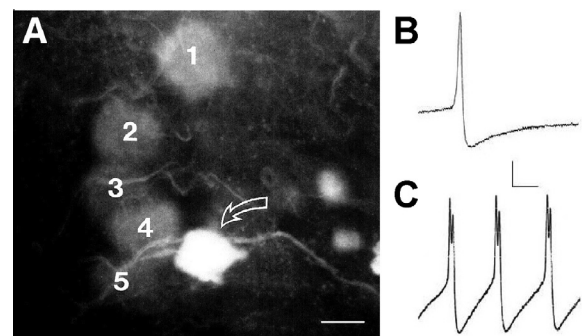

Fig. 2. Immunohistochemistry, Lucifer yellow injection and physiology of a DUM neuron in the abdominal neuromeres of the mesothoracic ganglionic mass of *R. prolixus*. (A) Lucifer yellow injection of a DUM neuron that does not exhibit serotonin-like immunoreactivity (arrow), and therefore is an FGLa/AST-like immunoreactive DUM neuron. Serotonin DUMs 1–5 are labeled 1–5. (B) Intracellular recording from the cell body of this neuron showing spontaneous overshooting action potentials and (C) multiple-component action potentials. Scale bars: A, 50 μm; B and C, 10 mV, 20 ms.
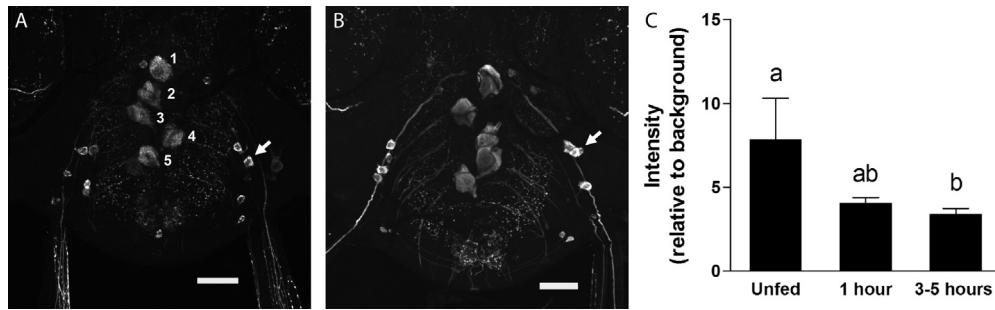
Fig. 3. FGLa/AST-like immunoreactivity in the DUM neurons in the posterior MTGM of unfed (A) and 3–5 h post-fed with saline (B) fifth-instar *R. prolixus*. The 5 DUM neurons are numbered in (A). The arrow in each figure shows cell-specific staining which indicates that the decrease in DUM neuron staining was not due to a procedure artifact. Scale bars: 50 μm. (C) Bar graph showing the average staining-intensity in DUM neurons relative to the background. Bars represent mean ± s.e.m. of 5–6 preparations. Note the decrease in staining-intensity in DUM neurons from 3–5 h post-fed insects ($P < 0.05$, unpaired *t*-test). Different letters denote significantly different.
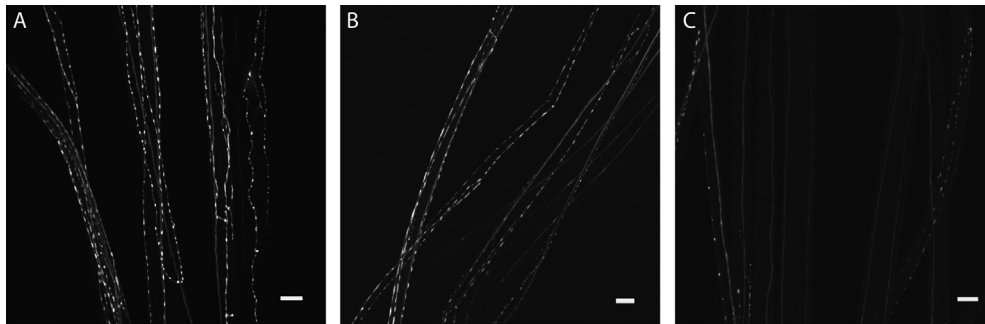

Fig. 4. FGLa/AST-like immunoreactivity in the abdominal nerves of unfed (A), 1 h post-fed with saline (B) and 3–5 h post-fed with saline (C) fifth-instar *R. prolixus*. Note the decrease in the number and intensity of neurohemal sites that stained after 3–5 h post-feeding. Scale bars: 20 μm.

3.6. Hindgut contraction bioassay

Rhopr-kinin-2 stimulated a sustained basal contraction of hindgut with phasic contractions superimposed (Fig. 8). Rhopr-FGLa/AST-2 and 3 at 10^{-6} M both reduced the amplitude of the basal contraction and inhibited the frequency and amplitude of the phasic contractions stimulated using 2.5×10^{-9} M Rhopr-kinin-2 (Fig. 8). Rhopr-FGLa/ASTs also increased the delay between time of peptide application and the first contraction (Fig. 8).

4. Discussion

There are 10 DUM neurons in the abdominal neuromeres of the MTGM of *R. prolixus*; 5 of which express FGLa/AST-like immunoreactivity, and the other 5, larger DUMs, express serotonin-like immunoreactivity. Both sets of DUMs produce neurohaemal sites on the abdominal nerves, indicating potential release of their content into the haemolymph. Interestingly, even though serotonin and FGLa/ASTs are not co-localized in these DUMs, other cells in the MTGM do show co-localization. This would indicate that the two neuroactive chemicals can be released from the same neurons as co-transmitters, but not in the case of the DUMs. The FGLa/AST-containing DUM neurons share electrophysiological properties with all other DUM neurons examined (electrically-excitable cell bodies, overshooting action potentials of long duration, pronounced after-hyperpolarization, multiple spike initiating zones), and so the electrophysiological properties are due to the morphology and membrane properties of DUM neurons and not to their amine/peptide content. The two sets of 5 DUMs are not electrically-coupled and so are not neurally-integrated as a common unit.

Serotonin is a regulator of feeding-related physiological events in *R. prolixus* (see Orchard, 2006) and is responsible, along with peptidergic diuretic hormones, for diuresis (see Orchard, 2006, 2009). These 5 serotonergic DUM neurons release serotonin into the haemolymph at the time of feeding. Serotonin then stimulates absorption of salts and water across the anterior midgut into the haemolymph, secretion of salts and water by the upper MTs, and reabsorption of KCl by the lower MTs. Serotonin controls other feeding-related tissues, and stimulates contractions of salivary glands, anterior midgut, hindgut and heart (see Orchard, 2006, 2009). The location of the 5 FGLa/AST DUM neurons alongside the 5 serotonergic DUM neurons in the MTGM presented the interesting possibility of neural integration of the two groups and therefore the possible involvement of Rhopr-FGLa/ASTs in feeding-related activities. Initially we examined the possible release of FGLa/ASTs from the DUM neuron cell bodies and their neurohaemal sites using immunohistochemistry. Immunohistochemistry can give an indication of release from neurohaemal sites, but also indications of synthesis and restocking. Feeding on either rabbit blood or saline (containing ATP) resulted in a reduction in staining of neurohaemal sites at 3–5 h post-feeding (towards the end of the time of diuresis), indicative of the release of FGLa/AST into the haemolymph in response to feeding. Interestingly the cell bodies were less intensely stained with axons becoming more visible, indicative of transport of FGLa/AST towards the release sites. The similar results with rabbit blood and saline containing ATP indicate that the stimulus for these post-feeding events does not lie in the nutrient component of the meal.

Previously, we have examined the serotonergic DUM neurons and shown that their neurohaemal sites are reduced in intensity 15 min after the start of a blood meal – a timing that matches the peak of serotonin titer in the haemolymph (Lange et al., 1989). Serotonin initiates anterior midgut absorption and MT secretion, which is maintained at a high rate by the presence of a peptidergic DH, corticotropin releasing-factor-related diuretic
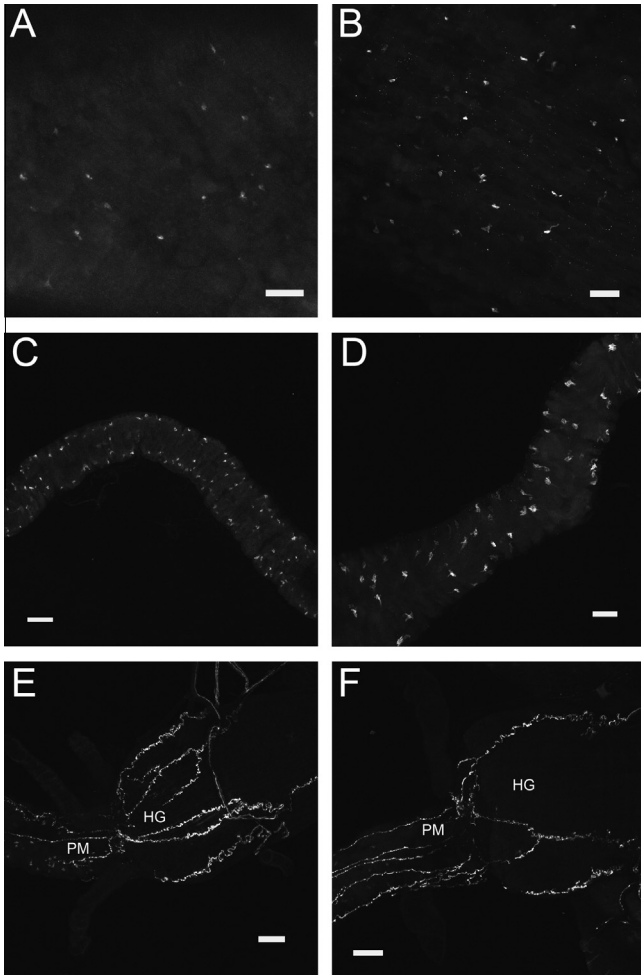
Fig. 5. FGLa/AST-like immunoreactivity in the gut of unfed (A, C and E) and 3–5 h post-fed with saline (B, D and F) fifth-instar *R. prolixus*; anterior midgut (A and B), posterior midgut (C and D) and hindgut (E and F). PM = posterior midgut, HG = hindgut. Scale bars: (A, B and D): 50 μm and (C, E and F): 100 μm.

Fig. 7. Effects of Rhopr-FGLa/ASTs on serotonin-stimulated Malpighian tubule (MT) secretion in fifth-instar *R. prolixus*. Secretion rates of MTs incubated in 10^{-7} M serotonin or serotonin plus Rhopr-FGLa/ASTs (10^{-7} M). Bars represent mean ± s.e.m. of 7 or more.

hormone (Rhopr-CRF/DH), acting with serotonin to continue post-prandial diuresis for about 3 h. The timing of apparent release of the FGLa/ASTs from abdominal nerve neurohaemal sites at 3–5 h implies a role for FGLa/AST distinct from diuresis itself. In addition, FGLa/AST-like immunoreactive endocrine cells remained brightly stained in anterior and posterior midgut at 3–5 h post-feeding, suggesting that release from these cells must occur later in the feeding cycle. Digestion takes place in the posterior midgut in *R. prolixus* some days after feeding, and so possibly FGLa/ASTs from these cells might be released at this later stage. Similarly, there was no difference in the staining-intensity of immunoreactive processes on the hindgut from unfed and fed insects at the times tested.

In light of the apparent release of FGLa/ASTs from abdominal nerve neurohaemal sites associated with feeding, we examined for physiological effects of Rhopr-FGLa/ASTs on feeding-related tissues. Initially we questioned whether Rhopr-FGLa/ASTs might be antidiuretic hormones, since release appears to occur at a time similar to the antidiuretic hormone, Rhopr-CAPA-2, which is released into the haemolymph at a time which signals the end of diuresis in *R. prolixus* (Paluzzi and Orchard, 2006); however Rhopr-FGLa/ASTs did not mimic Rhopr-CAPA-2 by inhibiting sero-tonin-stimulated absorption from anterior midgut, or serotonin-stimulated secretion from MTs. On the other hand, Rhopr-FGLa/ASTs were potent inhibitors of hindgut contractions induced by Rhopr-kinin-2. Hindgut contractions result in expulsion of urine during diuresis, and we have previously suggested that Rhopr-ki-nins are involved in the control of these contractions. Kinins are re-leased from abdominal nerve neurohaemal sites following feeding and are also in the nerve supply to the hindgut (see Orchard, 2009). We did not observe changes in the staining intensity of the FGLa/AST-like immunoreactive process on the hindgut following feed-ing. This observation might imply that any inhibition of hindgut contractions 3–5 h after feeding may be a result of FGLa/ASTs act-ing as neurohormones, released from the 5 DUM neurons. The FGLa/AST-like immunoreactive processes on the hindgut might therefore result in inhibition at other stages in the physiology of the insect. Anterior midgut contractions and heart-beat frequency are also inhibited by Rhopr-FGLa/ASTs (Zandawala et al., 2012). Thus, although Rhopr-FGLa/ASTs appear to play no direct role in
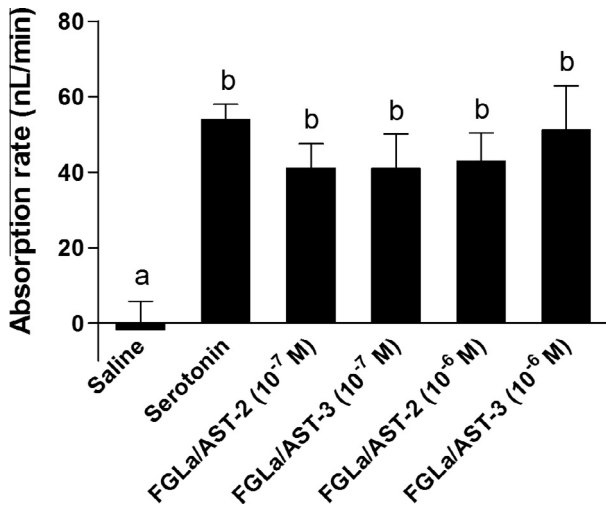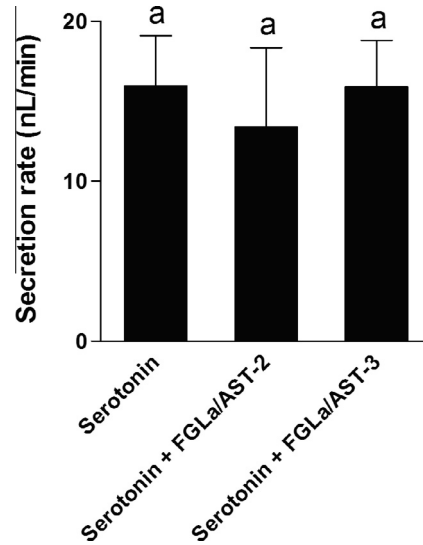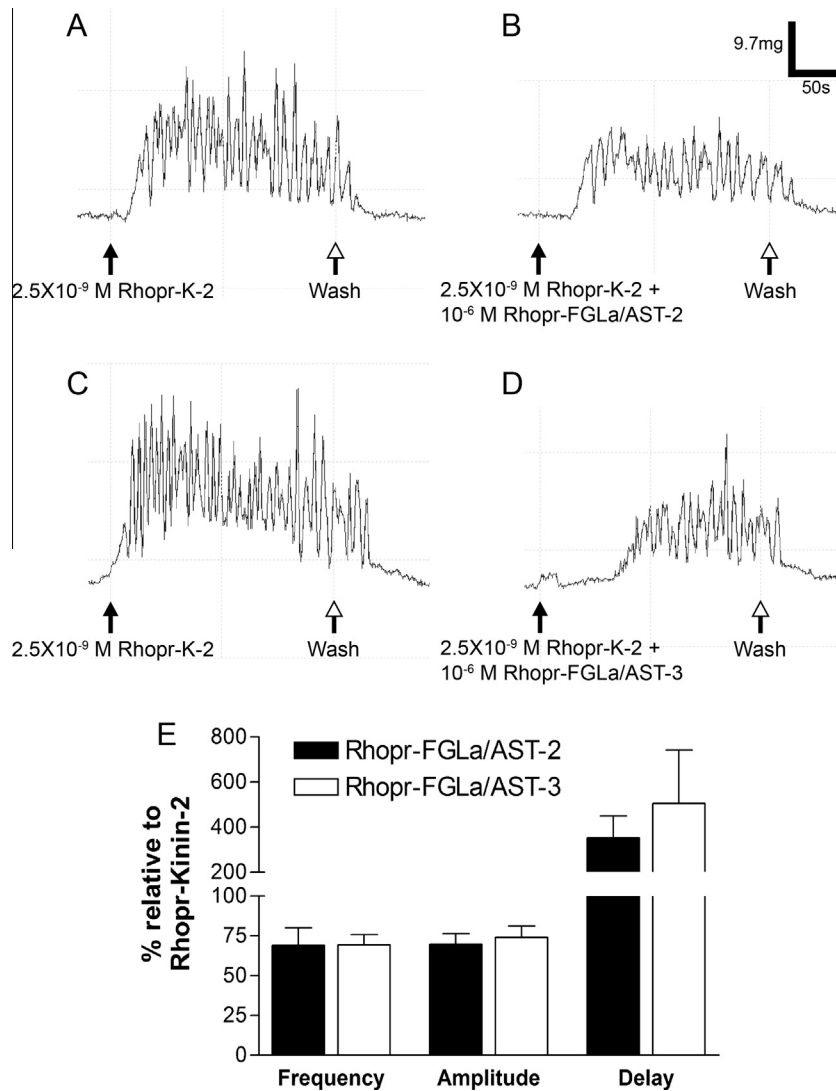

Fig. 6. Effects of Rhopr-FGLa/ASTs on serotonin-stimulated anterior midgut absorption in fifth-instar *R. prolixus*. Anterior midguts were incubated with saline, 10^{-7} M serotonin, or serotonin plus Rhopr-FGLa/ASTs (10^{-6} M and 10^{-7} M). Bars represent mean ± s.e.m. of 6–10 preparations. Different letters denote significantly different.

Fig. 8. Effects of Rhopr-FGLa/ASTs on Rhopr-kinin-2 (RK2)-induced contractions of *R. prolixus* hindgut. (A–D) Sample traces. Control contractions were induced by 2.5×10^{-9} M RK2 (A and C) prior to the application of 10^{-6} M Rhopr-FGLa/AST-2 (B) or Rhopr-FGLa/AST-3 (D). Each preparation was thoroughly washed (open arrow) between the control and experimental peptide application. Peptides were applied at the filled arrow. Note the inhibitory effects of Rhopr-FGLa/ASTs on the frequency and amplitude of RK2-induced contractions. (E) Bar graph showing the effects of Rhopr-FGLa/AST-2 (black bars) and Rhopr-FGLa/AST-3 (grey bars) on Rhopr-kinin-2-induced contractions of *R. prolixus* hindgut. The following aspects were analyzed: frequency, amplitude and delay – the time between peptide application and the first contraction. Bars represent mean ± s.e.m. of 5 preparations. Scale bars represent tension and time.

diuresis, they can play an indirect role by inhibiting contractions of muscles that are actively involved in feeding-related events; dorsal vessel contractions are vital for circulation of the haemolymph and the diuretic hormones released following feeding, the anterior midgut contracts to remove unstirred layers, mix the blood meal, and improve circulation of haemolymph, and hindgut contraction is used to expel the urine (see Orchard, 2006, 2009). Serotonin (released from 5 DUM neurons in the MTGM) is stimulatory on each of the above tissues, whereas Rhopr-FGLa/ASTs (released from a different set of 5 DUM neurons of the MTGM) are inhibitory. Rhopr-CAPA-2 is a potent inhibitor of serotonin-stimulated anterior midgut absorption and MT secretion (Ianowski et al., 2010; Paluzzi et al., 2008); however, unlike Rhopr-FGLa/ASTs, Rhopr-CAPA-2 does not inhibit contraction of muscles associated with feeding-related events. Hence, Rhopr-FGLa/ASTs and Rhopr-CAPA-2 seem to complement each other during the cessation of diuresis in *R. prolixus*, when most of the excess water and salts from the blood meal have been excreted, the release of diuretic hormones into the haemolymph has stopped, and urine production

needs to be terminated in order to conserve water and essential ions.

The act of blood feeding in *R. prolixus* initiates short-term physiological changes associated with digestion, and salt and water balance, but is also the stimulus for growth and development (see Orchard, 2009). Rhopr-FGLa/ASTs are biologically active inhibitors of tissues associated with feeding. These include anterior midgut, hindgut, and dorsal vessel. It is likely that there are other physiological states that are associated with inhibition of gut activity, including ecdysis or stress associated with starvation. Rhopr-FGLa/ASTs might be important messengers at these times, acting to fine-tune the contractile activity of visceral and cardiac muscle.

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