Insect Biochemistry and Molecular Biology 57 (2015) 1–10

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Identification and functional characterization of FGLamide-related allatostatin receptor in *Rhodnius prolixus*

Meet Zandawala*, Ian Orchard

Department of Biology, University of Toronto Mississauga, Mississauga, Ontario, Canada

ARTICLE INFO

Article history:
Received 5 October 2014
Received in revised form
11 November 2014
Accepted 2 December 2014
Available online 11 December 2014

Keywords:
Insect
Neuropeptide
Allatostatin
Galanin
G protein-coupled receptor
Juvenile hormone

ABSTRACT

FGLamide-related ASTs (FGLa/ASTs) are a family of brain/gut peptides with numerous physiological roles, including inhibition of juvenile hormone (JH) biosynthesis by the corpora allata and inhibition of visceral muscle contraction. FGLa/ASTs mediate their effects by binding to a rhodopsin-like G-protein coupled receptor that is evolutionarily related to the vertebrate galanin receptor. Here we determine the cDNA sequence encoding FGLa/AST receptor (FGLa/AST-R) from the Chagas disease vector, *Rhodnius prolixus* (*Rhopr-FGLa/AST-R*), determine its spatial expression pattern using quantitative PCR and functionally characterize the receptor using a heterologous assay. Our expression analysis indicates that *Rhopr-FGLa/AST-R* is highly expressed in the central nervous system. The receptor is also expressed in various peripheral tissues including the dorsal vessel, midgut, hindgut and reproductive tissues of both males and females, suggesting a role in processes associated with feeding and reproduction. The possible involvement of Rhopr-FGLa/ASTs in the inhibition of JH biosynthesis is also implicated due to presence of the receptor transcript in the *R. prolixus* corpora cardiaca/corpora allata complex. The functional assay showed that various Rhopr-FGLa/ASTs activate the receptor, with EC_{50} values for the response in the nanomolar range. Moreover, Rhopr-FGLa/AST-R can couple with Gq alpha subunits and cause an increase in intracellular calcium concentration. Lastly, we tested various FGLa/AST analogs in our heterologous assay. These compounds also activated the receptor and thus have the potential to serve as insect growth regulators and aid in pest control.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Allatostatins (ASTs) are insect neuropeptides that were first identified for their ability to inhibit juvenile hormone (JH) biosynthesis by the corpora allata (CA) (see Bendena and Tobe, 2012). Three families of ASTs exist and these are characterized by their conserved C-terminus sequences. These include the FGLamide-related ASTs (FGLa/ASTs), myoinhibitory peptide (MIP)/ASTs and PISCF-related ASTs (PISCF/ASTs); these are also commonly referred to as AST-A, AST-B and AST-C, respectively (Coast and Schooley, 2011). Although most insects have been shown to contain all three AST families, only one type of AST possesses allatostatic activity, if any, in any given insect. Hence, only the FGLa/ASTs have been shown to possess allatostatic activity in cockroaches, crickets, termites and locusts (see Bendena and Tobe, 2012). It is now evident that FGLa/ASTs have numerous other physiological roles and that their role as inhibitors of JH biosynthesis is only a secondary evolved function. One such major role is the inhibition of visceral muscle contraction. Consequently, myoinhibitory activity of FGLa/ASTs has been shown in various insects including *Diploptera punctata* (Fuse et al., 1999; Lange et al., 1993), *Locusta migratoria* (Robertson et al., 2012), *Drosophila melanogaster* (Vanderveken and O'Donnell, 2014) and *Rhodnius prolixus* (Sarkar et al., 2003; Zandawala et al., 2012) amongst others. Moreover, FGLa/ASTs influence processes associated with feeding (Aguilar et al., 2003; Hergarden et al., 2012; Wang et al., 2012; Zandawala and Orchard, 2013) and reproduction (Veelaert et al., 1996; Woodhead et al., 2003).

Regardless of which biological process they influence, FGLa/ASTs mediate their effects by binding to a G-protein coupled receptor (GPCR). FGLa/AST receptors (FGLa/AST-Rs) belong to the family of rhodopsin-like GPCRs. The first FGLa/AST-R was isolated and functionally characterized from *D. melanogaster* and was originally referred to as DAR-1 (Birgul et al., 1999). Soon after that, another

* Corresponding author. Tel.: +1 905 828 5333; fax: +1 905 828 3792.
E-mail address: meet.zandawala@utoronto.ca (M. Zandawala).

http://dx.doi.org/10.1016/j.ibmb.2014.12.001
0965-1748/© 2014 Elsevier Ltd. All rights reserved.

FGLa/AST-R (DAR-2) was also discovered in *D. melanogaster* (Larsen et al., 2001; Lenz et al., 2000). Both these receptors were shown to be evolutionarily related to the vertebrate galanin receptors. Since then only a single FGLa/AST-R has been isolated and functionally characterized from each of *Periplaneta americana* (Auerswald et al., 2001), *D. punctata* (Lungchukiet et al., 2008), *Bombyx mori* (Secher et al., 2001) and *Caenorhabditis elegans* (Bendena et al., 2008). Knockdown of *C. elegans* FGLa/AST-R and *D. melanogaster* DAR-1 via RNAi has been shown to affect foraging behavior in these species (Bendena et al., 2008; Wang et al., 2012).

In the present study, we have isolated and functionally characterized the FGLa/AST-R from *R. prolixus* (Rhopr-FGLa/AST-R). Rhopr-FGLa/AST-R was activated by various Rhopr-FGLa/ASTs and FGLa/AST analogs in a heterologous assay utilizing Chinese hamster ovary (CHO) cells, with EC_{50} values for the response in the nanomolar range. Phylogenetic analysis indicates that this receptor is more closely related to the previously characterized receptor from *P. americana* than to either of the two receptors from *D. melanogaster*. Most insects, with the exception of dipterans, appear to possess only one FGLa/AST-R. Quantitative PCR (qPCR) was also used to determine the spatial expression pattern of *Rhopr-FGLa/AST-R* and consequently unravel novel target tissues for this peptide.

2. Material and methods

2.1. Animals

Fifth-instar and adult *R. prolixus* of both sexes were taken from a long standing colony at the University of Toronto Mississauga. Insects were raised in incubators maintained at 60% humidity and 25 °C, and were artificially fed on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA; supplied by Cedarlane Laboratories Inc., Burlington, ON, Canada).

2.2. Isolation of the cDNA sequence encoding R. prolixus FGLa/AST receptor

Supercontig sequences representing the *R. prolixus* genome assembly (January 2012 release) were downloaded from VectorBase (https://www.vectorbase.org/). The sequences were imported to Geneious 4.7.6 and used to perform local BLAST searches. *D. melanogaster* FGLa/AST-R-1 (DAR-1) amino acid sequence was used as a query to search the *R. prolixus* genome. A forward gene-specific primer (Rpr-ASTR-FOR1: 5′ AGAACTTACTGAAAAAATAGTGGCG 3′) and a reverse gene-specific primer (Rpr-ASTR-REV1: 5′ GTTCTGGTGTTTTTGGTTAAGGC 3′) were designed based on the resultant hits and used to amplify the partial cDNA sequence encoding Rhopr-FGLa/AST-R. A fifth-instar *R. prolixus* CNS cDNA library (Paluzzi et al., 2008) was used as the template for this PCR. The resultant product was purified, cloned and sequenced, as described earlier (Zandawala et al., 2013). *R. prolixus* genome assembly was used to predict the splice sites upstream and downstream of the regions encoding this partial Rhopr-FGLa/AST-R so that additional sequence for the first and last exons could be obtained (see below for splice site prediction). Primers (Rpr-ASTR-FOR8: 5′ ATCGAGATGAACGGATCAC 3′ and Rpr-ASTR-REV4: 5′ TAACACGAACCTAGGCAGTACAG 3′) were designed with these predicted splice sites in mind so as to avoid them spanning an intron splice site. These were then used to amplify the complete cDNA sequence encoding Rhopr-FGLa/AST-R with a proof-reading Taq polymerase using the methods described earlier (Zandawala et al., 2013, 2011).

2.3. Sequence and phylogenetic analysis

The intron–exon boundaries of *Rhopr-FGLa/AST-R* were predicted using a combination of BLAST and Genie, a splice site prediction software (Reese et al., 1997). The following software programs were used for predicting various features of Rhopr-FGLa/AST-R: Geneious 4.7.6 for the membrane topology of the receptor, NetPhos 2.0 Server (Blom et al., 1999) for the potential phosphorylation sites and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/ – last accessed on June 8, 2014) for the potential N-linked glycosylation sites.

Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/ – last accessed on June 8, 2014) was used to align Rhopr-FGLa/AST-R (KM283241) with its homologs from *D. melanogaster* (NP_524700.1 and NP_524544.1), *P. americana* (AAK52473.1) and *B. mori* (AAG44631.1). The alignment figure was obtained using the BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html – last accessed on June 8, 2014). Additional sequences used for the phylogenetic analysis were obtained by performing a protein BLAST search using Rhopr-FGLa/AST-R sequence as the query. Additionally, galanin receptors from *Homo sapiens* (NP_001471.2, NP_003848.1 and NP_003605.1) were also included in the analysis and *D. melanogaster* AST-C receptor (NP_649039.4) was utilized as an outgroup. ClustalX2 was used to align these sequences and the alignment exported to MEGA5 (Tamura et al., 2011). A maximum likelihood tree was constructed using Close-Neighbor-Interchange (CNI) analysis and the bootstrap values obtained were based on 1000 replicates.

2.4. Preparation of expression vector and receptor functional assay

Primers (ASTR-KOZAK-FOR: 5′ GCCACCATGAACGGATCAC 3′ and Rpr-ASTR-REV3: 5′ TTGCTTTAAACAGTTGCTCACC 3′) were used to amplify the *Rhopr-FGLa/AST-R* ORF and introduce a Kozak translation initiation sequence at the 5′ end. A plasmid containing the largest cDNA fragment encoding Rhopr-FGLa/AST-R was used as a template for this PCR. The resulting amplicons were subcloned into pIRES2-ZsGreen1 expression vector (Clontech, Mountain View, CA, USA).

CHO cells stably expressing the human G-protein G16 (CHO/G16) were cultured in complete media (94% Dulbecco's Modified Eagle Medium Nutrient Mixture F12-Ham (DMEM/F12) (Life Technologies Corporation, Carlsbad, CA, USA), 5% heat-inactivated fetal bovine serum (FBS), and 1% penicillin and streptomycin) containing 200 μg/mL hygromycin B (Stables et al., 1997; Zandawala et al., 2013). Cells were transiently co-transfected with expression vectors for aequorin (a calcium bioluminescent reporter protein) and the receptor using X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA), as described previously (Zandawala et al., 2013). Transfection using the empty expression vector was also performed as a negative control.

To determine if Rhopr-FGLa/AST-R couples with Gq alpha subunits (and subsequently increases intracellular calcium concentration), CHO cells stably expressing aequorin were used (CHOK1-aeq) (Paluzzi et al., 2012). The cells were cultured in complete media containing 200 μg/mL Geneticin® and transiently transfected with expression vector for the receptor as mentioned above.

Bioluminescence assay was performed 48 h post-transfection as described earlier (Zandawala et al., 2013). Briefly, cells were harvested and resuspended in bovine serum albumin (BSA) media. The cells were incubated in coelenterazine h (Promega, Madison, WI, USA) for 3 h in the dark, diluted 4–5-fold using BSA media, and loaded using an automated injector into a 96-well plate which contained peptides at various concentrations. At least 3 technical replicates were performed for each peptide concentration.

Luminescence was recorded in 5 s intervals for a total of 15 s using a Wallac Victor2 plate reader (Perkin Elmer, San Diego, CA, USA).

Rhopr-FGLa/AST-2 (LPVYNFGL-NH_2), Rhopr-FGLa/AST-3 (AHNEGRLYSFGL-NH_2) and Rhopr-FGLa/AST-6 (SNPNGHRFSFGL-NH_2) used in the assay were custom synthesized by Bio Basic Inc. (Markham, ON, Canada). C-terminal pentapeptide YDFGL-NH_2 and FGLa/AST analogs K15, K24 and H17 (Kai et al., 2009, 2010), were a kind gift from Professor Stephen Tobe, University of Toronto. FGLa/AST analogs were dissolved in DMSO to make 10^{-2} M stock solutions. These were then diluted to various concentrations in BSA media. We also tested a library of one amine and representatives of 10 other neuropeptide families.

2.5. Quantitative PCR tissue profiling

Quantitative PCR (qPCR) was used to determine the spatial expression profile of *Rhopr-FGLa/AST-R* using the method described earlier (Zandawala et al., 2013). Briefly, tissues were individually-dissected in phosphate-buffered saline (PBS) and stored in RNA*later*® Stabilization Solution (Life Technologies Corporation, Carlsbad, CA, USA) until RNA extraction. Total RNA was extracted from these tissues using PureLink® RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, USA) which was then used to synthesize cDNA with iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The resulting cDNA was diluted 10-fold and used as template for qPCR.

A 237 bp *Rhopr-FGLa/AST-R* fragment was amplified using the forward primer, ASTR-qPCR-FOR (5′ AGTGGCTTTTCAGGTTTCATTC 3′) and the reverse primer, ASTR-qPCR-REV (5′ CCGATACTTTTCATTACCAGAATCAC 3′). MX4000 Quantitative PCR System (Stratagene, Mississauga, ON, Canada) was used to perform all the experiments. Temperature-cycling profile consisted of an initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 24 s; a melt curve analysis was also included. Delta–delta Ct method was used to determine the relative expression of *Rhopr-FGLa/AST-R*, which was normalized via the geometric averaging of the transcript levels of alpha-tubulin, beta-actin and ribosomal protein 49 (Zandawala et al., 2013).

3. Results

3.1. Rhopr-FGLa/AST-R

We have isolated a 1550 bp cDNA sequence (*Rhopr-FGLa/AST-R*) encoding Rhopr-FGLa/AST-R (Fig. 1). The open reading frame (ORF) is 1215 bp long which encodes a receptor comprised of 404 amino acids. The ORF spans three exons which are separated by two large introns (Fig. 2). Exons 1 and 3 are at least 753 bp and 599 bp, respectively, while exon 2 is 198 bp long. A polyadenylation signal sequence is absent in the 3′ UTR suggesting that it is incomplete. The 5′ UTR is also partial and despite the absence of an in-frame stop codon upstream of the start codon, we have sufficient evidence to believe that the ORF is complete (see below under discussion). As is typical of all functional GPCRs, Rhopr-FGLa/AST-R is predicted to have an extracellular N-terminus, an intracellular C-terminus and seven transmembrane domains. The receptor is also predicted to undergo phosphorylation at five residues in its

```
                                                                                   5' - ATCGAG   6

ATG AAC GGA TCA CCA GCG ACC GCA ATT GTC GAA GCT ATC GGC AGT ATG CCT CCC ATA AAA TAC GAT CCG AAT AAT AAT TTC ACA AAT AAT   96
MET Asn Gly Ser Pro Ala Thr Ala Ile Val Glu Ala Ile Gly Ser Met Pro Pro Ile Lys Tyr Asp Pro Asn Asn Asn Phe Thr Asn Asn   30

ACT ATA AAT TTT AAT AAT AAT ATT CAT AAT TTC AAT AAT AAC AAT ATC GAT ATG AGA AAT TTC TAT TCA AAT GTA ACT GAT ACA GAA ATA  186
Thr Ile Asn Phe Asn Asn Asn Ile His Asn Phe Asn Asn Asn Asn Ile Asp Met Arg Asn Phe Tyr Ser Asn Val Thr Asp Thr Glu Ile   60

TTT ATG GAA GAA ATT TCA CCA GAA CTT ACT GAA AAA ATA GTG GCG ATT GTC GTA CCG GTT CTA TTC GGT ATA ATT GTG ATA CTC GGC CTT  276
Phe Met Glu Glu Ile Ser Pro Glu Leu Thr Glu Lys Ile Val Ala Ile Val Val Pro Val Leu Phe Gly Ile Ile Val Ile Leu Gly Leu   90

TTT GGT AAC GCT TTG GTG GTC ATT GTC GTA GCG GTC AAC CAA CAG ATG CGC AGC ACC ACA AAT ATT TTA ATA ATA AAT TTA GCT ATA GCA  366
Phe Gly Asn Ala Leu Val Val Ile Val Val Ala Val Asn Gln Gln Met Arg Ser Thr Thr Asn Ile Leu Ile Ile Asn Leu Ala Ile Ala  120

GAT CTA TTA TTT ATT GTC TTC TGC GTG CCA TTC ACA GCC ACA GAC TAT ATA TTC AGA TTT TGG CCT TTT GGT GAC ACC TGG TGC AAA ATG  456
Asp Leu Leu Phe Ile Val Phe Cys Val Pro Phe Thr Ala Thr Asp Tyr Ile Phe Arg Phe Trp Pro Phe Gly Asp Thr Trp Cys Lys Met  150

GTA CAA TAT TTA ATT GTG GTC ACT GCG TAC GCG TCC GTA TAT ACC CTG GTA CTG ATG TCG TTG GAC AGG TTT CTG GCT GTT GTA CAT CCA  546
Val Gln Tyr Leu Ile Val Val Thr Ala Tyr Ala Ser Val Tyr Thr Leu Val Leu Met Ser Leu Asp Arg Phe Leu Ala Val Val His Pro  180

ATA GCG TCC ATG TCA ATA AGA ACG GAA AAG AAT GCC ATA TCC GCT ATA TTG GTC ACA TGG ATA GTA ATC GTC ATT TCG AAT ATA CCA GTA  636
Ile Ala Ser Met Ser Ile Arg Thr Glu Lys Asn Ala Ile Ser Ala Ile Leu Val Thr Trp Ile Val Ile Val Ile Ser Asn Ile Pro Val  210

TTT TTA TGC CAC GGT GAA GTA ACT TTC AAT TAT TCA TCT TCA GAA CAC ACA GTC TGT ATC TTT CTA GAA ATG GAC CCA TTG ATA AGA CCT  726
Phe Leu Cys His Gly Glu Val Thr Phe Asn Tyr Ser Ser Ser Glu His Thr Val Cys Ile Phe Leu Glu Met Asp Pro Leu Ile Arg Pro  240

GAT GGT TTC AAC AAA GTG GCT TTT CAG GTT TCA TTC TTT GCA ACA GCC TAT GTG ATA CCA TTG GCT CTG ATA TGC GGC CTG TAT CTA GTG  816
Asp Gly Phe Asn Lys Val Ala Phe Gln Val Ser Phe Phe Ala Thr Ala Tyr Val Ile Pro Leu Ala Leu Ile Cys Gly Leu Tyr Leu Val  270

ATG CTG GTG AGA CTG TGG GGC GGT GCT GCA CCC GGT GGA AGG TGT TCC GCT GAA TCC AGA CGA GGA AAG AGA AGA GTA ACA AGA ATG GTG  906
Met Leu Val Arg Leu Trp Gly Gly Ala Ala Pro Gly Gly Arg Cys Ser Ala Glu Ser Arg Arg Gly Lys Arg Arg Val Thr Arg Met Val  300

CTT GTG GTC GTG GCC ATA TTT GCT ATT TGC TGG TGT CCC ATT CAA GTG ATT CTG GTA ATG AAA AGT ATC GGC CAA TAC GAA ATC ACG CCA  996
Leu Val Val Val Ala Ile Phe Ala Ile Cys Trp Cys Pro Ile Gln Val Ile Leu Val Met Lys Ser Ile Gly Gln Tyr Glu Ile Thr Pro  330

ACC TCG GTG ATG GTG CAG ATA GTG TCG CAC GTG CTG GCC TAC ATG AAC TCT TGC GTT AAT CCA ATT CTC TAC GCA TTT CTA TCG GAA AAT 1086
Thr Ser Val Met Val Gln Ile Val Ser His Val Leu Ala Tyr Met Asn Ser Cys Val Asn Pro Ile Leu Tyr Ala Phe Leu Ser Glu Asn  360

TTC AGA AAA GCA TTT CGA AAA GTT ATC TAC TGT GGG CCG GAA GGA GGT TCG CAT CCA CCA CAC CTC AAC GGC CGC CAA ATT GAT GCT GAA 1176
Phe Arg Lys Ala Phe Arg Lys Val Ile Tyr Cys Gly Pro Glu Gly Gly Ser His Pro Pro His Leu Asn Gly Arg Gln Ile Asp Ala Glu  390

AAA TCA GCC TTA ACC AAA AAC ACC AGA ACT ACT GAC ATT CTC TGA ACCAGGTGAGCAACTGTTTAAAGCAATTATTTTTTGTTGTTGTTGTTTGCTTTAATTAA  1280
Lys Ser Ala Leu Thr Lys Asn Thr Arg Thr Thr Asp Ile Leu  *                                                                404

CTGCCTTTGATCAGCCCGGTAGAAGTTTATCGTTAATTGCAAAAAATTTAGATGAGTAAGAGGAAAGCACAAAGCTCTCGTTAATTCAAAAACAGAGAGCAAAAAAATTCAAGAGTGAA  1399
TACTTGAAAAGAGTGTTTTCTCTACAAAATCAAAACCATTTTTTTTTTAAGTTATAAAAGGCTTTGAAAAAAATTCTAACTATAAATCGAGATGTCAAATGGGGTACCTTTACTAACCA  1518
AGTAGATTACTGTACTGCCTAGGTTCGTGTTA - 3'                                                                                     1550
```

Fig. 1. *Rhopr-FGLa/AST-R* cDNA sequence and the deduced amino acid sequence. The numbering for each sequence is shown at right. Within the nucleotide sequence, the exon–exon boundaries are shaded in gray. Within the amino acid sequence, the initial methionine start codon has been capitalized, the potential phosphorylation sites are shaded in black, the potential N-linked glycosylation sites are boxed and the seven putative transmembrane domains are underlined.

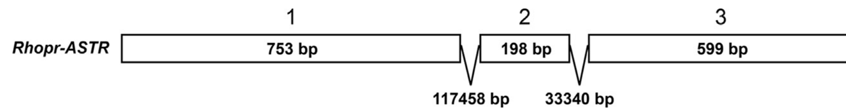

Fig. 2. *Rhopr-FGLa/AST-R* gene structure based on BLAST analysis and splice site prediction. The gene comprises three exons (represented by boxes) that are separated by two introns.

intracellular loops and N-linked glycosylation at four sites in its N-terminus and extracellular loop.

3.2. Sequence and phylogenetic analysis

Rhopr-FGLa/AST-R amino acid sequence was aligned along with its orthologs from *D. melanogaster*, *B. mori* and *P. americana* that have been previously cloned (Fig. 3). The region between the seven transmembrane domains is highly conserved across all sequences, with very little conservation observed in the N- and C-termini. The five phosphorylation sites predicted in Rhopr-FGLa/AST-R are also conserved across all the other receptors which suggests that they may very well be functional. The predicted N-linked glycosylation sites, on the other hand, are less conserved across the receptors examined here.

A comprehensive phylogenetic analysis of FGLa/AST-Rs and galanin receptors confirms the evolutionary relatedness between invertebrate FGLa/AST-Rs and vertebrate galanin receptors (Fig. 4); the clade comprising the three human galanin receptor subtypes is sister to the clade comprising all the invertebrate FGLa/AST-Rs. Rhopr-FGLa/AST-R is sister to a clade which includes receptors from *Nilaparvata lugens* and *P. americana*. It is also interesting to note that most insects, with the exception of dipterans, possess one FGLa/AST-R. Most dipteran species, including *Anopheles gambiae*, *D. melanogaster* and several other *Drosophila* species, have two FGLa/AST-Rs that form two distinct monophyletic clades. This suggests that these two receptors arose from a recent duplication in dipterans. Lastly, the four FGLa/AST-Rs found in *Ixodes scapularis* represents an independent duplication event in this species.

3.3. Functional receptor assay

In order to functionally characterize Rhopr-FGLa/AST-R, we utilized a previously established heterologous assay (Zandawala et al., 2013). Rhopr-FGLa/AST-R expressed in CHO/G16 cells was activated by Rhopr-FGLa/ASTs (2, 3 and 6) in a dose-dependent manner (Fig. 5A). The efficacy of these three peptides varied with Rhopr-FGLa/AST-3 having the lowest EC_{50} ($EC_{50} = 9.62 \times 10^{-10}$ M), followed by Rhopr-FGLa/AST-6 ($EC_{50} = 1.23 \times 10^{-9}$ M) and Rhopr-FGLa/AST-2 ($EC_{50} = 2.94 \times 10^{-9}$ M) (Table 1). Moreover, all three peptides produced a rapid response, with the peak response observed between 0 and 5 s (Fig. 5B). With regards to the controls, Rhopr-FGLa/AST-R could not be activated by any other peptide or amine from our library (Fig. 5C) (see Table S1 for a list of these peptides and their sequences). In addition, Rhopr-FGLa/ASTs failed to elicit any response in cells that had been transfected with empty vector (data not shown).

C-terminal pentapeptide and FGLa/AST analogs (K15, K24 and H17) all activated Rhopr-FGLa/AST-R expressed in CHO/G16 cells, albeit with different potencies (Fig. 6). H17 had the lowest EC_{50} ($EC_{50} = 2.14 \times 10^{-8}$ M), followed by K15 ($EC_{50} = 2.95 \times 10^{-8}$ M), K24 ($EC_{50} = 5.35 \times 10^{-8}$ M) and C-terminal pentapeptide ($EC_{50} = 1.51 \times 10^{-7}$ M). Although the analogs were less potent compared to the endogenous peptides, they still activated the receptor in the nanomolar range (Table 1).

Rhopr-FGLa/AST-R expressed in CHOK1-aeq was also activated by Rhopr-FGLa/ASTs (2, 3 and 6) (Fig. 7); however, the EC_{50} values for the response are approximately 30-fold higher than those obtained in CHO/G16 cells (Table 1).

3.4. Spatial expression profile of Rhopr-FGLa/AST-R

To identify the possible target tissues of Rhopr-FGLa/ASTs, spatial expression profile of *Rhopr-FGLa/AST-R* was determined via qPCR. Within the fifth-instar, *Rhopr-FGLa/AST-R* was highly expressed in the CNS and expressed at moderate levels in the dorsal vessel and posterior midgut (Fig. 8). Low levels of the transcript were also detected in the foregut, salivary glands, anterior midgut and hindgut (Fig. 8). Looking at the expression in adult reproductive tissues, *Rhopr-FGLa/AST-R* was highly expressed in female reproductive tissues, comprised of bursa, oviducts, spermatheca and cement gland (Fig. 9). The receptor transcript was moderately expressed in the male reproductive tissues, comprised of vas deferens, seminal vesicle, accessory glands and ejaculatory duct, while low levels of the transcript were detected in both testes and ovaries (Fig. 9).

In order to determine the possible involvement of Rhopr-FGLa/ASTs in JH biosynthesis in *R. prolixus*, we examined the presence of the receptor transcript in the CC/CA complex using qPCR. *Rhopr-FGLa/AST-R* is expressed in the CC/CA complex of both fifth-instars and adults although at lower levels than in fifth instar and adult CNS (Fig. 10).

4. Discussion

We have isolated and characterized a cDNA sequence encoding FGLa/AST-R from the Chagas disease vector, *R. prolixus* (*Rhopr-FGLa/AST-R*). Even though an in-frame stop codon upstream of the start codon is absent in our sequence, we suggest that the ORF is complete. This is based on the fact that the receptor topology prediction yields seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. Moreover, the multiple sequence alignment of FGLa/AST-Rs shows that the predicted N-linked glycosylation sites in the N-terminus of Rhopr-FGLa/AST-R are also conserved across other receptors and Rhopr-FGLa/AST-R does not appear to be any shorter than other sequences. Lastly and most importantly, the receptor is activated by Rhopr-FGLa/ASTs in our functional assay, with EC_{50} values of the response in the low nanomolar range. Hence, we believe the isolated Rhopr-FGLa/AST-R is complete and functional. When comparing the efficacy (as determined by EC_{50} values) of different Rhopr-FGLa/ASTs in the functional assay, Rhopr-FGLa/AST-3 was the most-effective, followed by Rhopr-FGLa/AST-6, and Rhopr-FGLa/AST-2 was the least-effective. This is in agreement with our previous results where Rhopr-FGLa/AST-3 was shown to be more effective than Rhopr-FGLa/AST-2 at inhibiting frequency and amplitude of anterior midgut and hindgut contractions in *R. prolixus* (Zandawala et al., 2012).

Insect growth regulators (IGRs) are chemicals that are used in pest management since they inhibit the life cycle of insects. Thus FGLa/ASTs can also be considered as potential IGRs for species in which they inhibit the synthesis of JH, a major hormone involved in insect molting. Several studies in *D. punctata* have examined the biological activities (both *in vitro* and *in vivo*) of synthetic analogs

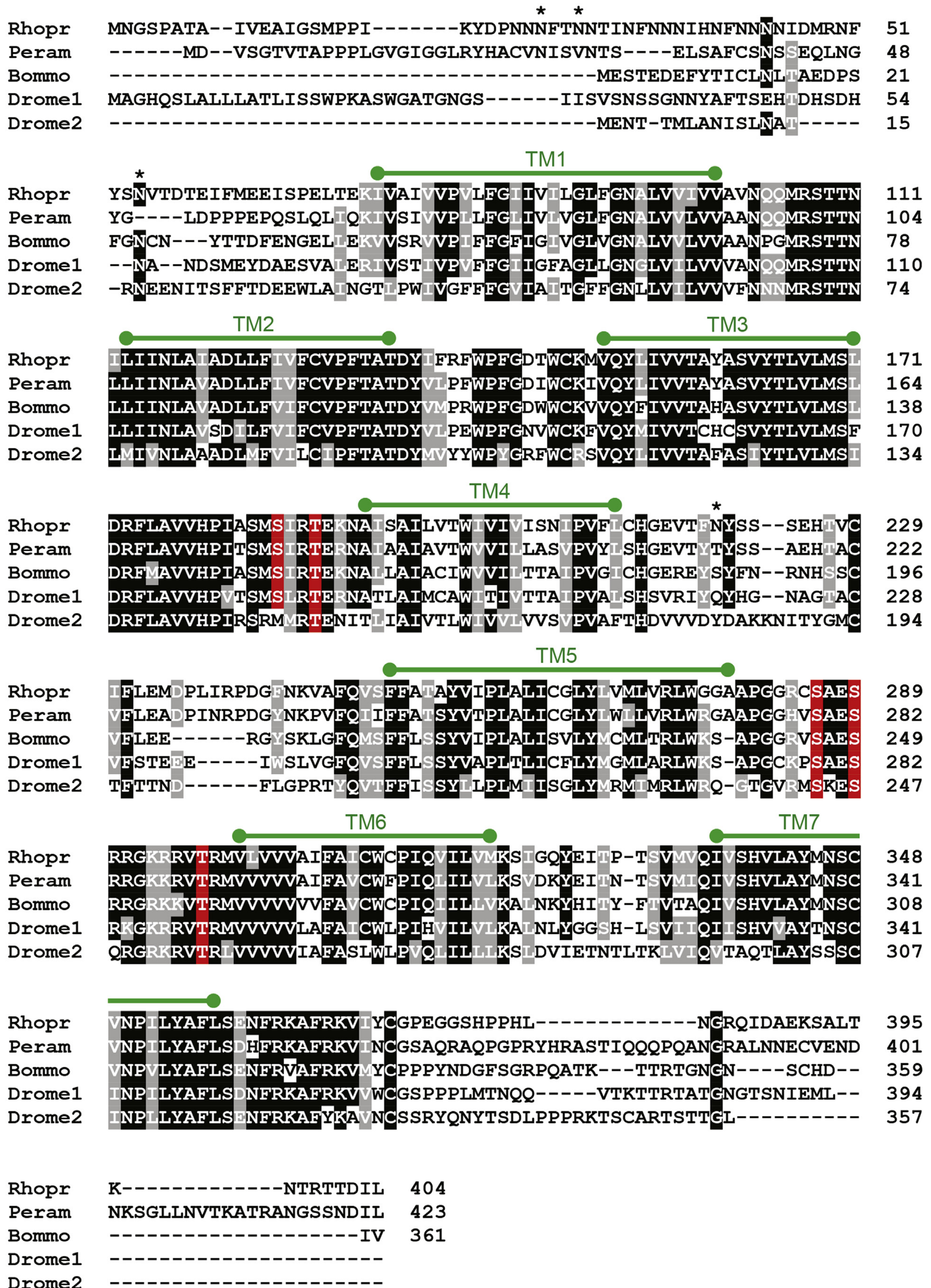

Fig. 3. Multiple sequence alignment of select insect FGLa/AST receptors. Identical and similar amino acids across 80% of the sequences have been highlighted in black and gray, respectively. The predicted locations of the seven transmembrane domains of Rhopr-FGLa/AST-R have been indicated using green lines. The five predicted phosphorylation sites that are conserved across all sequences are highlighted in red and the four predicted N-linked glycosylation sites are indicated using an asterisk. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

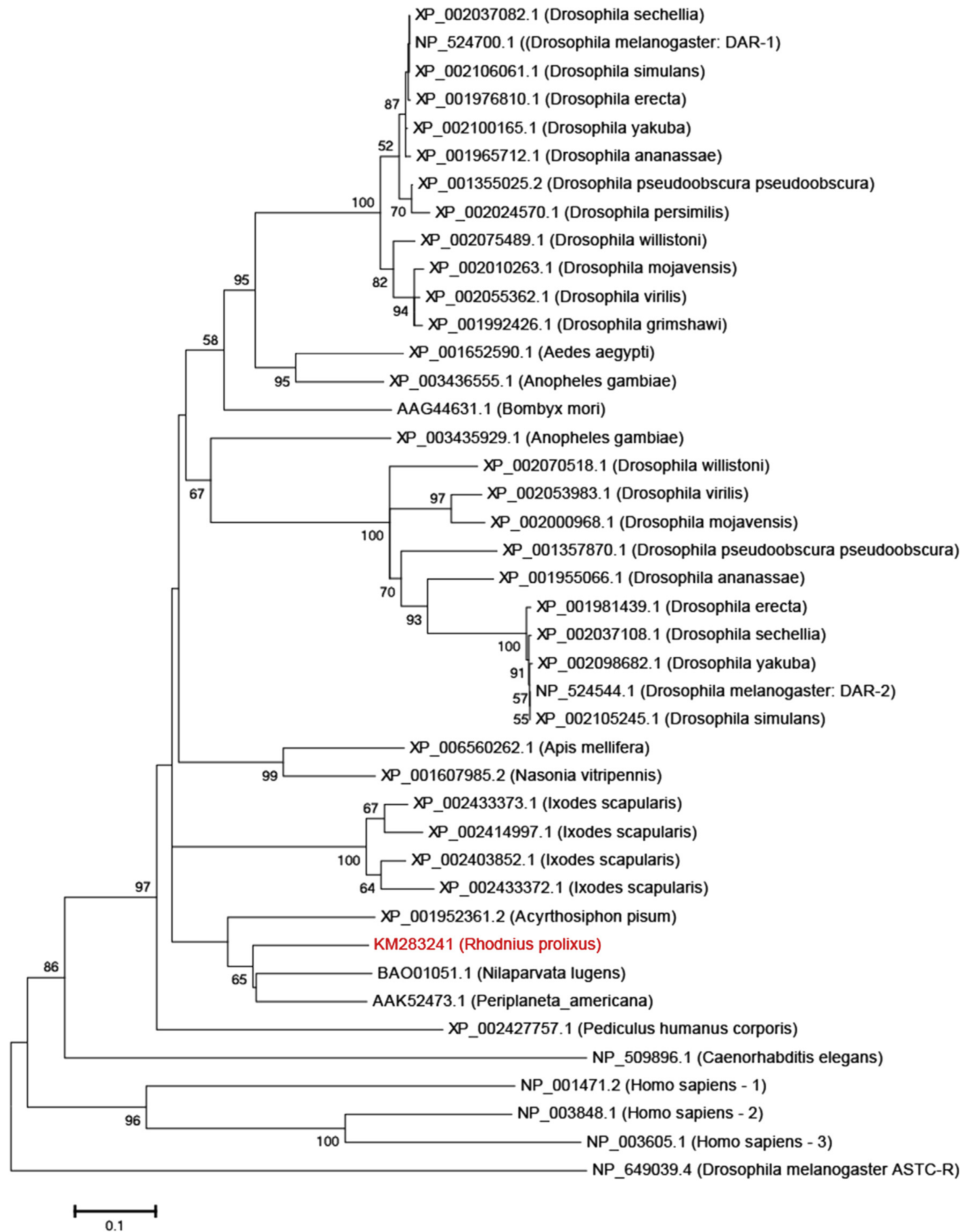

Fig. 4. A phylogram of FGLa/AST and galanin GPCRs obtained following a maximum likelihood analysis (1000 bootstrap replicates). The taxa are labelled using GenBank accession numbers and the species names. The number following the species name indicates the receptor subtype (only those that are experimentally verified). *Drosophila melanogaster* allatostatin C receptor (ASTC-R) was utilized as outgroup.

containing the FGLamide motif (Kai et al., 2009, 2010, 2011; Nachman et al., 1999; Xie et al., 2011). These studies have identified several lead compounds that are potent inhibitors of JH synthesis in *D. punctata* and could thus be used to control cockroach populations. We tested a subset of three such compounds (K15, K24 and H17) in our heterologous assay to see if they were also potent activators of Rhopr-FGLa/AST-R. Indeed all three compounds were potent agonists of Rhopr-FGLa/AST-R, with EC_{50} values in the low nanomolar range. These compounds were also more potent than the C-terminal pentapeptide which is the 'active core' region of FGLa/ASTs. Hence these compounds could be used as IGRs for *R. prolixus* if FGLa/ASTs possess allatostatic activity in this species (see below).

Phylogenetic analysis indicates that Rhopr-FGLa/AST-R is more closely related to the previously characterized receptor from *P. americana* than to either of the two previously characterized

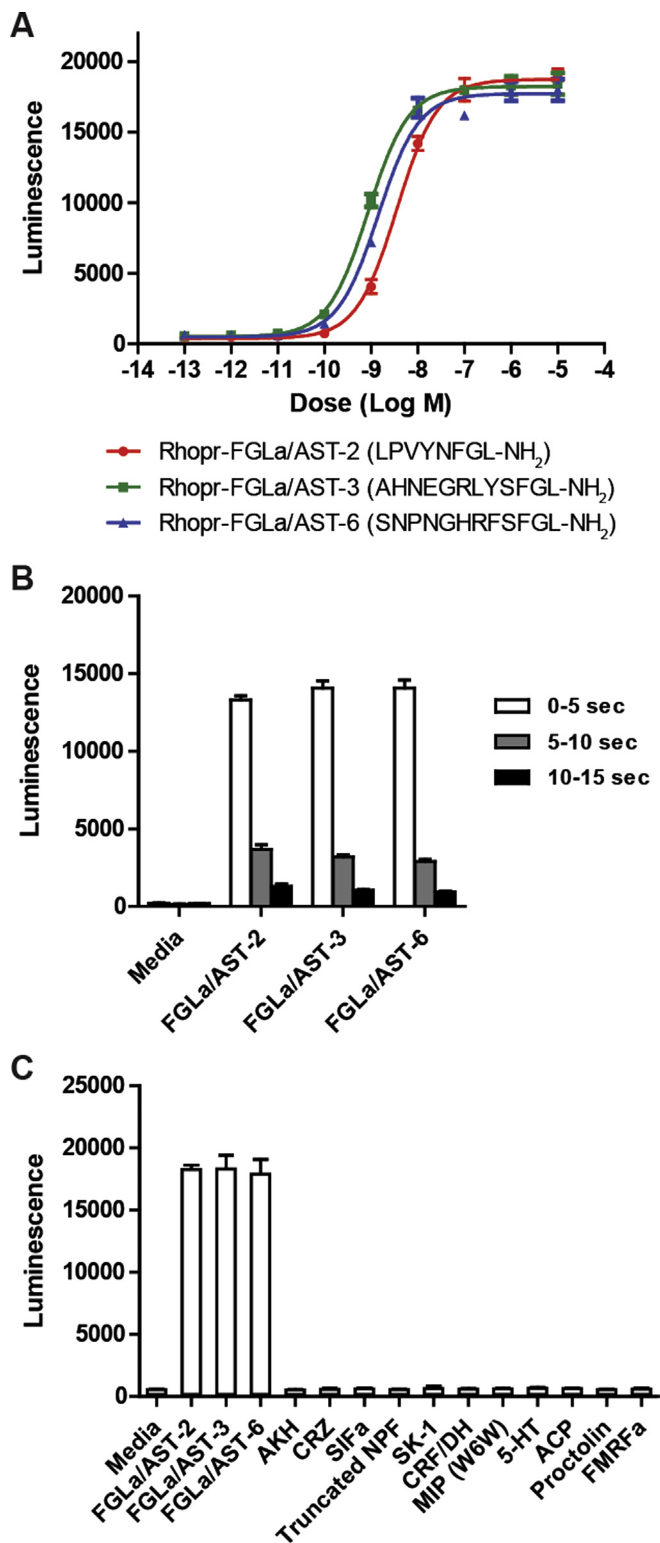

Fig. 5. Functional assay of Rhopr-FGLa/AST-R transiently expressed in CHO/G16 cells. (A) Dose-dependent effect on the bioluminescence response after addition of Rhopr-FGLa/AST-2, 3 and 6. (B) Kinetics of the bioluminescence response of CHO/G16 cells expressing Rhopr-FGLa/AST-R. Bioluminescence was recorded for every 5 s for 15 s following the addition of media or 10^{-6} M peptide. (C) Bioluminescence response following the addition of various ligands (amine and neuropeptides) at 10^{-6} M.

receptors from *D. melanogaster*. Moreover, the analysis also shows that most dipteran species examined here have two FGLa/AST-Rs that form two distinct monophyletic clades, suggesting that these two receptor subtypes may have originated from a recent duplication in dipterans. These two receptor types are functionally different as evident from their responses in Chinese Hamster Ovary (CHO) cells (Larsen et al., 2001). The two receptors, DAR-1 and DAR-2, when activated by FGLa/ASTs, lead to multiple signaling pathways, including the Gi/o alpha subunit mediated pathway that involves the inhibition of adenylate cyclase. However, these receptors show different preferences for coupling to specific G alpha subunits. Recent functional characterization of Dippu-FGLa/AST-R in multiple cell lines showed that this receptor couples with both the Gq and Gs alpha subunits (Huang et al., 2014). However, it did not couple with Gi alpha subunits, unlike the *D. melanogaster* FGLa/AST-Rs. Dippu-FGLa/AST-R was not included in our phylogenetic analysis as its sequence has not been submitted to GenBank. However, previous analysis indicated that Dippu-FGLa/AST-R is closely related to the receptor from another cockroach, *P. americana* (Lungchukiet et al., 2008). Functional characterization of Rhopr-FGLa/AST-R in CHOK1-aeq cells demonstrates that this receptor can couple with Gq alpha subunits and cause an increase in intracellular calcium concentration. Since Rhopr-FGLa/AST-R is more closely related to the cockroach receptors than to the *D. melanogaster* receptors, it probably also couples with Gs alpha subunits as opposed to the Gi alpha subunits.

Spatial expression analysis of *Rhopr-FGLa/AST-R* via qPCR identified several target tissues of FGLa/ASTs in *R. prolixus*. The receptor is expressed in the dorsal vessel, anterior midgut and hindgut, all tissues in which contractility is inhibited by Rhopr-FGLa/ASTs (Zandawala et al., 2012; Zandawala and Orchard, 2013). The receptor is also expressed in the foregut, salivary glands and posterior midgut. FGLa/ASTs have been shown to inhibit spontaneous contractions of the foregut in *Leucophaea maderae* (Duve et al., 1995), proctolin-induced foregut contractions in *L. migratoria* (Robertson et al., 2012), proctolin-induced midgut contractions in *D. punctata* (Fuse et al., 1999) and gut contractions in *D. melanogaster* (Vanderveken and O'Donnell, 2014). Hence Rhopr-FGLa/ASTs may also inhibit contractions of the foregut, posterior midgut and salivary glands, all of which are surrounded by muscles in *R. prolixus*. Moreover, FGLa/ASTs inhibit K^+ absorption across *D. melanogaster* midgut (Vanderveken and O'Donnell, 2014), K^+ absorption across *L. migratoria* hindgut (Robertson et al., 2014) and may also affect ion transport in *Aedes aegypti* midgut (Onken et al., 2004). Therefore, Rhopr-FGLa/ASTs may also influence digestion of the blood meal and/or the subsequent absorption of K^+ across the *R. prolixus* posterior midgut. Not surprisingly, the receptor is not expressed in Malpighian tubules (MTs), which is in agreement with the previous findings where Rhopr-FGLa/ASTs failed to inhibit serotonin-stimulated MT secretion (Zandawala and Orchard, 2013). This expression pattern of their receptor further supports the theory that Rhopr-FGLa/ASTs play a role in feeding-related physiological events.

Rhopr-FGLa/AST-R is also expressed in male reproductive tissues without the testes and female reproductive tissues without the ovaries. The male reproductive tissue sample includes the vas deferens, seminal vesicle, accessory glands and ejaculatory duct while the female reproductive tissue sample includes the bursa, oviducts, spermatheca and cement gland. This suggests that Rhopr-FGLa/ASTs could influence reproductive physiology. Indeed, they have been shown to inhibit spontaneous contractions of *R. prolixus* oviduct and bursa, *in vitro* (Sedra, personal communication), and thus may eventually contribute to the inhibition of egg-laying. The effects of Rhopr-FGLa/ASTs on male reproductive tissues have not yet been examined. One prediction is that they may affect the

Table 1
Effectiveness of various FGLa/ASTs on Rhopr-FGLa/AST-R expressed in CHO/G16 and CHOK1-aeq cells. EC_{50} values presented here are averages of multiple independent replicates.

Peptide name	Structure	EC_{50} (M)	
		CHO/G16	CHOK1-aeq
Rhopr-FGLa/AST-2	LPVYNFGL-NH_2	2.94×10^{-9}	8.81×10^{-8}
Rhopr-FGLa/AST-3	AHNEGRLYSFGL-NH_2	9.62×10^{-10}	2.50×10^{-8}
Rhopr-FGLa/AST-6	SNPNGHRFSFGL-NH_2	1.23×10^{-9}	3.48×10^{-8}
C-terminal pentapeptide	YDFGL-NH_2	1.51×10^{-7}	Not tested
K15	See Kai et al. (2010)	2.95×10^{-8}	Not tested
K24	See Kai et al. (2010)	5.35×10^{-8}	Not tested
H17	See Kai et al. (2009)	2.14×10^{-8}	Not tested

transfer of sperm and seminal fluids to females during copulation. Moreover, FGLa/AST-like immunoreactivity is not associated with male or female reproductive tissues of *R. prolixus* (Sedra and Lange, unpublished). Hence the effect, if any, of Rhopr-FGLa/ASTs on reproductive tissues will most-likely be mediated via a hormonal route.

Previous studies have not been able to provide any conclusive evidence for Rhopr-FGLa/ASTs involvement, or lack thereof, in JH biosynthesis in *R. prolixus*. FGLa/AST-like immunoreactive processes are not associated with the CA of fifth-instar and adult *R. prolixus*, as is the case in species in which FGLa/ASTs are allatostatic, but are associated with the CC and aorta close by (see Nassel, 2002). Moreover, it is usually the brain neurons associated with nervi corpori cardiaci II (NCCII) that are responsible for this neural inhibition of CA; however, transecting the NCCII in fifth-instar *R. prolixus* does not affect the insect's normal progression into an adult stage (Chiang, 2000), although transecting them in adults does influence egg production (Chiang, 1998). Lastly, it has not been possible to obtain any direct evidence confirming that Rhopr-FGLa/ASTs possess allatostatic activity as the chemical identification of *R. prolixus* JH is still unknown. Hence to address this question, we investigated the presence of *Rhopr-FGLa/AST-R* in the CC/CA complex via qPCR. Our analysis indicates that the receptor is indeed expressed in the CC/CA of both fifth-instars and adults. Thus Rhopr-FGLa/ASTs may be acting on the CC/CA of *R. prolixus* to inhibit JH production; however, other possibilities must also be taken into consideration. For instance, Rhopr-FGLa/ASTs and their receptor may also influence the release of other hormones found in the CC, such as the adipokinetic hormone, as has been shown in *L. migratoria* (Clark et al., 2008). Additional investigations, possibly

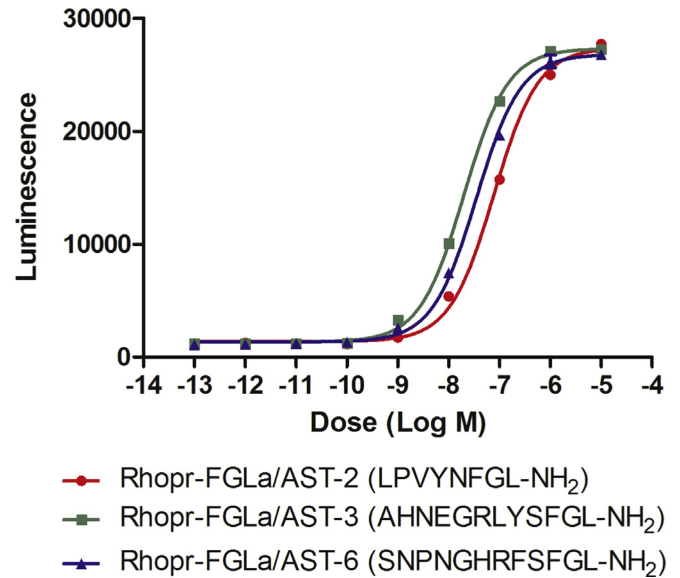

Fig. 7. Functional assay of Rhopr-FGLa/AST-R transiently expressed in CHOK1-aeq cells. Dose–response curves demonstrating the activities of Rhopr-FGLa/AST-2, 3 and 6 on Rhopr-FGLa/AST-R.

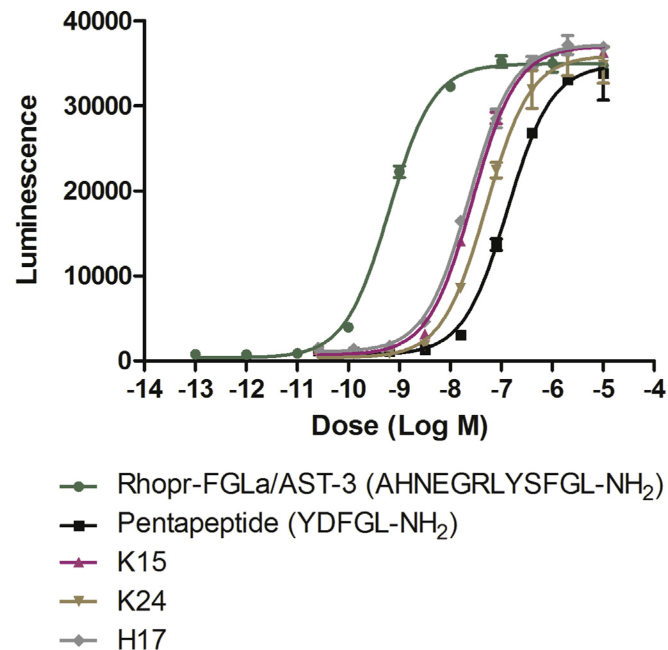

Fig. 6. Activity of structurally related analogs containing the FGLamide motif on Rhopr-FGLa/AST-R transiently expressed in CHO/G16 cells. All the analogs tested activate the receptor.

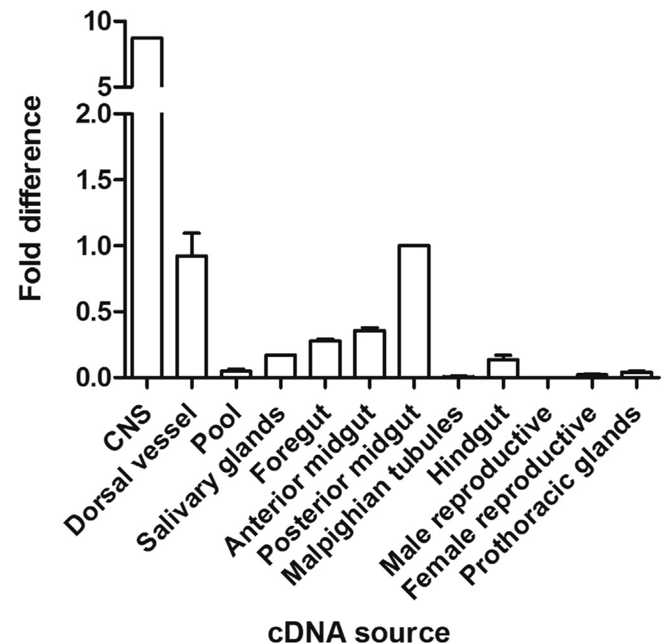

Fig. 8. Spatial expression analysis of *Rhopr-FGLa/AST-R* in fifth instar *R. prolixus* determined using quantitative PCR. Expression was analyzed in the following tissues: CNS (central nervous system), dorsal vessel, Pool (fat bodies, abdominal nerves and diaphragm), foregut, salivary glands, anterior midgut, posterior midgut, Malpighian tubules, hindgut, male reproductive tissues, female reproductive tissues and prothoracic glands (and associated fat bodies). Expression is shown relative to transcript levels in posterior midgut cDNA.

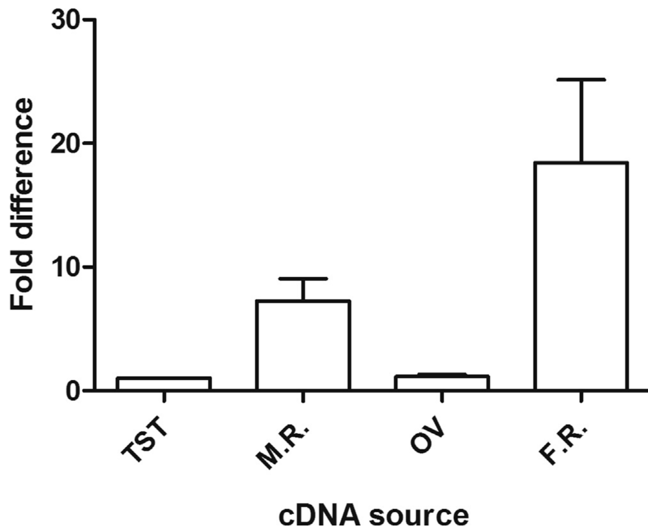

Fig. 9. Spatial expression analysis of *Rhopr-FGLa/AST-R* in *R. prolixus* adult reproductive tissues determined using quantitative PCR. Expression was analyzed in the following tissues: TST (testes), M.R. (rest of the male reproductive tissues), OV (ovaries) and F.R. (rest of the female reproductive tissues). Expression is shown relative to transcript levels in TST cDNA.

involving the knockdown of *Rhopr-FGLa/AST-R* via RNAi, are needed to further address this question and thus determine the factor(s) controlling JH production and development in *R. prolixus*.

In conclusion, we have isolated and functionally characterized the *R. prolixus* FGLa/AST receptor. Spatial expression profile of this receptor reaffirms the multifunctional nature of this signaling system, with the receptor expressed in various tissues. Hence FGLa/ASTs could influence JH biosynthesis and reproductive physiology, aside from their previously documented myoinhibitory roles in *R. prolixus*.

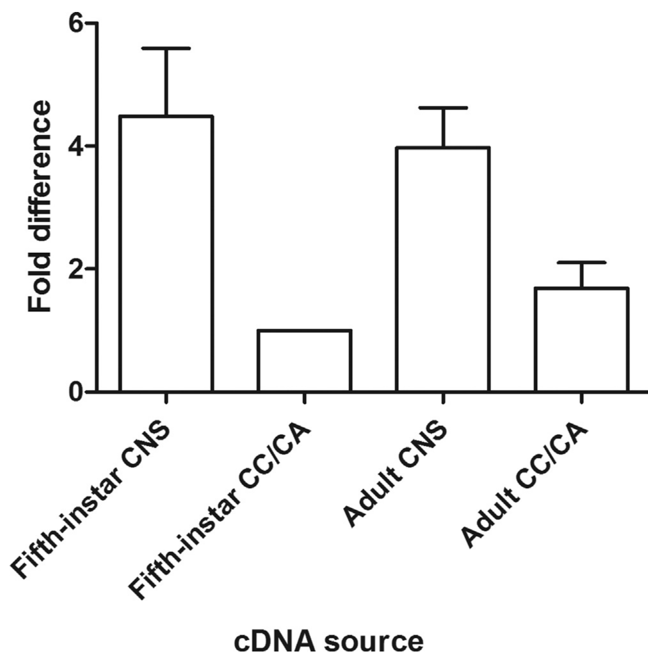

Fig. 10. Spatial expression analysis of *Rhopr-FGLa/AST-R* in *R. prolixus* corpora cardiaca/corpora allata (CC/CA) complex determined using quantitative PCR. Expression was analyzed in the CNS (central nervous system) and CC/CA from fifth-instar and adult *R. prolixus*. Expression is shown relative to transcript levels in fifth-instar CC/CA cDNA.

Acknowledgements

The authors wish to thank Nikki Sarkar for maintaining the colony and Dr. Amir Haddad for technical support. We are also grateful to Dr. Steve Tobe, Zhen-peng Kai and Juan Huang for providing FGLa/AST analogs. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to I.O. (NSERC RGPIN 8522-12) and an NSERC Canadian Graduate Scholarship to M.Z.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ibmb.2014.12.001.

References

Aguilar, R., Maestro, J.L., Vilaplana, L., Pascual, N., Piulachs, M.D., Belles, X., 2003. Allatostatin gene expression in brain and midgut, and activity of synthetic allatostatins on feeding-related processes in the cockroach *Blattella germanica*. Regul. Pept. 115, 171–177.

Auerswald, L., Birgul, N., Gade, G., Kreienkamp, H.J., Richter, D., 2001. Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. Biochem. Biophys. Res. Commun. 282, 904–909.

Bendena, W.G., Boudreau, J.R., Papanicolaou, T., Maltby, M., Tobe, S.S., Chin-Sang, I.D., 2008. A *Caenorhabditis elegans* allatostatin/galanin-like receptor NPR-9 inhibits local search behavior in response to feeding cues. Proc. Natl. Acad. Sci. U. S. A. 105, 1339–1342.

Bendena, W.G., Tobe, S.S., 2012. Families of allatoregulator sequences: a 2011 perspective. Can. J. Zool. 90, 521–544.

Birgul, N., Weise, C., Kreienkamp, H.J., Richter, D., 1999. Reverse physiology in *Drosophila*: identification of a novel allatostatin-like neuropeptide and its cognate receptor structurally related to the mammalian somatostatin/galanin/opioid receptor family. EMBO J. 18, 5892–5900.

Blom, N., Gammeltoft, S., Brunak, S., 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J. Mol. Biol. 294, 1351–1362.

Chiang, R.G., 1998. Partial localization of a brain factor inhibiting egg production in the blood-feeding insect, *Rhodnius prolixus*. Arch. Insect Biochem. Physiol. 39, 126–131.

Chiang, R.G., 2000. Neural inhibition of the corpus allatum in the last larval instar differs from that in adults in the blood-feeding insect, *Rhodnius prolixus*. Arch. Insect Biochem. Physiol. 44, 1–6.

Clark, L., Lange, A.B., Zhang, J.R., Tobe, S.S., 2008. The roles of Dippu-allatostatin in the modulation of hormone release in *Locusta migratoria*. J. Insect Physiol. 54, 949–958.

Coast, G.M., Schooley, D.A., 2011. Toward a consensus nomenclature for insect neuropeptides and peptide hormones. Peptides 32, 620–631.

Duve, H., Johnsen, A.H., Scott, A.G., Thorpe, A., 1995. Isolation, identification and functional significance of [Hyp2]Met-callatostatin and des Gly-Pro Met-callatostatin, two further post-translational modifications of the blowfly neuropeptide Met-callatostatin. Regul. Pept. 57, 237–245.

Fuse, M., Zhang, J.R., Partridge, E., Nachman, R.J., Orchard, I., Bendena, W.G., Tobe, S.S., 1999. Effects of an allatostatin and a myosuppressin on midgut carbohydrate enzyme activity in the cockroach *Diploptera punctata*. Peptides 20, 1285–1293.

Hergarden, A.C., Tayler, T.D., Anderson, D.J., 2012. Allatostatin-A neurons inhibit feeding behavior in adult *Drosophila*. Proc. Natl. Acad. Sci. U. S. A. 109, 3967–3972.

Huang, J., Marchal, E., Hult, E.F., Zels, S., Broeck, J.V., Tobe, S.S., 2014. Mode of action of allatostatins in the regulation of juvenile hormone biosynthesis in the cockroach, *Diploptera punctata*. Insect Biochem. Mol. Biol. 54, 61–68.

Kai, Z.P., Huang, J., Tobe, S.S., Yang, X.L., 2009. A potential insect growth regulator: synthesis and bioactivity of an allatostatin mimic. Peptides 30, 1249–1253.

Kai, Z.P., Huang, J., Xie, Y., Tobe, S.S., Ling, Y., Zhang, L., Zhao, Y.C., Yang, X.L., 2010. Synthesis, biological activity, and hologram quantitative structure–activity relationships of novel allatostatin analogues. J. Agric. Food Chem. 58, 2652–2658.

Kai, Z.P., Xie, Y., Huang, J., Tobe, S.S., Zhang, J.R., Ling, Y., Zhang, L., Zhao, Y.C., Yang, X.L., 2011. Peptidomimetics in the discovery of new insect growth regulators: studies on the structure–activity relationships of the core pentapeptide region of allatostatins. J. Agric. Food Chem. 59, 2478–2485.

Lange, A.B., Chan, K.K., Stay, B., 1993. Effect of allatostatin and proctolin on antennal pulsatile organ and hindgut muscle in the cockroach, *Diploptera punctata*. Arch. Insect Biochem. Physiol. 24, 79–92.

Larsen, M.J., Burton, K.J., Zantello, M.R., Smith, V.G., Lowery, D.L., Kubiak, T.M., 2001. Type A allatostatins from *Drosophila melanogaster* and *Diplotera punctata* activate two *Drosophila* allatostatin receptors, DAR-1 and DAR-2, expressed in CHO cells. Biochem. Biophys. Res. Commun. 286, 895–901.

Lenz, C., Williamson, M., Grimmelikhuijzen, C.J., 2000. Molecular cloning and genomic organization of a second probable allatostatin receptor from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. 273, 571–577.
Lungchukiet, P., Donly, B.C., Zhang, J., Tobe, S.S., Bendena, W.G., 2008. Molecular cloning and characterization of an allatostatin-like receptor in the cockroach *Diploptera punctata*. Peptides 29, 276–285.
Nachman, R.J., Garside, C.S., Tobe, S.S., 1999. Hemolymph and tissue-bound peptidase-resistant analogs of the insect allatostatins. Peptides 20, 23–29.
Nassel, D.R., 2002. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Prog. Neurobiol. 68, 1–84.
Onken, H., Moffett, S.B., Moffett, D.F., 2004. The anterior stomach of larval mosquitoes (*Aedes aegypti*): effects of neuropeptides on transepithelial ion transport and muscular motility. J. Exp. Biol. 207, 3731–3739.
Paluzzi, J.P., Russell, W.K., Nachman, R.J., Orchard, I., 2008. Isolation, cloning, and expression mapping of a gene encoding an antidiuretic hormone and other CAPA-related peptides in the disease vector, *Rhodnius prolixus*. Endocrinology 149, 4638–4646.
Paluzzi, J.P., Young, P., Defferrari, M.S., Orchard, I., Carlini, C.R., O'Donnell, M.J., 2012. Investigation of the potential involvement of eicosanoid metabolites in anti-diuretic hormone signaling in *Rhodnius prolixus*. Peptides 34, 127–134.
Reese, M.G., Eeckman, F.H., Kulp, D., Haussler, D., 1997. Improved splice site detection in Genie. J. Comput. Biol. 4, 311–323.
Robertson, L., Donini, A., Lange, A.B., 2014. K^+ absorption by locust gut and inhibition of ileal K^+ and water transport by FGLamide allatostatins. J. Exp. Biol..
Robertson, L., Rodriguez, E.P., Lange, A.B., 2012. The neural and peptidergic control of gut contraction in *Locusta migratoria*: the effect of an FGLa/AST. J. Exp. Biol. 215, 3394–3402.
Sarkar, N.R., Tobe, S.S., Orchard, I., 2003. The distribution and effects of Dippu-allatostatin-like peptides in the blood-feeding bug, *Rhodnius prolixus*. Peptides 24, 1553–1562.
Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., Hansen, G.N., Svane, P., Grimmelikhuijzen, C.J., 2001. Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. J. Biol. Chem. 276, 47052–47060.
Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., Sautel, M., Milligan, G., Lee, M., Rees, S., 1997. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal. Biochem. 252, 115–126.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Vanderveken, M., O'Donnell, M.J., 2014. Effects of diuretic hormone 31, drosokinin, and allatostatin A on transepithelial K(+) transport and contraction frequency in the midgut and hindgut of larval *Drosophila melanogaster*. Arch. Insect Biochem. Physiol. 85, 76–93.
Veelaert, D., Devreese, B., Schoofs, L., Van Beeumen, J., Vanden Broeck, J., Tobe, S.S., De Loof, A., 1996. Isolation and characterization of eight myoinhibiting peptides from the desert locust, *Schistocerca gregaria*: new members of the cockroach allatostatin family. Mol. Cell. Endocrinol. 122, 183–190.
Wang, C., Chin-Sang, I., Bendena, W.G., 2012. The FGLamide–allatostatins influence foraging behavior in *Drosophila melanogaster*. PLoS ONE 7, e36059.
Woodhead, A.P., Thompson, M.E., Chan, K.K., Stay, B., 2003. Allatostatin in ovaries, oviducts, and young embryos in the cockroach *Diploptera punctata*. J. Insect Physiol. 49, 1103–1114.
Xie, Y., Kai, Z.P., Tobe, S.S., Deng, X.L., Ling, Y., Wu, X.Q., Huang, J., Zhang, L., Yang, X.L., 2011. Design, synthesis and biological activity of peptidomimetic analogs of insect allatostatins. Peptides 32, 581–586.
Zandawala, M., Li, S., Hauser, F., Grimmelikhuijzen, C.J., Orchard, I., 2013. Isolation and functional characterization of calcitonin-like diuretic hormone receptors in *Rhodnius prolixus*. PLoS ONE 8, e82466.
Zandawala, M., Lytvyn, Y., Taiakina, D., Orchard, I., 2012. Cloning of the cDNA, localization, and physiological effects of FGLamide-related allatostatins in the blood-gorging bug, *Rhodnius prolixus*. Insect Biochem. Mol. Biol. 42, 10–21.
Zandawala, M., Orchard, I., 2013. Post-feeding physiology in *Rhodnius prolixus*: the possible role of FGLamide-related allatostatins. Gen. Comp. Endocrinol. 194, 311–317.
Zandawala, M., Paluzzi, J.P., Orchard, I., 2011. Isolation and characterization of the cDNA encoding DH(31) in the kissing bug, *Rhodnius prolixus*. Mol. Cell. Endocrinol. 331, 79–88.