Cloning of the cDNA, localization, and physiological effects of FGLamide-related allatostatins in the blood-gorging bug, *Rhodnius prolixus*

Meet Zandawala*, Yuliya Lytvyn, Daria Taiakina, Ian Orchard

Department of Biology, University of Toronto Mississauga, Mississauga, ON, Canada L5L 1C6

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ABSTRACT

Allatostatins (ASTs) are insect neuropeptides that were first identified as inhibitors of juvenile hormone biosynthesis by the corpora allata. There are three families of ASTs in insects, defined by their C-terminus conserved regions, one of which is FGLamide. Here we determine, for the first time in a hemipteran, the complete 1013 bp cDNA sequence encoding the *Rhodnius prolixus* FGLa/ASTs (Rhopr-FGLa/ASTs), and confirm the transcript size using northern blot. Phylogenetic analysis suggests that the Rhopr-FGLa/AST prepropeptide is most similar to the FGLa/AST precursors identified in Hymenoptera. Reverse-transcriptase PCR demonstrates that the *Rhopr-FGLa/AST* transcript is highly expressed in the central nervous system (CNS) in unfed fifth-instar *R. prolixus*, and is reduced in expression in CNS dissected from one day old blood-fed insects. Fluorescent *in situ* hybridization shows transcript expression in neurons in each ganglion of the CNS, but also in cells located on peripheral nerves. Rhopr-FGLa/ASTs dose-dependently inhibit contractions of the anterior midgut and hindgut, suggesting a role in feeding-related physiological events.

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1. Introduction

Allatostatins (ASTs) were first identified by their ability to inhibit the biosynthesis of juvenile hormone (JH) by the corpora allata (CA) in insects (see Bendena et al., 1997, 1999; Tobe and Bendena, 2006). There are three types of ASTs that differ in their C-terminus conserved regions: the cockroach type (FGLa/ASTs), cricket type (MIP/ASTs), and moth type (PISCF/ASTs). The FGLa/ASTs were the first to be identified when they were isolated from *Diploptera punctata* (Pratt et al., 1991; Woodhead et al., 1989). This neuropeptide family inhibits JH biosynthesis in only cockroaches, crickets, termites and locusts (see Elliott et al., 2010). FGLa/ASTs are now known to be present in numerous other insect orders (although currently not known in Coleoptera), where they possess other physiological roles, especially as inhibitors of visceral muscle contraction (see Tobe and Bendena, 2006). Indeed, it is now considered that their role as inhibitors of JH biosynthesis is a secondarily evolved function, and that inhibition of visceral muscle contraction is likely the ancestral function (see Tobe and Bendena, 2006). 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 (Fusé et al., 1999) and spontaneous and proctolin-induced hindgut muscle contractions of *D. punctata* (Lange et al., 1993, 1995). FGLa/AST-like immunoreactivity is found in neurons within the central nervous system (CNS), but is also associated with the foregut, midgut, and hindgut, as well as open-type midgut endocrine cells in a variety of insects (see Robertson and Lange, 2010), which suggest that ASTs play an important role in feeding-related physiological events.

FGLa/AST encoding genes (*FGLa/ASTs*) have also been identified in a variety of insect orders, including Orthoptera (Vanden Broeck et al., 1996; Belles et al., 1999; Vos et al., 2001), Diptera (East et al., 1996; Veenstra et al., 1997; Lenz et al., 2000), Lepidoptera (Duve et al., 1997; Davey et al., 1999; Secher et al., 2001; Latief et al., 2004), and Blattaria (Donly et al., 1993; Ding et al., 1995; Belles et al., 1999). The FGLa/AST peptides are all derived from a single polypeptide precursor, a prepropeptide, except for the blowfly, *Calliphora vomitoria*, which has two *FGLa/ASTs* (Bendena et al., 1997; Davey et al., 1999; see Tobe and Bendena, 2006).

Recently, a putative FGLa/AST (Rhopr-FGLa/AST) prepropeptide, as well as some Rhopr-FGLa/AST peptides, were identified in the

* Corresponding author. Department of Biology, University of Toronto Mississauga, Room 3016A/B, William Davis Building, 3359 Mississauga Road N., Mississauga, ON, Canada L5L 1C6. Tel.: +1 905 828 5333; fax: +1 905 828 3792.
E-mail addresses: meet.zandawala@utoronto.ca, meetzandawala@hotmail.com (M. Zandawala), julia.lytvyn@utoronto.ca (Y. Lytvyn), daria.taiakina@utoronto.ca (D. Taiakina), ian.orchard@utoronto.ca (I. Orchard).

medically-important bug, *Rhodnius prolixus* (Ons et al., 2009, 2011). These are the first to be reported for any hemipteran, but are particularly important in this case because of the involvement of FGL/ASTs in insect feeding activities coupled to the blood-gorging feeding habits of *R. prolixus*, and the fact that the transmission of Chagas' disease follows feeding on the human host.

In the present study we have extended the work of Ons et al. (2009, 2011) and used a CNS cDNA library of *R. prolixus* to clone the complete *Rhopr-FGLa/AST* cDNA sequence; the first time in a hemipteran. Reverse-transcriptase PCR (RT-PCR) was performed to determine the spatial and temporal expression pattern of *Rhopr-FGLa/AST* in fifth-instar *R. prolixus*, and fluorescent *in situ* hybridization (FISH) was also performed to localize cell-specific expression of *Rhopr-FGLa/AST* within the CNS. Two of the Rhopr-FGL/ASTs were found to be potent inhibitors of both anterior midgut and hindgut contractions in *R. prolixus*, confirming the involvement of this family of peptides in feeding-related activities.

2. Methods

2.1. Animals

Male and female fifth-instar *R. prolixus* were obtained from a long standing colony at the University of Toronto Mississauga. Insects were reared at high relative humidity at 25 °C in incubators and routinely fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada). All tissues were dissected from 4 to 6 weeks post-fed fifth-instar insects, in nuclease-free phosphate-buffered saline (PBS) (Sigma–Aldrich, Oakville, ON, Canada), or in saline for the physiological assays. For temporal expression analysis, CNSs were dissected from the following fifth-instar insects: unfed fifth-instars 3 weeks prior to and immediately prior to feeding the remaining insects; 1 day post-feeding, 2 days post-feeding and 10 days post-feeding. Tissues were stored at −20 °C in RNA later™ RNA stabilization reagent (Qiagen Inc., Mississauga, ON, Canada) prior to use.

2.2. Screening of fifth-instar CNS cDNA library

Rhopr-FGLa/AST peptide sequences (Ons et al., 2009) were used to search the *R. prolixus* preliminary genome assembly using BLAST (Altschul et al., 1990). Gene-specific primers, RhoprAST-FOR1 and RhoprAST-REV1 (Supplementary Table 1), were designed and used to amplify the *Rhopr-FGLa/AST* open reading frame (ORF). A fifth-instar *R. prolixus* CNS cDNA library (Paluzzi et al., 2008) was used as the template. All PCR reactions were performed using s1000 thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada) with the following temperature-cycling profile: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by 10 min final extension at 72 °C. The PCR product was gel extracted using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, ON, Canada). The gel extracted product was cloned using the pGEM-T Vector System (Promega, Madison, WI, USA). Plasmid DNA, isolated from the overnight culture using EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic Inc., Markham, ON, Ontario), was sequenced at the Centre of Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, Ontario, Canada).

Next, a modified 3′ and 5′ rapid amplification of cDNA ends (RACE) PCR approach using fifth-instar *R. prolixus* CNS cDNA library was employed to obtain the complete *Rhopr-FGLa/AST* cDNA sequence. Series of forward and reverse gene-specific primers for 3′ and 5′ RACE, respectively, were designed using the ORF and used in combination with plasmid-specific primers (Supplementary Tables 2 and 3). For 3′ RACE, nested PCR was performed using

one plasmid-specific reverse primer (pDNR-LIB3 -88REV) and two gene-specific forward primers (AST5 FOR1, AST5 FOR2). One plasmid-specific forward primer (pDNR-LIB FOR1) and three gene-specific reverse primers (AST3 REV1, AST3 REV2, and AST3 REV3) were used for 5′ RACE. The primers were used in succession to amplify a specific product using a nested PCR approach. PCR product of each reaction was column purified using EZ-10 Spin Column PCR Product Purification Kit (Bio Basic Inc., Markham, ON, Canada) and used for the subsequent PCR reaction. The products from the final RACE reactions were gel extracted, cloned, and sequenced as described earlier.

Lastly, a forward primer, RhoprAST-FOR5 and a reverse primer, RhoprAST-REV5 (Supplementary Table 4) were used to amplify the largest possible *Rhopr-FGLa/AST* cDNA fragment and thus confirm the full cDNA sequence obtained through RACE. The *R. prolixus* CNS cDNA library was used as a template in a PCR reaction with 55 °C annealing temperature. The resulting PCR product was cloned and sequenced as described earlier. Sequencing results were confirmed from several independent clones to ensure base accuracy.

2.3. cDNA sequence analysis

The deduced Rhopr-FGLa/AST prepropeptide sequence was examined for a potential signal peptide for processing in a secretory pathway using SignalP 3.0 (Bendtsen et al., 2004). The intron–exon boundaries were predicted using a BLAST search of the *R. prolixus* genome assembly and confirmed using Genie, online software for splice site prediction (Reese et al., 1997). Dibasic arginine and lysine cleavage sites were predicted using ProP 1.0 online software (Duckert et al., 2004). ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/ − last accessed on June 26, 2011) was used to align the Rhopr-FGLa/AST prepropeptide (JN559385), with its homologs from: *Drosophila melanogaster* (AAF97792.1), *Glossina morsitans morsitans* (ADD20145.1), *Aedes aegypti* (AAB08870.1), *Anopheles gambiae* (XP_313511.3), *D. punctata* (AAA18260.1), *Blaberus craniifer* (AAC72893.1), *Blattella germanica* (AAC72892.1), *Supella longipalpa* (AAC72894.1), *Periplaneta americana* (CAA62500.1), *Blatta orientalis* (AAC72895.1), *Reticulitermes flavipes* (ACN42938.1), *Helicoverpa armigera* (AAB94674.1), *Spodoptera frugiperda* (Q5ZQK7), *Bombyx mori* (NP_001037036.1), *Schistocerca gregaria* (CAA91232.1), *Apis mellifera* (ADE45320.1), *Gryllus bimaculatus* (CAC83078.1), *Harpegnathos saltator* (EFN80332.1), *Camponotus floridanus* (EFN68211.1), and *Acromyrmex echinatior* (EGI57352.1). An alignment figure was obtained using the BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html − last accessed on June 26, 2011). Multiple sequence alignment was performed and exported to produce a phylogenetic tree using MEGA5 (Tamura et al., 2011). Neighbor-joining (NJ) analysis was performed to obtain an unrooted phylogram. Bootstrap values were obtained based on 1000 replicates.

2.4. Northern blot hybridization

Northern blot hybridization was performed with total RNA isolated from the CNS using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON, Canada). 1 μg of CNS RNA was used to perform northern hybridization using the protocol described earlier (Zandawala et al., 2011), with the following modifications. To prepare the RNA probe, a 679 bp partial *Rhopr-FGLa/AST* cDNA fragment was amplified using RhoprAST-FOR1 and RhoprAST-REV1 primers (Supplementary Table 1) and cloned into pGEM-T Vector System. Plasmid DNA was then isolated, linearized using *NcoI* and purified using EZ-10 Spin Column PCR Product Purification Kit (Bio Basic Inc., Markham, ON, Canada). DIG-labeled anti-sense RNA probe was synthesized from this linearized recombinant plasmid

DNA via *in vitro* transcription using SP6 RNA polymerase supplied with the DIG RNA labeling kit SP6/T7 (Roche Applied Science, Mannheim, Germany). Hybridization was performed using hybridization solution containing denatured DIG-labeled RNA probe (~1 μg/mL). The blots were exposed at room temperature for various times ranging from 1 min to 1 h using Bioflex Scientific Imaging Films (Clonex Corporation, Markham, ON, Canada).

2.5. RT-PCR tissue profiling

The following tissues were used for spatial expression analysis: (1) dorsal vessel, fat body, abdominal nerves, diaphragms and trachea, (2) salivary glands, (3) immature ovaries, (4) immature testes, (5) foregut, (6) anterior midgut, (7) posterior midgut, (8) Malpighian tubules (MT), (9) hindgut, and (10) CNS. Moreover, different parts of the CNS were also dissected separately: (1) brain and suboesophageal ganglion (SOG), (2) prothoracic ganglion (PRO), and (3) mesothoracic ganglionic mass (MTGM). For temporal expression analysis, CNSs were dissected at different times pre- and post-feeding, as described earlier (see Section 2.1). Manufacturer supplied protocol was followed to isolate total RNA using RNeasy Mini Kit (Qiagen Inc., Mississauga, ON, Canada) and at least 20 ng of total RNA was used as a template for cDNA synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). An aliquot of this single-stranded cDNA was used as a template in a subsequent PCR reaction. A 679 bp segment of *Rhopr-FGLa/AST* was amplified using the forward primer, RhoprAST-FOR1 and the reverse primer, RhoprAST-REV1 (Supplementary Table 1). A 318 bp fragment of *Rhopr-β-actin* (housekeeping gene) was also amplified using the forward primer, ACTIN FOR1 and the reverse primer, ACTIN REV1 (Supplementary Table 5). The temperature-cycling profile used was similar to that described earlier, but amplification was performed for 30 cycles instead of 35 (see Section 2.2).

2.6. Expression localization using fluorescent in situ hybridization (FISH)

Cell-specific expression in CNS of unfed fifth-instar *R. prolixus* was assessed using FISH protocols described earlier (Paluzzi et al., 2008; Paluzzi and Orchard, 2010; Zandawala et al., 2011), with the following adaptations. DIG-labeled anti-sense RNA probe was synthesized as described earlier for northern hybridization; however, a different set of primers (RhoprAST-FOR3 and RhoprAST-REV2) were used to amplify a 155 bp *Rhopr-FGLa/AST* cDNA fragment (Supplementary Table 6). Similarly, DIG-labeled sense RNA probe was synthesized for a negative control using the same recombinant plasmid that was linearized with *Nde*I instead of *Nco*I. CNSs were dissected in PBS and fixed in a freshly prepared 4% paraformaldehyde (in PBS) solution for no longer than 2 h at room temperature; female and male tissues were kept separate throughout the entire protocol. Following the quenching of the endogenous peroxidase activity, tissues were incubated in 4% Triton X-100 (Sigma–Aldrich, Oakville, Ontario, Canada) in PBS for 1.5 h at room temperature. Prehybridization was performed for 2 h at 56 °C and hybridization was performed in hybridization solution containing probe at a final concentration of approximately 1.75 ng/μL. Biotin-SP-conjugated IgG fraction monoclonal mouse anti-digoxin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used for *in situ* hybridization signal detection. Samples were incubated with Alexa Fluor 568 tyramide solution diluted 1:200 with amplification buffer. ZEN 2009 LE software (Zeiss, Jena, Germany) was used to obtain images which were then analyzed using ImageJ (Collins, 2007).

2.7. Radioimmunoassay (RIA) quantification of FGLa/ASTs

Peptides were extracted from CNS and an RIA was performed as previously described (see Yagi et al., 2005) using an anti-Dippu-AST-7 antiserum. Data was expressed as Dippu-AST-7 equivalents.

2.8. Muscle contraction assays

The anterior midgut, hindgut and dorsal vessel from unfed fifth-instar *R. prolixus* were set up for monitoring muscle contractions as previously described (Te Brugge and Orchard, 2002; Te Brugge et al., 2002, 2009; Sarkar et al., 2003). The amplitude and frequency of hindgut contractions were monitored before and after application of synthetic Rhopr-FGLa/ASTs by way of a force transducer (Aksjeselskapet Mikro-elektronikk, Horten, Norway). The contractions were recorded using BIOPAC MP100 system hardware and AcqKnowledge MP100 Manager software (BIOPAC Systems, Inc, Santa Barbara, CA, USA). The frequency of anterior midgut and dorsal vessel contractions was monitored before and after application of Rhopr-FGLa/ASTs by way of an impedance monitor (UFI, Morro Bay, CA, USA). Rhopr-FGLa/AST-2 (LPVYNFGLamide) and Rhopr-FGLa/AST-3 (AHNEGRLYSFGLamide) were custom synthesized by GenScript (Piscataway, NJ, USA) at >95% purity.

3. Results

3.1. Rhopr-FGLa/AST transcript

The complete cDNA encoding *Rhopr-FGLa/AST* was cloned and sequenced (Fig. 1A). The sequence is at least 1013 bp long, which includes a 612 bp ORF. The size of the RNA transcript was confirmed using northern hybridization, which estimated a transcript size, including the poly(A) tail, of approximately 1300 bp (Fig. 1C). The 5′ and 3′ UTRs are 44 bp and 357 bp long, respectively. Within the 3′ UTR is a poly(A) tail that is at least 29 bp long. There are 5 polyadenylation consensus sequences (AATAAA) in the 3′ UTR of *Rhopr-FGLa/AST* (Fig. 1A). Proteins that cleave the 3′ end of RNA recognize and bind this consensus sequence, following which the poly(A) tail is added (Murthy and Manley, 1995; Zhao et al., 1999). Molecular organization of *Rhopr-FGLa/AST* was determined using a combination of a BLAST search and splice site prediction. *Rhopr-FGLa/AST* comprises 4 exons which are 33 bp, 375 bp, 193 bp and 383 bp long (Fig. 1B). These exons are separated by 3 introns which are ~30670 bp, ~4984 bp, and 1818 bp long. The complete gene is predicted to be approximately 38.5 kb long. The large size of *Rhopr-FGLa/AST* is a result of its large introns.

3.2. Sequence and phylogenetic analysis of Rhopr-FGLa/AST prepropeptide

The *Rhopr-FGLa/AST* ORF encodes a 203 amino acid long prepropeptide. The prepropeptide contains a highly predicted signal peptide, which is most likely cleaved between alanine at position 20 and isoleucine at position 21. The prepropeptide contains 7 predicted Rhopr-FGLa/AST peptides that could result following post-translational cleavage at dibasic and tribasic, lysine and arginine residues (Table 1). The first Rhopr-FGLa/AST peptide (Rhopr-FGLa/AST-1) has an unusual LGL-NH$_2$ C-terminus. Rhopr-FGLa/AST-1 is flanked by the signal peptide at the N-terminus and lysine and arginine dibasic amino acids at the C-terminus. All the subsequent Rhopr-FGLa/AST peptides are flanked by lysine and arginine dibasic amino acids at the N-terminus and glycine followed by lysine and arginine dibasic amino acids at the C-terminus. The glycine residue at the C-terminus is predicted to undergo amidation. In addition to the common lysine and arginine cleavage sites, there are two

A

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                                5' - CTCAGTTAACTTGGTGACTACGCTACCAGAAAGAATCCGCTACA        44

ATG ATG CTG CCA TTC ATT GTC CTG CTA GTG GTA GAT GTT TTT GCC CTT GGT GCA CAA GCC        104
MET Met Leu Pro Phe Ile Val Leu Leu Val Val Asp Val Phe Ala Leu Gly Ala Gln Ala         20

ATT AAC GAC AGA GAA GAT GAT TTC AAC AAA AAA CTT ACC GAA CTG GGA TTA GGA AAA CGT        164
Ile Asn Asp Arg Glu Asp Asp Phe Asn Lys Lys Leu Thr Glu Leu Gly Leu Gly Lys Arg         40

GCC GCA TAT TCA TAC GTT TCT GAG TAC AAA AGG CTG CCA GTT TAT AAT TTT GGT CTT GGC        224
Ala Ala Tyr Ser Tyr Val Ser Glu Tyr Lys Arg Leu Pro Val Tyr Asn Phe Gly Leu Gly         60

AAA AGA GCA CAT AAT GAG GGG CGT CTG TAT TCA TTT GGT CTA GGC AAA CGT GAT TAT GAT        284
Lys Arg Ala His Asn Glu Gly Arg Leu Tyr Ser Phe Gly Leu Gly Lys Arg Asp Tyr Asp         80

TCT GGT GAA GAA ATG GAA TAT TTA GAC GAT GAA TTA GCA ATT AGA GAT GAA CTT GCT AAA        344
Ser Gly Glu Glu Met Glu Tyr Leu Asp Asp Glu Leu Ala Ile Arg Asp Glu Leu Ala Lys        100

CGG GCC GCA AAA ATG TAT TCA TTT GGC CTA GGA AAA CGA CTG CCT TCA ATT AAA TAC CCA        404
Arg Ala Ala Lys Met Tyr Ser Phe Gly Leu Gly Lys Arg Leu Pro Ser Ile Lys Tyr Pro        120

GAA GGT AAA ATG TAC TCT TTT GGT CTG GGA AAA AGG GTC CCA TTT GCA GAC CAA GCA TAC        464
Glu Gly Lys Met Tyr Ser Phe Gly Leu Gly Lys Arg Val Pro Phe Ala Asp Gln Ala Tyr        140

TTC CTG GAC GAT AAT GAT TCA TCA GAA GAA AGC AAG CGA TCC AAT CCA AAT GGC CAT AGA        524
Phe Leu Asp Asp Asn Asp Ser Ser Glu Glu Ser Lys Arg Ser Asn Pro Asn Gly His Arg        160

TTT TCA TTT GGC CTT GGA AAG CGG GAC GAG CAG GAG ATG AAC GAG AAG AGA AAA GGG GAA        584
Phe Ser Phe Gly Leu Gly Lys Arg Asp Glu Gln Glu Met Asn Glu Lys Arg Lys Gly Glu        180

AGA AGT ATG CAA TAC AGC TTT GGA TTA GGA AAA AGA ACT CAG CTA GAT CCA GCC AAT AAC        644
Arg Ser Met Gln Tyr Ser Phe Gly Leu Gly Lys Arg Thr Gln Leu Asp Pro Ala Asn Asn        200

TTA CAC AAT TGA AAGTAAATTCAAATTAAATTCAAAAGAAACCTTCCCTCAACATCCAGCAAAATCAATCCCTTC        719
Leu His Asn ---                                                                         203

CACATTAACTGAATAAGCACAATAAATATAATACAATAAGTTAAGTAATTTTTGATAATTTTAGATTAAAATAAATCAT        798
CTAATTTTTATTTATATATTTTTGTTAAATAATAAGAATATTTTTTAGTTCCATTAGAAAGTTAGTATTTTATTGATTA        877
ATTTAGTATTTTAAATGAAATATGAAAAAATAAATAATGTACGTAATTCTCTAAATTATACAGGATTTATTTGTTCTCA        956
AAATTAAAAATAAATAAATTTTTTGATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA - 3'                      1013
```
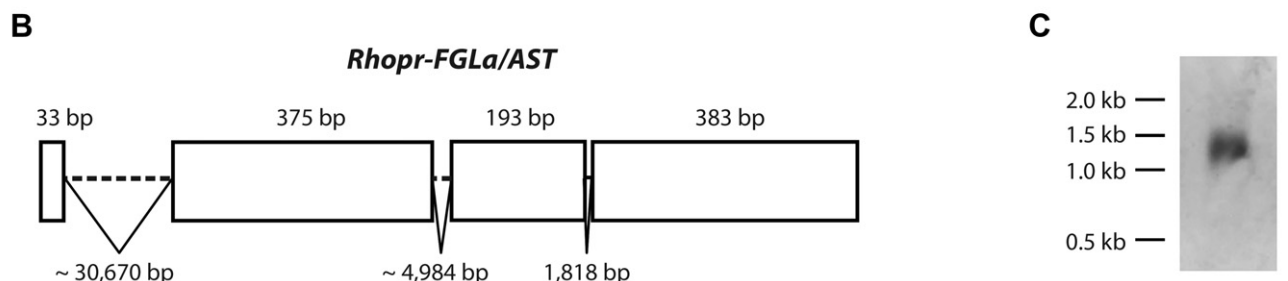
B

Rhopr-FGLa/AST

33 bp   375 bp   193 bp   383 bp

~ 30,670 bp   ~ 4,984 bp   1,818 bp

C

2.0 kb —
1.5 kb —
1.0 kb —

0.5 kb —

Fig. 1. *Rhopr-FGLa/AST* sequence and structure. (A) *Rhopr-FGLa/AST* cDNA and its deduced amino acid sequence. The numbering for each sequence is on the right. Nucleotides highlighted in black denote exon–exon boundaries. Polyadenylation consensus sequences are underlined, while the partial poly(A) tail is dashed underlined. Capitalized methionine codon indicates the translation start site. Amino acid sequences of the 7 predicted Rhopr-FGLa/AST peptides are bolded. The dibasic and tribasic, lysine and arginine residues, denoting post-translation cleavage sites, are shaded in gray. The lysine–lysine cleavage site (underlined) is not predicted to get cleaved while the lysine–arginine–lysine cleavage site (dashed underlined) could get cleaved at either the first two or all three residues. Glycine residues needed for amidation are boxed. The predicted site for signal sequence cleavage is shown with an arrow. (B) *Rhopr-FGLa/AST* structure determined using BLAST and intron prediction. Boxes represent exons and lines or dashed lines (estimated length) represent introns. (C) Northern blot analysis of *Rhopr-FGLa/AST*. Hybridization was performed with an anti-sense probe complimentary to the *Rhopr-FGLa/AST* ORF. The size of RNA molecular weight markers is indicated.

Table 1
Rhopr-FGLa/AST predicted peptides.

No.	Peptide sequence
1	INDREDDFNKKLTELGL-NH$_2$[a]
2	LPVYNFGL-NH$_2$
3	AHNEGRLYSFGL-NH$_2$
4	AAKMYSFGL-NH$_2$
5	LPSIKYPEGKMYSFGL-NH$_2$
6	SNPNGHRFSFGL-NH$_2$
7	KGERSMQYSFGL-NH$_2$[b]

[a] Non-functional KK dibasic cleavage site.
[b] K residue that is partially cleaved.

unconventional cleavage sites present in the prepropeptide; there is a rare lysine–lysine dibasic cleavage site and a lysine–arginine–lysine tribasic cleavage site (Fig. 1A). The lysine–lysine site is predicted to be non-functional in this case as it lacks a cysteine residue at −3 position (Veenstra, 2000). As a result, this dibasic residue pair is part of Rhopr-FGLa/AST-1 (Table 1). The lysine–arginine–lysine tribasic cleavage site is also unique in the sense that it is predicted to undergo cleavage at either the lysine–arginine pair or at all 3 residues. Depending on the position of the cleavage, the length of Rhopr-FGLa/AST-7 would be affected (Table 1); the partial cleavage could result in some peptides with lysine at the N-terminus and some without. Peaks corresponding to the predicted masses for both these peptides are obtained with mass spectrometry analysis, suggesting partial cleavage at this site, although there was not sufficient material for sequencing to confirm this. The 7 predicted Rhopr-FGLa/AST peptides consist of 8 (Rhopr-FGLa/AST-2) to 17 (Rhopr-FGLa/AST-1) amino acids.

Predicted or cloned FGLa/AST prepropeptide sequences from other insect species were aligned to study conservation of the FGLa/AST prepropeptide. Identical and similar amino acids in the aligned sequences were highlighted with a 50% conservation cut-off (Fig. 2). The alignment shows that FGLa/AST prepropeptide is not well conserved across insects. The FGLa/AST prepropeptide sequences in insects are well conserved only at the C-terminus of each encoded FGLa/AST peptide. However, Rhopr-FGLa/AST-2 (peptide #2 on the alignment) is most highly conserved across all the species observed. Interestingly, the prepropeptide region between Rhopr-FGLa/AST-1 and Rhopr-FGLa/AST-2 is well conserved in all species, except the dipterans. There is also a great variation in the length of FGLa/AST prepropeptide sequences across various insects. The sequence lengths vary from 151 amino acids in *D. melanogaster*, to 380 amino acids in *R. flavipes*, with *R. prolixus* sequence (203 amino acids) lying in the middle of the spectrum (Supplementary Table 7). There is also variation in the number of FGLa/AST peptides that could result from the prepropeptide; four FGLa/AST peptides are predicted in several dipteran and hymenopteran species, including *D. melanogaster*, and 14 peptides in several cockroach species including *B. craniifer* and *B. germanica*. *R. prolixus* is in the middle of the range, where 7 Rhopr-FGLa/AST peptides are predicted.

For phylogenetic analysis, 21 FGLa/AST prepropeptide sequences from various insects across several orders were used. Apart from Rhopr-FGLa/AST prepropeptide sequence, no other hemipteran FGLa/AST sequences are fully available. Our analysis indicates that Rhopr-FGLa/AST prepropeptide sequence is sister to a monophyletic group comprising hymenopteran sequences (Fig. 3). Moreover, the clade comprising the *R. prolixus* and

hymenopteran sequences is sister to all other insect sequences. Dipteran sequences form a monophyletic group and so do the lepidopteran and orthopteran sequences. Interestingly, sequences representing the cockroach species (Blattaria) do not form a monophyletic group. The FGLa/AST precursor sequence of *B. germanica* (Blattaria) is most similar to the *R. flavipes* (Isoptera) sequence.

3.3. Rhopr-FGLa/AST expression profiling

Rhopr-FGLa/AST expression profiling was performed using RT-PCR. *Rhopr-FGLa/AST* is highly expressed in the CNS and expressed at a lower level in a pool of tissues comprising dorsal vessel, fat bodies, trachea, diaphragms and abdominal nerves (Fig. 4A). Within the CNS, *Rhopr-FGLa/AST* is expressed at similar levels in brain and SOG, PRO and MTGM (Fig. 4B). Weak expression was also associated with peripheral tissues such as the ovaries, anterior midgut, posterior midgut, Malpighian tubules (MTs) and hindgut. Lastly, *Rhopr-FGLa/AST* expression in the CNS was determined at different time points pre- and post-feeding. Interestingly, low *Rhopr-FGLa/AST* expression was observed in the CNS one day post-feeding, while similar levels of expression were observed for all the other time points, pre- and post-feeding (Fig. 4C).

3.4. Rhopr-FGLa/AST expression in fifth-instar CNS

FISH was performed to localize *Rhopr-FGLa/AST* expression within the CNS (Fig. 5A–C). The diagrammatic representation of *Rhopr-FGLa/AST* expression shows that it is expressed in approximately 105 neurons throughout all ganglia of the CNS, that are organized in a bilaterally symmetrical manner (Fig. 6), except for 5 dorsal unpaired median (DUM) neurons in the MTGM (Figs. 5C and 6). None of these neurons stain in control CNSs hybridized with DIG-labeled sense RNA probe (not shown). Moreover, no obvious staining differences were observed between males and females. Approximately 58 cells in the brain show *Rhopr-FGLa/AST* expression. The SOG contains 16 *Rhopr-FGLa/AST*-expressing cells, including a pair along the midline on the ventral side which are strongly stained. The PRO contains 8 bilaterally-paired cells, of which 2 are located on the dorsal side and 6 on the ventral side. The MTGM contains *Rhopr-FGLa/AST* expression in 23 cells, of which 16 bilaterally-paired cells are located ventrally. Strong staining was observed in 5 DUM neurons in the distal region of the posterior MTGM. Peripheral *Rhopr-FGLa/AST*-expressing cells were also observed on abdominal nerves (Fig. 5D).

3.5. RIA quantification

FGLa/AST content of the CNS was measured by RIA using an antiserum against the cockroach Dippu-AST-7. The whole CNS contains about 800 ± 50 fmol of Dippu-AST-7 equivalents. This amount is distributed amongst the various ganglia, with the brain containing about 500 ± 120 fmol, the SOG 130 ± 8 fmol, the PRO 150 ± 10 fmol and the MTGM 200 ± 10 fmol when assayed separately. Dippu-AST-7 equivalents were also measured in the abdominal nerves, with about 50 fmol present.

Fig. 2. Multiple sequence alignment of the Rhopr-FGLa/AST prepropeptide from different insects. ClustalW2 was used to align FGLa/AST prepropeptide sequences from the fruit fly, *D. melanogaster* (AAF97792.1), the tsetse fly, *G. morsitans morsitans* (ADD20145.1), the yellow fewer mosquito, *A. aegypti* (AAB08870.1), the African malaria mosquito, *A. gambiae* (XP_313511.3), the Pacific beetle cockroach, *D. punctata* (AAA18260.1), the death's head cockroach, *B. craniifer* (AAC72893.1), the German cockroach, *B. germanica* (AAC72892.1), the brown-banded cockroach, *S. longipalpa* (AAC72894.1), the American cockroach, *P. americana* (CAA62500.1), the oriental cockroach, *B. orientalis* (AAC72895.1), the Eastern subterranean termite, *R. flavipes* (ACN42938.1), the kissing bug, *R. prolixus* (JN559385), the cotton bollworm, *H. armigera* (AAB94674.1), the fall army worm, *S. frugiperda* (Q5ZQK7), the silkworm, *B. mori* (NP_001037036.1), the desert locust, *S. gregaria* (CAA91232.1), the Western honey bee, *A. mellifera* (ADE45320.1), the field cricket, *G. bimaculatus* (CAC83078.1), the Jerdon's jumping ant, *H. saltator* (EFN80332.1), the Florida carpenter ant, *C. floridanus* (EFN68211.1), and the leaf-cutter ant, *A. echinatior* (EGI57352.1). Identical and similar amino acids in the aligned sequences are highlighted with a 50% majority rule. Location of the 7 Rhopr-FGLa/AST peptides has been indicated.

```
                                                     1                                                                2
R.prolixus      --------------MMLPFIVLLVVDVFALGAQAIN------------------------------------------DREDLFNKKLTBLGLGKRAAYSYVSEY---KRLPV   54
D.melanogaster  -----MNSLHAHLILLAVCGVGYLASSPVIGQDQRSGD---------------SDADVLLAAD-----EMADNGG-DNILKRVERYAFGLGRR-AYMYTNGGPGMKRLPV       83
G.morsitans     ----MMTINFTCLLVLLLCWLNMFCCQPNYGDDDTNRNDI----------HDASQLNSLFSINAGTNMEMDDDHDEKNNYLKRVERVAFGLGRR-GYITYTNGN-GMKRLPV      94
A.aegypti       MRPSTTPMVLLSYLAFVLCLACVAYGSSALGSSSGSSDQSLFGGGAGGGGGSASAESDIGDDR--GQREISQATFQHMLAVRSPKYNFGLGLGKR-RYIIEDVP-GAKRLPH    106
A.gambiae       ------MLALSAYVTLSLCLS-VSWSLPACGGTAGSSSSS----------SNDLDMDDLSRDRVSGQGEISTSQFQHMLAVRSPKYNFGLGLGKR-RYIIEDVP-GAKRLPH     91
D.punctata      ---MSGPR-TCFCLPSALVL-VLLSLSTSALGTAEP---------SGVHEESPAG--GGTDLLPHPEDLSASDNPDLEFVKRLYDFGLGKR-AYSYVSEY---KRLPV         89
B.craniifer     ---MPGPR-TYITLPAALLL-VLISTTALGTATEP---------SGVHEESPAG--GGAELLPHPEELSASDNSDLEFVKRLYDFGLGKR-AYSYVSEY---KRLPV          89
B.germanica     ---MPGPR-TWYSLQAAFVLSLLLKLSSSAFATTTSAG---------THAVQEESSAG--GGAEILPRLEEL--ADNSELDLVKRLYDFGLGKR-AYSYVSEY---KRLPV      90
S.longipalpa    ---MYPDSR-TCISLQAVFLLALLLLQLANSAFGTATAP---------AGSPEEASSN--AGSELLSHLEDS---ENPELLFVKRLYDFGLGKR-AYSYVSEY---KRLPV       87
P.americana     ---------MGFLQLILLSIILLHLSTGSLATAPANSG-------HNGAPEETPSGAATGSGLLPHLEESSVNDNSELLFVKRLYDFGLGKR-AYSYVSEY---KRLPV        89
B.orientalis    ----------------------------------------------------VNDLSELLFIKRLYDFGLGKR-AYSYVSEY---KRLPV                          34
R.flavipes      ---MYPGLQPCSRYSQVVFVCVLLLQLSVLALGTVPPNSG-------SHGTPEESSSGSGSGVGLLPHNEETSAADNTELLFVKRLYDFGLGKR-AYSYVSEY---KRLPV     96
H.armigera      ---MLY-------SSLPVCFLVLGAALCAPERMQNEA-------EPHDLQPHE-AEPHSDH-------VAPLAKRSPH------YDFGLGKR-AYSYVSEY---KRLPV         74
S.frugiperda    ---MLY-------PSIPVCFLVIGVALCAPERMQNEP------DPHDTPVHEGTEPHSDH-------IAPLEKRSPH------YDFGLGKR-AYSYVSEY---KRLPV          75
B.mori          ---MLS--------ACLPILLVLGAALAAEQVAEHG--------------AEQVAEH-------AAPLEKRSPQ------YDFGLGKR-AYSYVSEY---KRLPV            63
S.gregaria      -MGMYTSRSSSSEAARLPLPALVLLLCTSPATPQEVPGDAMTGGGPASAPVSTASEAAAASPPGSASTGAAPMDAESEYELYKRLCDFGVGKR-AYTYVSEY---KRLPV      105
A.mellifera     ---MRSRTSVLTSSLAFLYFFGIVGRSALAMEETPAS----------------------------SMNLQHYNNMLNPMVELDTVPEKRAYIYVSEY---KRLPV           71
G.bimaculatus   -------------------------PASDAAAAQEAAGEL-------LERLENEAGSG---------------ATPDDELEFYKRLYDFGVGKR-AYSYVSEY---KRLPV        60
H.saltator      ----------------MLCLISVVGRSTAAMEDMPSS--------------------------SLHMPRLNPLLNIVEYEE-PSEKRSYAYVSEY---KRLPL             57
C.floridanus    ---MKTS-SLIAMRLIIFYLLSVVGRSTAAVEEAPAS--------------------------SLHIPRLNPLSSNLEYDE-PSERAYAYISEY---KRLPL             69
A.echinatior    ---MKTTTSLITMRIIMFYLLSVVGR-AVAIEEAASS--------------------------SLHIQQLHPLLNNMEYEE-PIKK----AYILAEY---KRLPL            66

                       3
R.prolixus      YNFGLGKRA-------------HNEGRLYSFGLGK-------------------RDYD----SGEEMEYLDDE---------------LAIRD--------------      96
D.melanogaster  YNFGLGKRSRP---------------YSFC------------------------------------------------------------LGKRSDYD-YDQDNEIDYR   116
G.morsitans     YNFSLSKR-AP---------------YTFD-------------------------------------------------------------LDKRSGYNRLDDELLAKQE  127
A.aegypti       YNFGLGKRAR---------------NNLLEYD----------------------DDSAPSWS-EDYS---SLIPRLG-LDYDGDKDKSAE                       154
A.gambiae       YNFGLGKRGSPM---------------GGNDYEYDGLM----------------GGNLQWGNDNDYT---NLITKLGQEDYDAEKEKDAA                       147
D.punctata      YNFGLGKRS----KMYGFGLGKR---DGRMYSFGLGK------------------RDYD-YYGEEDEDDQQAIGDEDIEESDVG--DLMDKRDRLYSFGLGKRARPY      168
B.craniifer     YNFGLGKRS----KMYGFGLGKR---DGRMYSFGLGK------------------RDYD-YYGE-DEDDQLANGDEDIEDSEVG--DLIDKRDRLYSFGLGKRARPY      167
B.germanica     YNFGLGKRS----KMYGFGLGKRAGSDGRLYSFGLGK-----------------RDYDDYYGDDDEDHQTSADEDIEDADSV--DLMDKRDRLYSFGLGKRARPY       173
S.longipalpa    YNFGLGKRS----KMYGFGLGKRAGSDSRLYSFGLGK-----------------RDYDDYYEEDEDEDQQSSG-EDIEDSDAV--DLVDKRERLYSFGLGKRARPY      169
P.americana     YNFGLGKRS----KMYGFGLGKRSGNDGRLYSFGLGK-----------------RDYDDYIQEDELEDEDISSGD-DDVDNSEYE--DLMDKRDRMYSFGLGKRARPY     171
B.orientalis    YNFGLGKRS----KMYGFGLGKRSGNDGRLYSFGLGK-----------------RDYDDYIQEDELEDEDISSGD-DDVDNSEYE--DLMDKRDRMYSFGLGKRARPY     116
R.flavipes      YNFGLGKRN----KIYGFGLGKRAGADGRLYSFGLGK-----------------RDYDDYIQEDE--DVIDDE-DDLDAD-V--DLMBKRDRLYSFGLGKRAKPY       175
H.armigera      YNFGLGKRS-------------RPYSFGLGKRS--------------VDEDQSNDEQQLTTSDLLQQAALAELFDQYEDA--------EKRARPYSFGLGKRFADD       144
S.frugiperda    YNFGLGKRS-------------RPYSFGLGKRS--------------VDEDQSSESQPLT-SDLLQQAALAEFFDQYEDA--------YEKRARPYSFGLGKRFADD      146
B.mori          YNFGLGKRS-------------RPYLFGLGKRS--------------AGAEQLDD--DIS-NEALQNTLDELFDQYEDSAAVPTGYVEKRARPYSFGLGKRFA-E       137
S.gregaria      YNFGLGKRATGAASLYSFGLGKR---GPRTYSFGLGKRGDDEPNDYSEQELFADVDGDSEDALPVAVEALERELPEAAEEMPGVFT--ELMDKRGRLYSFGLGKRARPY   210
A.mellifera     YNFGIGKRWID---------TNDNKRGRDYSFGLGKR--------------------------RQYSFGLGKRNDNADYPLRLNLDYLPVDNP-AFHSQ            134
G.bimaculatus   YNFGLGKRAGG--ROYGFGLGKRAG--GRQYGFGLGKRP------------------PGDEDDYYFDEPEEDVPED--NLDDSD-----SVDKRDRLYSFGLGKRARPY    141
H.saltator      YNFGIGKRWVD---------DNEDKRTRPFSFGIGKRL------------------RDYRFGIGKRNS-----HPLNLDYLPADNLEAYHSR                    117
C.floridanus    YNFGIGKRWID---------NSEDKRTRPFSFGIGKRL------------------RDYRFGIGKRNSGY---RPLGMDFS-VDNM-DFHSR                   129
A.echinatior    YTFGIGKRWID---------NNEDKRSRQFSFGIGKRL------------------RNYDFGIGKRNNEY---HPLNLDY-EEDNMGDYQSH                   126

                     4                  5                                        6
R.prolixus      --ELAKRAA-----KMYSFGLGKRLPS-------IKYPEGKMYSFGLGKRVPFADQAY----------FLDDNDSSE------ESKRSNPNGHRFSFGLGKRDEQ--EMN  174
D.melanogaster  VP---------PANYLAAERAVR-----------------PGRQ--------------------------------------------NKRTTRPQPENFGLGRR---------  151
G.morsitans     LD----------KAFNTATMESKR--------------EPSED-------------------------------------------------VSHRASYPMRYGFGLSR--------- 163
A.aegypti       KR----------ASAYRYHFGLGKR-------------RVYDFGLGKRVYE----------------------------------DKRLPNRYNFGLGKR            197
A.gambiae       KRTASGNCA---GSAYRYHFGLGKR-------------RAYDFGLGKRYFDA---------------------------------EDFNKRLPNRYNFGLGKR          201
D.punctata      SFGLGKRAP-SGAQRLYGFGLGKRGGS-LYSFGLGKRGDGRLYAFGLGKRPVNSGRSSGSRENFGLGKRSDDIDFRELEEKFAED-KRYPQEHRFSFGLGKREVEPSELE  275
B.craniifer     SFGLGKRAP-SGTQRLYAFGLGKRAGSSLYSFGLGKRGELRLYGFGLGKRPVNSGRSSGSRFNFGLGKRSEDIDIRDLEEKFAEEE-KRYPQEHRFAFGLGKREVAPSELE 276
B.germanica     SFGLGKRAP-SSAQRLYGFGLGKRA--LYSFGLGKRAGGRLYAFGLGKRPVNSGRQSGSRFNFGLGKRSDDFDIRELEGKFAEED-KRFPQDHRFSFGLGKREVAPSELE  279
S.longipalpa    SFGLGKRAPSSSGVQNRLYGFGLGKRGGS-LYSFGLGKRGGGRLYAFGLGKRPVNSGRQTGSRFNFGLGKRSEDFDL-------EEDKRFPQDHRFSFGLGKRKLHPVSLE 270
P.americana     SFGLGKRSPS-GMQRLYGFGLGKRADGRLYGFGLGKRGGS-MYSFGLGKRPVSSARQTGSRFNFGLGKRSDEIDLKEIEEEIAEEGKRSPQSHRFSFGLGKREVAPSELE  279
B.orientalis    SFGLGKRSPS-GMQRLYGFGLGKRADGRLYAFGLGKRGGS-MYSFGLGKRPVSSARQTGSRFNFGLGKRSDEIDLKEIEEEIAEEGKRSPQSHRFSFGLGKREVAPSELE  224
R.flavipes      SFGLGKRSPSSGNQRLYGFGLGKRGGS-LYSFGLGKRG-GRLYSFGLGKRPVNSGRSGSRFNFGLGKRSDESDWDEYEEDIAEDEKRSPQGHRFAFGLGKREVAPGELE   283
H.armigera      ETSEEKRA------RAYDFGLGKR--------------LPMYNFGLGKRARS-------YNFGLGKRYSKFNFGL-------EKRRERDMHRFSFGLGKRSGDDVSAD      217
S.frugiperda    ETSEEKRA------RAYDFGLGKR--------------LPMYNFGLGKRARS------STLAWASD-------------------------------------------   186
B.mori          EPAEDKRA------RMYSFGLGKR--------------ARSYSFGLGKRLSSK-------BNFGLGKR-----------------------QRDMHRFSFGLGKRSEDDT---  197
S.gregaria      SFGLGKRSPG-PAPSRLYSFGLGKRE------------GRMYSFGLGKRPVYGGDRR--FSFGLGKR----------------------APAEHRFSFGLGKRDARSALSQ  283
A.mellifera     E-NTDDFLEEKRGRQPYSFGLGKR--------------AVHYSGG-QPLGSK-------------------------------------RPNDMLSQRYHFGLGKRMSEDEDES 195
G.bimaculatus   GFGLGKRAG------MYSFGLGKRAQH-QYSFGLGKRGE-GRMYSFGLGKRP-NYERMAGSRENFGLGKRADANPAYLLSD--LGEEKRGPD-HRFAFGLGKREVSPNELE   240
H.saltator      EDNADDLMEEKRSNQPFSFGIGKR--------------GWKLAG--ATARRP--------------------------NADVVAAFRYLLSLGKGIGKDEEVA          178
C.floridanus    EDNLDDFIDDKRGGQPFSFGIGKR--------------GWKLPVCEMAVSGR--------------------------RLNDVVGPKYLLGLGKGLSENENLI          192
A.echinatior    EDNSNDFMENKRSNHQEGFGIGKR--------------VWKLATGETTVSGR--------------------------RLNDAIVPKYWFSLLAKELENENLN          189

                  7
R.prolixus      EKRKG-----------------------ERSMQ------------------------------YSFGLGKRTQLDPANNLHN---                            203
D.melanogaster  ---------------------------------------------------------------------------------                               
G.morsitans     ---------------------------------------------------------------------------------                               
A.aegypti       ---------------------------------------------------------------------------------                               
A.gambiae       ---------------------------------------------------------------------------------                               
D.punctata      AVRNE-------EKDN---SSVHDKKNNTND--MHSGERIKRSLHYPFGIRKL-ESSYDLNSASSLNSEENDDITPEEFSRMVRRPFNFGLGKRIPMYDFGIGKRSER      370
B.craniifer     AVKNE-------ERDS---ASVHDKRNNTND--LHSGERIKRSLHYPFGIRKQ-ESSYDLNSASSLNSEENDDITPEEFSRMIRRPFNFGLGKRIPMYDFGIGKRSER     371
B.germanica     AVKNE-------EKDS---VSNQEKKNNTNDAHIHNGERVKRSLHYPFGFGKQ-DSGFDLHS-SSLSSEENDDIGPEEFARMVRRPFEYARQKQVPMYDFGIGKRSER     375
S.longipalpa    AVRDE-------EKDN---ESESKDVSVQEKKNSTTGERVKRSLS--------ASPYDTSA---SEED----VDEFARLIRRPYNFGLGKRIPMYDFGIGKRSER       350
P.americana     AVRNE-------ERDK-GKHQDETRKNGTSESYHHTGERVKRSLHYAFGLGKRGGSPYDFESSPSFESDEDEEMGTDEFSRLIRRPYNFGLGKRIPMYDFGIGKRSEH     379
B.orientalis    AVRNE-------ERDK-GKHQDETRKNGTSGE-YHHTGERVKRSLHYAFGLGKRGGSPYDFESSPSFESDEDEEMGTDEFSRLIRRPYNFGLGKRIPMYDFGIGKRDFG------  316
R.flavipes      AIRNE-------ERDKETKNNNESRKNGTAESSHHSGERVKRSLHYAFGIGKR---AYDSELS-TPDDSDEDNEAGVEEFARIVRRPYNFGLGKRLPMYDFGIGKRSEY    380
H.armigera      DSDNYFDV-------------------------------------------------------------------------                                225
S.frugiperda    ---------------------------------------------------------------------------------                               
B.mori          -SENYIDA-------------------------------------------------------------------------                               204
S.gregaria      ---------------------------------------------------------------------------------                               
A.mellifera     SQ-------------------------------------------------------------------------------                                197
G.bimaculatus   AVREEQLHHDKEAQQHELAEEAAPAPEREPNDAHANGKHAVKRSLHYGFGIGKRTSDAFGLDVD---PEEDDRDAISEDFTRYIRRPYSFGLGKRVPMYDFGIGKRADR    345
H.saltator      Q--------------------------------------------------------------------------------                                179
C.floridanus    Q--------------------------------------------------------------------------------                                193
A.echinatior    Q--------------------------------------------------------------------------------                                190
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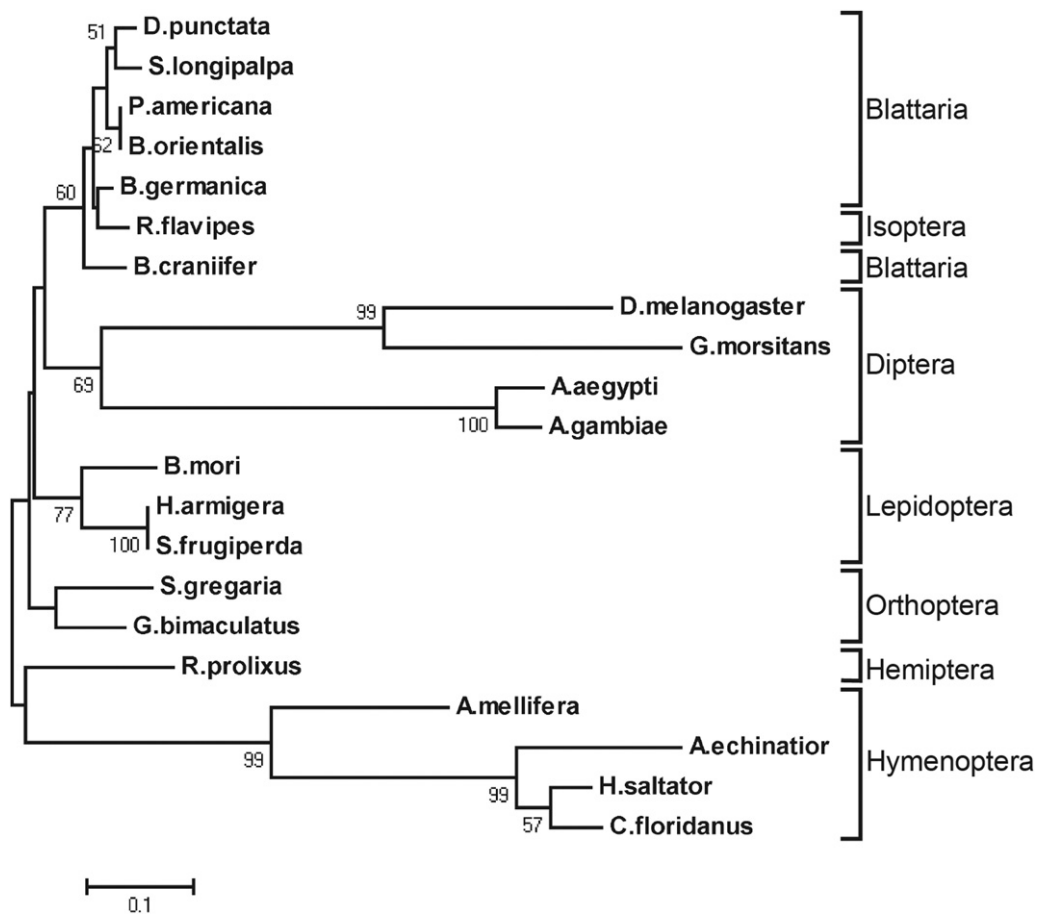
Fig. 3. An unrooted phylogram obtained using neighbor-joining analysis, showing evolutionary relationships of FGLa/AST prepropeptide from 21 species. Boot strap values (above 50) based on 1000 replicates are indicated at the split of the branches. The tree is depicted to scale, with branch lengths proportional to the evolutionary distances used to infer the phylogenetic tree.

## 3.6. Effects of Rhopr-FGLa/ASTs on muscle contraction

Anterior midguts from fifth-instars that still contained blood content from the previous feed as fourth instars consistently produced phasic peristaltic contractions. Both Rhopr-FGLa/AST-2 and 3 resulted in a dose-dependent inhibition in the frequency of these spontaneous contractions (Figs. 7 and 8). The effects were reversible upon washing in physiological saline. As can be seen from the dose–response curves, Rhopr-FGLa/AST-3 is more potent than Rhopr-FGLa/AST-2, with the threshold of the former being between 10^{-13} and 10^{-12} M, and IC_{50} at approximately 10^{-11} M. Total inhibition is achieved at doses of approximately 10^{-9} M.
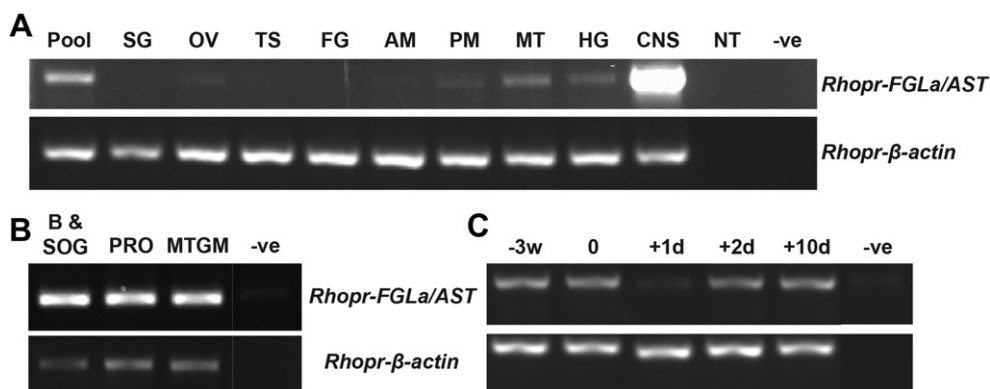
Fig. 4. Expression analyses of *Rhopr-FGLa/AST* in fifth-instar *R. prolixus*. (A) Spatial expression pattern. The following tissues were dissected from 10 insects: Pool (dorsal vessel, fat body, abdominal nerves, diaphragms and trachea), SG (salivary glands), OV (ovaries), TS (testes), FG (foregut), AM (anterior midgut), PM (posterior midgut), MT (Malpighian tubules), HG (hindgut) and CNS (central nervous system). NT represents an additional negative control with no template used for the cDNA synthesis reaction ($n = 4$ replicates). (B) Expression pattern within the CNS. The following tissues were dissected from 10 insects: B and SOG (brain and suboesophageal ganglion), PRO (prothoracic ganglion), and MTGM (mesothoracic ganglionic mass) ($n = 3$). (C) Temporal expression pattern within the CNS. CNS was dissected from insects at −3 w (3 weeks before feeding), 0 (right before feeding), +1 d (1 day post-feeding), +2 d (2 days post-feeding) and +10 d (10 days post-feeding) ($n = 2$). For all experiments, −ve represents a negative control, where no template was used for the PCR reaction, and *Rhopr-β-actin* was amplified as a positive control to test the quality of the cDNA.
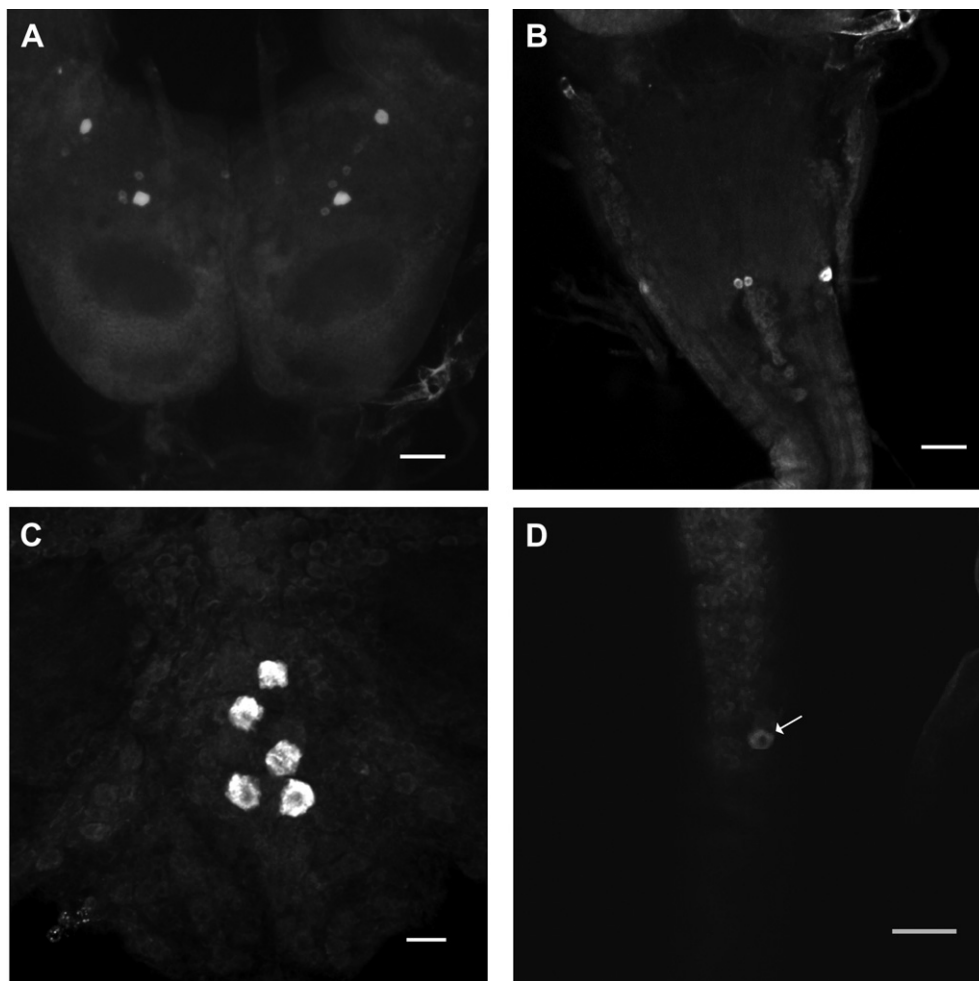
Fig. 5. Fluorescent *in situ* hybridization portraying *Rhopr-FGLa/AST* expression in fifth-instar *R. prolixus* dorsal CNS: (A) brain, (B) suboesophageal ganglion (C) mesothoracic ganglionic mass and (D) abdominal nerve. Arrow indicates the cell-specific staining in the abdominal nerve. Scale bars: A, B: 50 μm and C, D: 25 μm.

Hindguts from *R. prolixus* are not spontaneously myoactive; however, consistent longitudinal muscle contractions can be produced by the addition of insect kinins, such as leucokinin 1 (see Te Brugge and Orchard, 2002). Leucokinin 1 (LK1) produces a change in basal tonus and an increase in frequency and amplitude of superimposed phasic contractions (Fig. 9). Rhopr-FGLa/AST-2 and 3 are both capable of inhibiting 10^{-8} M LK1-induced hindgut contractions in a dose-dependent manner (Figs. 9 and 10). Again, Rhopr-FGLa/AST-3 is more potent with threshold for inhibition of amplitude of phasic contractions between 10^{-11} and 10^{-10} M, and IC$_{50}$ at approximately 10^{-8} M. Maximum inhibition is achieved at doses of approximately 10^{-5} M. Rhopr-FGLa/AST-3 is also more potent than Rhopr-FGLa/AST-2 when measuring the frequency of contractions (Fig. 11). Rhopr-FGLa/AST-2 was also capable of inhibiting the frequency of heart-beat, with threshold between 10^{-10} M and 10^{-9} M and EC$_{50}$ at 1.5×10^{-8} M (not shown).

4. Discussion

We have isolated and cloned the complete *Rhopr-FGLa/AST* cDNA sequence from *R. prolixus* – the first time in a hemipteran. Previously, Ons et al. (2011) submitted the AST A precursor mRNA sequence (GQ856315.1) to GenBank, that is referred to as *Rhopr-FGLa/AST*; however our extended sequence contains the 3′ UTR. Our sequencing results differ slightly from that of Ons et al. (2011) by 1 nucleotide at position 185, which is T rather than C confirmed in

multiple sequenced clones and from the *R. prolixus* genome. The 1013 bp cDNA sequence contains 5 polyadenylation signal consensus sequences (AATAAA). Since only 1 band was observed in the northern blot analysis, it is most likely that only 1 signal sequence is used for cleavage and addition of a poly(A) tail.
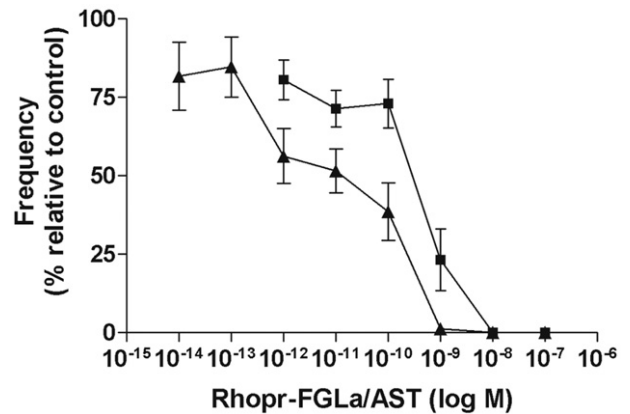
The 203 amino acid long Rhopr-FGLa/AST prepropeptide contains 7 predicted FGLa-like peptides, one of which is an unusual LGLa. Such a peptide has been found in only one other species; that of *C. floridanus*. Five of these 7 peptides have been *de novo* sequenced, namely Rhopr-FGLa/AST-2,3,4,5 and 6 (Ons et al., 2011). Rhopr-FGLa/AST-2 is highly conserved in all species examined. Rhopr-FGLa/AST-7 has an ambiguous cleavage site (KRK) that might result in the presence or absence of an N-terminus K residue.

Expression of *Rhopr-FGLa/AST* was predominantly found in the CNS of both female and male insects. Weak expression was observed in the ovaries, anterior midgut, posterior midgut, hindgut, MTs and the pool of dorsal vessel, fat body, diaphragms, abdominal nerves and trachea. The midgut data is predictable, since previously we have shown FGLa/AST-like immunoreactive endocrine cells in this tissue (Sarkar et al., 2003), as is found in a variety of other insects (see Tobe and Bendena, 2006). The other tissues are more intriguing, although interestingly expression of *Dippu-FGLa/AST* has been shown in the oviducts of *D. punctata*, and has also been shown to change over the reproductive cycle (Garside et al., 2002). Caution must be exercised, however, in attributing expression to the actual tissue. For example, we have found peripheral cells expressing the
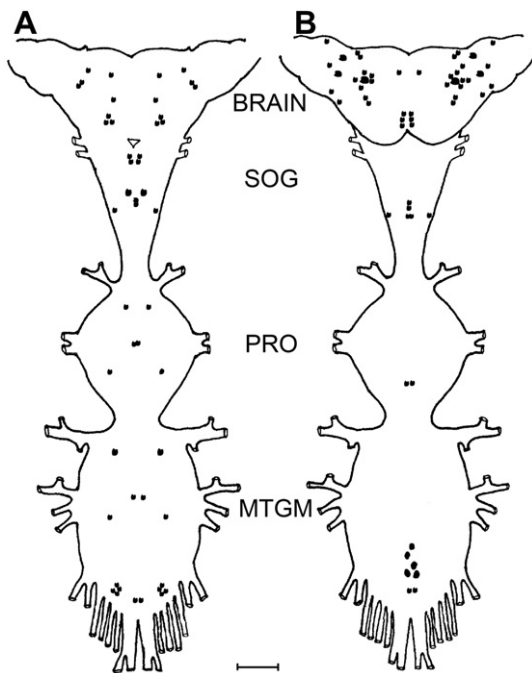
Fig. 6. Diagrammatic representation of *Rhopr-FGLa/AST* expression on (A) the ventral and (B) dorsal aspect of the CNS: brain, suboesophageal ganglion (SOG), prothoracic ganglion (PRO), and mesothoracic ganglionic mass (MTGM). The map is based on at least 20 preparations. Scale bar: 200 μm.

Fig. 8. Dose–response curves for the effects of Rhopr-FGLa/AST-2 (■) and Rhopr-FGLa/AST-3 (▲) on the frequency of spontaneous contractions of *R. prolixus* anterior midgut. Results are expressed as the percentage relative to the saline controls.

transcript on peripheral nerves, and we do not know the extent of their distribution. Peripheral neurons expressing a variety of neuropeptides (including the FGLa/ASTs) are well described in insects (see Fifield and Finlayson, 1978; Cantera and Nassel, 1992; Yoon and Stay, 1995; Žitňan et al., 1996; O'Brien and Taghert, 1998; Te Brugge et al., 2001). Thus, peripheral nerves are likely present in our dissected tissues and any peripheral cells might contaminate the tissue. In addition, *FGLa/AST in situ* distribution has been detected in a small population of hemocytes in *D. punctata* (Skinner et al., 1997), although the function of these AST-producing hemocytes is unknown. It is possible that weak *Rhopr-FGLa/AST* expression could be due to hemocyte contamination of tissues prior to RNA extraction, assuming that *R. prolixus* hemocytes might also produce Rhopr-FGLa/ASTs. Clearly additional investigations are needed to clarify these data. Lastly, the relative expression levels need to be confirmed using quantitative RT-PCR as the classical RT-PCR method used here is only qualitative.
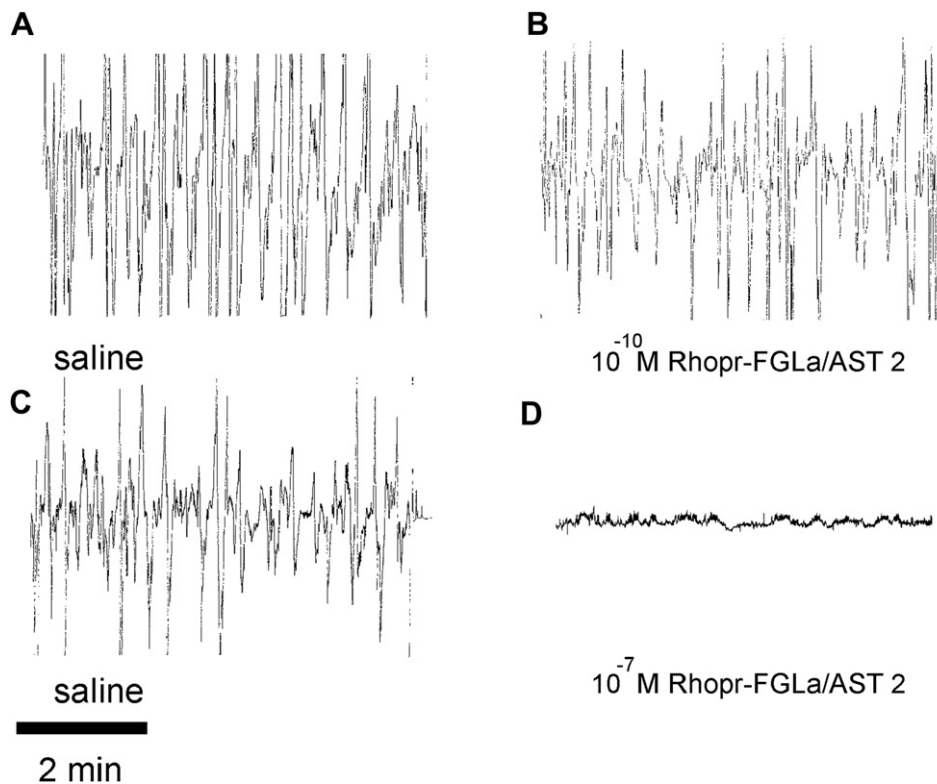
Fig. 7. Sample traces showing the effects of Rhopr-FGLa/AST-2 on spontaneous contractions of *R. prolixus* anterior midgut. (A, B) Before and after application of 10^{-10} M Rhopr-FGLa/AST-2. (C, D) Before and after application of 10^{-7} M Rhopr-FGLa/AST-2. Note the inhibitory effect of the peptide.
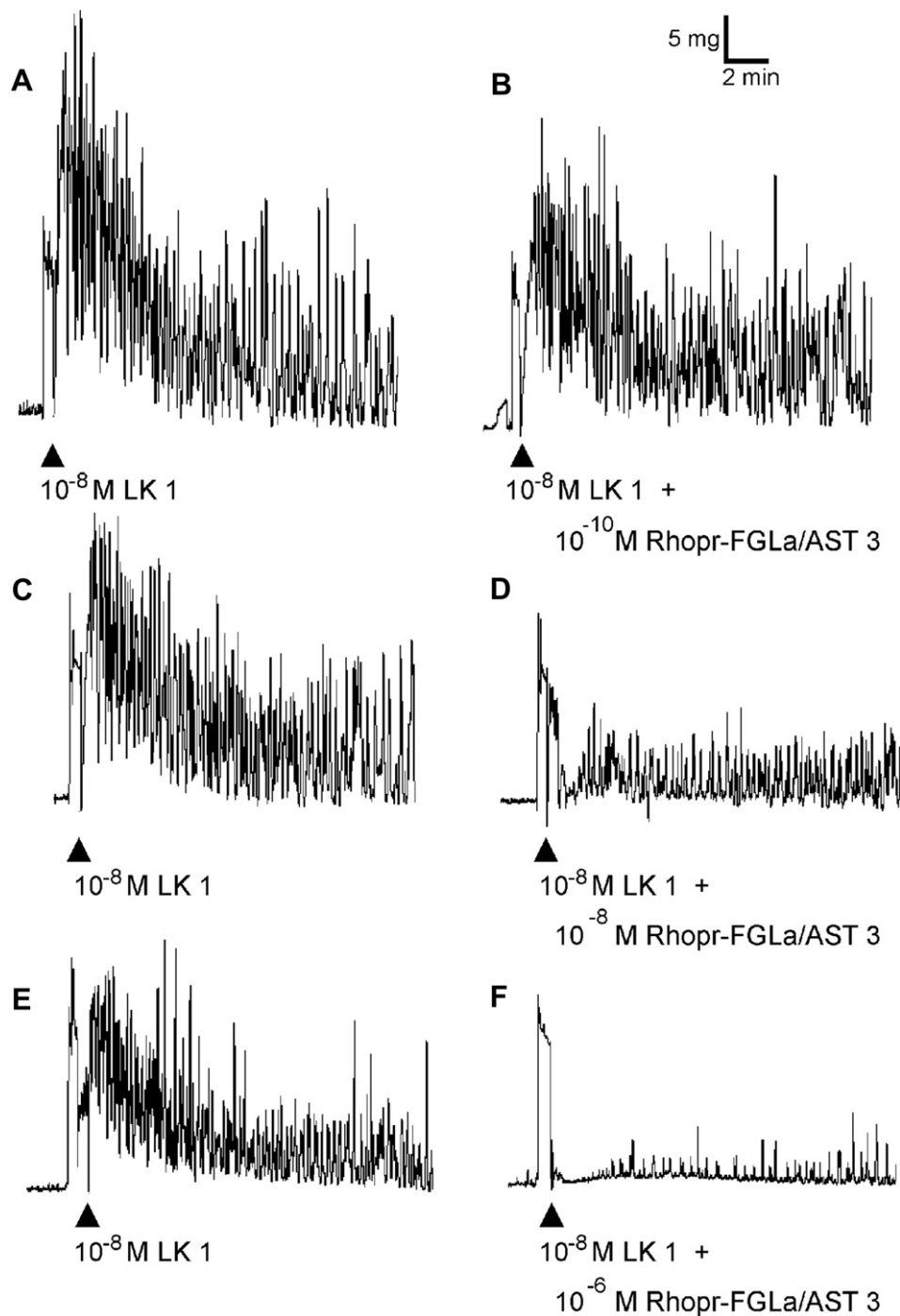
Fig. 9. Sample traces showing the effects of Rhopr-FGLa/AST-3 on leucokinin 1 (LK1)-induced contractions of *R. prolixus* hindgut. Control contractions were induced by 10^{-8} M LK1 (A, C, E) prior to the application of increasing doses of Rhopr-FGLa/AST-3 (B, D, F). Each preparation was thoroughly washed between the control and experimental peptide application. Peptides applied at the arrow heads. Note the inhibitory effect of Rhopr-FGLa/AST-3 on the frequency of contractions and amplitude of induced basal contraction.

FISH was performed to localize *Rhopr-FGLa/AST* expression within the CNS. Expression was consistent with RT-PCR analysis and RIA data, which showed expression in all parts of the CNS: brain, SOG, PRO, and MTGM. Such a wide distribution is typical in insects (see Stay et al., 1994; Tobe and Bendena, 2006). In addition, expression is consistent with the clusters of cells that are also FGLamide-like immunoreactive (Sarkar et al., 2003). All of the cells detected by FISH were also observed in immunohistochemistry. The immuno-histochemical analysis (Sarkar et al., 2003) reveals a few more cells than are localized by FISH. This might indicate low transcript expression in these cells. Among the brightest neurons stained by

FISH were the lateral neurosecretory cells that project over the corpus cardiacum/CA to the dorsal vessel, and 5 DUM neurons in the MTGM. These DUM neurons produce neurohaemal sites over the abdominal nerves, and are distinct from 5 DUM neurons in the MTGM that are serotonergic (see Orchard et al., 1989; Sarkar et al., 2003) and responsible for the regulation of diuresis (see Orchard, 2006, 2009). These latter serotonergic DUM neurons release serotonin at the time of feeding that stimulates absorption of salts and water across the anterior midgut, secretion of salts and water by the upper MTs, and reabsorption of KCl by the lower MTs. Serotonin also stimulates contraction of anterior midgut and hindgut (see Orchard,
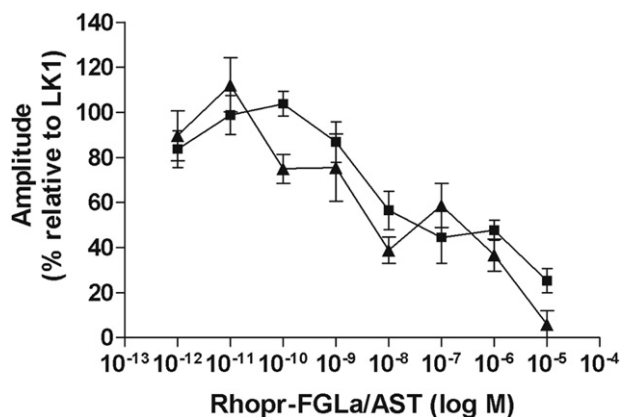
Fig. 10. Dose–response curves for the effects of Rhopr-FGLa/AST-2 (■) and Rhopr-FGLa/AST-3 (▲) on the amplitude of 10^{-8} M LK1-induced contractions of *R. prolixus* hindgut. Results are expressed as a percentage relative to 10^{-8} M LK1 alone.

2006, 2009). The location of the 5 Rhopr-FGLa/AST-expressing DUM neurons alongside the 5 serotonergic DUM neurons in the MTGM presents the interesting possibility of neural integration of the two groups and therefore the possible involvement of Rhopr-FGLa/ASTs in feeding-related activities (see later). There is about 800 fmol of Dippu-AST-7 equivalents in the CNS, and also about 50 fmol associated with the abdominal nerves and presumably their neurohaemal sites. These values are not dissimilar to those reported for other insects (e.g. Yu et al., 1993; Yagi et al., 2005). The absolute values cannot be determined because the antiserum is generated against a peptide that is an FGLamide, but does not have the same amino acid sequence as the native Rhopr-FGLa/ASTs, and therefore relies upon cross-reactivity. Clearly, though, Rhopr-FGLa/ASTs are present throughout the CNS, and are likely to act as neurohormones, in addition to their involvement as brain-gut peptides.

There is currently no evidence for a functional AST controlling JH production in *R. prolixus* (see Sarkar et al., 2003). Indeed, severing the nervi corpori cardiaci II in fifth-instars does not inhibit progression to the adult stage (Chiang, 2000), and there are no AST-like immunoreactive processes associated with the CA, other than those running over the surface to the dorsal vessel (Sarkar et al., 2003). Previously it has been suggested that species in which FGLa/ASTs are allatostatic have CAs that are directly innervated by FGLa/AST neurons (see Nässel, 2002). Interestingly though, the *Rhopr-FGLa/AST* temporal expression profile shows reduced
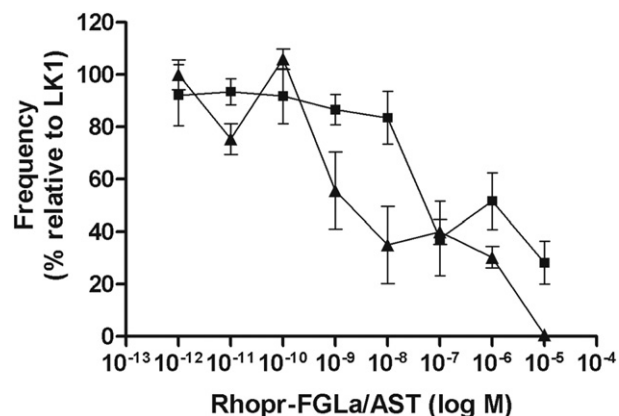

Fig. 11. Dose–response curves for the effects of Rhopr-FGLa/AST-2 (■) and Rhopr-FGLa/AST-3(▲) on the frequency of 10^{-8} M LK1-induced phasic contractions of *R. prolixus* hindgut. Results are expressed as the percentage relative to 10^{-8} M LK1 alone.

expression within one day post-feeding, with the levels recovering on the second day post-feeding. This indicates that the *Rhopr-FGLa/AST* expression might be down-regulated and then up-regulated after feeding, thereby altering the amount of peptide available for release. Blood feeding is the stimulus for growth and development in *R. prolixus*, but also initiates short term physiological changes associated with digestion, and salt and water balance (see Orchard, 2009). Interestingly, with this in mind, we have demonstrated that Rhopr-FGLa/ASTs are biologically active on tissues associated with feeding, namely the anterior midgut and hindgut, in addition to the dorsal vessel. This confirms some previous data on the myoinhibitory nature of non-native FGLs/ASTs in *R. prolixus* (Sarkar et al., 2003). Gut contractions during feeding produce mixing of the ingested blood meal as well as the surrounding haemolymph. *R. prolixus* MTs have been shown to have large unstirred layers surrounding them *in vitro*, while *in vivo* these unstirred layers are significantly reduced (see Orchard, 2009). It has been suggested that contractions of the anterior midgut and hindgut reduce the unstirred layers, as well as increase the circulation of hormones within the haemolymph (see Orchard, 2009). These tissues may be considered accessory hearts. Presumably the contractions of anterior midgut might also aid in the absorption of salts and water. Release of Rhopr-FGLa/ASTs into the haemolymph might therefore occur at the cessation of diuresis in *R. prolixus*, when diuretic hormone release has stopped and urine production is being halted. In this physiological state, the insect has already concentrated the blood meal in the anterior midgut and now needs to begin the process of digestion (which takes several days) and to also conserve water. The rapid mixing of haemolymph and gut-contents during diuresis is no longer required and a decrease in gut contractions might return the insect to a more stable physiological state. It is not known if Rhopr-FGLa/ASTs actually participate in physiological activities involved in the cessation of diuresis in *R. prolixus*; however, our results suggest this may be a role for Rhopr-FGLa/ASTs in *R. prolixus*, and this possibility deserves further investigation. There are also other potential physiological states that may require a decrease in gut activity, such as during and after ecdysis or starvation stress. Clearly though, these Rhopr-FGLa/ASTs would appear to be well suited to act as instruments for fine-tuning the contractile activity of the insect's gut. FGLa/ASTs have previously been shown to be inhibitors of visceral muscle contraction in insects (Duve and Thorpe, 1994; Lange et al., 1995; see Bendena et al., 1999) where they directly innervate the tissue, and it has been suggested that this may be their ancestral function (see Tobe and Bendena, 2006). In addition they are inhibitors of heart-beat frequency, and so may fine-tune circulation induced by the heart.

The C-terminal FGLamide sequence is highly conserved and therefore implies an important physiological relevance, whereas the N-terminus is not particularly well conserved in insects. This is consistent with previous studies showing that N-terminally truncated peptides retain their activity provided that the minimal pentapeptide is preserved (Stay et al., 1994; see Tobe and Bendena, 2006). Considerable variation of the N-terminus has previously been shown to account for difference in relative potencies of FGLa/ASTs in their effect on JH biosynthesis and on muscle activity (Stay et al., 1994; Lange et al., 1995). A similar phenomenon occurs in *R. prolixus* whereby Rhopr-FGLa/AST-2 and 3 vary in their effectiveness in inhibiting contractions of the anterior midgut and hindgut in *R. prolixus*.

Clearly *R. prolixus* possesses the FGLa/AST family of peptides which can be considered brain-gut peptides, as well as hormones as suggested by their presence in neurosecretory and endocrine cells, as also seen in other insects. At present, there is no direct evidence that Rhopr-FGLa/ASTs possess allatostatic activity in *R. prolixus*, and studies to investigate this will have to await the chemical identification of the *R. prolixus* JH which is currently unknown.

Acknowledgments

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2011.10.002.

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