



Allatostatin-C receptors in mosquitoes

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ABSTRACT

In the present work we describe the functional and molecular characterization of two *Aedes aegypti* allatostatin-C receptor paralogs (AeAS-CrA and AeAS-CrB) and provide a detailed quantitative study of the expression of the AS-C receptor genes in an adult insect. The tissue distribution of the two AS-C receptors differed significantly; the mRNA levels of AeAS-CrB in the Malpighian tubules were the highest detected, while transcripts for AeAS-CrA were relatively low in this tissue. In addition, the transcript levels of both receptors were different in the thoracic and abdominal ganglia, corpora allata (CA) and the testis of the male. In the CA, the AeAS-CrB mRNA levels were constant from 0 to 72 h after female emergence, while the AeAS-CrA levels increased at 72 h. To complement the receptor expression studies, we analyzed the tissue specificity for allatostatin-C mRNA in female mosquitoes. Expression was high in abdominal ganglia and brain. Transcript levels of allatostatin-C in the head of females were elevated at eclosion and there were no major changes during the first week of adult life or after blood feeding. Fluorometric Imaging Plate Reader (FLIPR) recordings of calcium transients in HEK293T cells transiently expressing both putative receptors showed that they both responded selectively to allatostatin-C stimulation in the nanomolar concentration range. However, the peptide showed slightly greater affinity for AeAS-CrB than AeAS-CrA. Our studies suggest that some of the pleiotropic effects of allatostatin-C in mosquitoes could be mediated by the different receptor paralogs. Transcriptional regulation of the AS-C receptors may not have a critical role in the changes of CA responsiveness to the peptide that we previously described.

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1. Introduction

Allatostatins are neuropeptides originally described as inhibitors of juvenile hormone (JH) synthesis in insects (reviewed by Stay and Tobe [34]). However, these pleiotropic peptides are also involved in the suppression of muscular activity in different tissues, inhibition of vitellogenesis and modulation of the activity of certain midgut digestive enzymes [10,3]. Insect allatostatins can be grouped into three families, YXFGL-amide-allatostatins (cockroach or type-A) (AS-A), W₂W₉-allatostatins (cricket or type-B) (AS-B) and PISCF-allatostatins (type-C) (AS-C) [4,33]. Allatostatin-C is a 15 amino acid peptide originally isolated from the brain tissue of the lepidopteran *Manduca sexta* [17]. Using a combination of high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) we previously reported the isolation and characterization of an AS-C

from the female mosquito *Aedes aegypti* [23]. Maximum AS-C levels in the head of sugar-fed mosquitoes were found at 3 days after eclosion [23]; this maximum value correlates with an increase in the sensitivity of the corpora allata (CA) to inhibition by AS-C, as well as with a decrease in the CA synthetic activity [23].

The identification and characterization of allatostatin receptors is crucial for understanding the regulatory effects of these peptides on the synthesis of JH [34]. Two AS-C receptors were described in *Drosophila melanogaster* using a reverse pharmacological approach [18]. These receptors are insect homologs of the mammalian somatostatin receptors, a family of G-protein-coupled receptors (GPCRs). The GPCRs comprise a large and ancient superfamily of integral cell membrane proteins that play fundamental roles in signal transduction, including the response to light, odor, taste, neurotransmitters and hormones; they are activated by a diverse array of structurally diverse ligands, including small organic molecules, lipids, ions, hormones, short and large polypeptides and glycoproteins [31]. GPCRs share a seven hydrophobic transmembrane α -helical domain structure and transduce signals through coupling to heterotrimeric guanine nucleotide-binding regulatory

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proteins. The seven transmembrane domains are linked by three extracellular loops that alternate with three intracellular loops. The extracellular amino terminus is usually glycosylated and the cytoplasmic carboxy terminus is generally phosphorylated.

AS-C receptors have not been studied in other insects, and there is a lack of information on the tissue specificity and developmental changes in receptor transcript expression. In the present work we describe the functional and molecular characterization of two AS-C receptors expressed in the female *A. aegypti* mosquito.

2. Materials and methods

2.1. Insects

A. aegypti of the Rockefeller strain were reared at 28 °C and 80% relative humidity under a photoperiod of 16 h light:8 h dark. Mated adults were offered a cotton pad soaked in 3% sucrose solution. The cotton pad sucrose-fed adults are referred to as sugar-fed. Three-day-old female mosquitoes were fed porcine blood equilibrated to 37 °C. Adenosine triphosphate was added to the blood meal to a final concentration of 1 mM immediately before use [29].

2.2. RNA extraction and molecular cloning

Mosquito tissues were dissected in a drop of sterile DNA-RNase free phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (Halt™ Protease Inhibitor Cocktail, Pierce). Total RNA was isolated using RNA-binding glass powder as previously described [28]. Contaminating genomic DNA was removed using DNA-free™ kit (Ambion, Austin, TX, USA). First strand cDNA synthesis was carried out using SuperScript® II first strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, CA, USA). PCR was performed in a Mastercycler gradient (Eppendorf, Westbury, NY, USA) using Taq DNA polymerase (Promega, Madison, WI, USA). PCR products were cloned in pCR®2.1-TOPO and sequenced by the DNA Core Sequence Facility at Florida International University (Miami, FL, USA).

D. melanogaster sequences DROSTAR1 (CG7285) and DROSTAR2 (CG13702) [18] were used to query against the *A. aegypti* database VectorBase (<http://www.vectorbase.org/index.php>) and revealed two similar sequences with the accession numbers AAEL012356 and AAEL012920. Based on these two sequences, primers were designed to amplify the full length of the two putative *A. aegypti* allatostatin-C receptor paralogs that were designated AeAS-CrA and AeAS-CrB, respectively. For functional experiments, the AeAS-CrA and AeAS-CrB were cloned into the plasmid pCDNA5/FRT (Invitrogen). For immunocytochemical localization experiments we fused our target receptors with the fluorescent protein YFP (Yellow Fluorescent Protein) using the vector pEYFP-N1 (BD Biosciences Clontech, San Jose, CA, USA).

2.3. Real time PCR

RNA samples were treated with rDNaseI using DNA-free™ kit (Ambion, Austin, TX, USA) according to manufacturer's recommendations. Reverse transcription was carried out using the Reverse-IT™ 1st Strand Synthesis Kit (ABgene, Epsom, UK) using oligo dT and 300 ng of RNA. Real time PCR was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® Gene Expression Assays together with TaqMan® Universal PCR Master Mix (Applied Biosystems). The primers and probes for the house keeping gene 60S ribosomal protein rpl32 (AAEL003396), the AeAS-CrA, AeAS-CrB and *Aedes* AS-C genes were as follows:

rpl32 forward: 5'-CCATCAGTCCGATCGCTATGA-3'
rpl32 reverse: 5'-GTTGTCAATACCTTTTCGGCTTACG-3'
rpl32 probe: 5'-CAAGCTTGCCCCAACTG-3'
AeAS-CrA forward: 5'-GGCAATTCAACGAAAATCCTCCAT-3'
AeAS-CrA reverse: 5'-GCCGTATCGTTGGGATGAGT-3'
AeAS-CrA probe: 5'-CTGATCGCCAACTTC-3'
AeAS-CrB forward: 5'-GACCACATCGAAAGTGAACATC-3'
AeAS-CrB reverse: 5'-TGCCGCGGTCTGTGT-3'
AeAS-CrB probe: 5'-CACGACGGCCACCACG-3'
AeAS-C forward: 5'-GATCGGTTACAGCTGCAACTG-3'
AeAS-C reverse: 5'-TCCCAACCGCCAGCTC-3'
AeAS-C probe: 5'-TCCGATGGCATTGCTTT-3'

Primer/probes were synthesized by Applied Biosystems and reactions were carried out in 20 µl volume according to the manufacturer's recommendations for Custom TaqMan® Gene Expression Assays. Reactions were run in duplicate using 1 µl of cDNA per reaction. Standard curves to quantify relative gene copy number were made from 10-fold serial dilutions of plasmids containing rpl32 or the gene of interest (from 300,000 to 30 copies of a plasmid per reaction). Real time data were collected by 7300 System SDS Software and analyzed in Microsoft Excel. AS-C receptors and peptide transcript levels were normalized with rpl32 transcript levels in the same sample. Relative AS-C receptors and peptide transcript levels are expressed as a number of copies of transcript per 10,000 copies of rpl32. Each RT-PCR data point is average of at least three independent biological replicates of 3–20 tissue samples.

2.4. Functional expression of the two receptors

For this purpose we used three different cell lines, HEK293 cells, HEK293 cells stably expressing Gα15, and HEK293T cells expressing Gα16gust44. Gα15 is a promiscuous G-protein that couples various GPCRs to phospholipase C activity [30]. The chimeric G-protein Gα16gust44 has been shown to couple TAS2R bitter taste receptors robustly to the same effector [35,26]. Cells were seeded into 96-well black-wall, clear bottom microtiter plates (Greiner Bio One, Frickenhausen, Germany) and grown for 24 h to about 80% confluence. Then the cells were transiently transfected with AeAS-CrA and AeAS-CrB encoding plasmids using Lipofectamine2000 (Invitrogen). After an additional 24 h, the cells were loaded with the calcium-sensitive dye Fluo4-AM (Invitrogen) and incubated for 1 h at 37 °C. Cells were then washed three times with C1 buffer (130 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 10 mM glucose, pH 7.4) and stimulated with the appropriate ligands in a Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA). Calcium-dependent increase of Fluo4-fluorescence was recorded at 1 Hz and 510 nm simultaneously from each well after excitation at 488 nm [6]. Responses of three wells containing cells expressing the same receptor and receiving the same stimulus were averaged. For dose–response curve calculation, the changes in fluorescence after the ligand was added were corrected for fluorescence changes in mock-transfected cells (transfected with a plasmid without receptor DNA) and normalized to background fluorescence ($\Delta F/F = (F - F_0)/F_0$). Our calculations were based on at least three independent transfection experiments.

2.5. Test solutions

Custom made peptides were provided by Alpha Diagnostics International (San Antonio, TX, USA), purified by reverse phase liquid chromatography and assessed to be ≥99% pure by analytical reversed phase liquid chromatography, matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS)

and amino acid analysis. Stock aqueous solutions of *A. aegypti* allatostatin-C (QIRYRQCYFNPISCF) [23] and *A. aegypti* allatotropin (APFRNSEMMTARGF) [21] were prepared at a concentration of 1 mM and stored in aliquots at -80°C . For each assay, a new aliquot was used. Stock solutions of linear synthetic peptides were oxidized overnight shaking at 4°C [18]. Assay concentrations were ranging from $3\ \mu\text{M}$ to $30\ \mu\text{M}$.

2.6. Immunocytochemistry

HEK293/G α 16gust44 cells were seeded on coverslips coated with poly-D-lysine and, after 24 h, transfected with constructs for AeAS-CrA-pEYFP-N1 or AeAS-CrB-pEYFP-N1 using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were washed with PBS, cooled on ice and incubated for 1 h on ice with $5\ \mu\text{g}/\text{ml}$ biotin-labeled concanavalin A (Sigma). After washing with PBS five times, cells were fixed for 2 min with methanol:acetone (1:1). To reduce non-specific binding, the coverslips were incubated in 3% goat serum. We added streptavidin conjugated with Alexa Fluor 633 (1:1000) and incubated in darkness for 1 h at RT. After washing three times with PBS and once with double distilled water, cells were mounted using Fluorescent Mounting Medium (DakoCytomation, CA, USA). Cells were analyzed using a Leica TCS SP2 Laser Scan Inverted microscope.

2.7. Phylogenetic analysis

Allatostatin-C receptor sequences were obtained from the databases and used for the alignments and phylogenetic analysis. Poorly aligned variable regions at the amino and carboxy terminals were excluded and the analysis was performed using Bayesian methods for phylogenetic analysis of the peptide sequences using the program MRBAYES 3.1.2 [32]. We set the prior model to the mixed setting, allowing the Markov chain Monte Carlo (MCMC chain) to integrate over the 10 fixed amino acid rate matrices available in MRBAYES. Four Markov chains for 1 million generations were employed, with trees sampled every 100th generation, and discarding the first 15% of sampled trees as burn-in. Two simultaneous runs were initiated from different random starting trees, and convergence was inferred when the average standard deviation of split frequencies was below 0.01, and the potential scale reduction factor for all parameters approached 1.0. Posterior probabilities above 0.50 are reported for all nodes in the tree.

2.8. Statistical analysis

The results are expressed as the means \pm S.E. Student's *T*-test was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and considered significantly different at $P \leq 0.05$. Regression curves and EC₅₀ values were obtained by fitting the data using non-linear regression analysis using the GraphPad Prism software. Values expressed as percentage were normalized using the arc sine transformation of their square root prior to statistical analysis by one-way ANOVA followed by a comparison of means (Tukey's test).

3. Results

3.1. Identification and phylogenetic analysis of two *A. aegypti* AS-C receptors

Screening of the *A. aegypti* genome using the two *D. melanogaster* AS-C receptors [18] resulted in the identification of two sequences with a high degree of identity, corresponding to the predicted genes AELO12356 (AeAS-CrA) and AELO12920 (AeAS-CrB). These receptors are localized in the contigs 22,801 and 24,172 of the two distant supercontigs 1.683 and 1.760. Vectorbase did not list introns for

AELO12356; but predicted a 28 kb intron close to the 3'-end for AELO12920. 3'-Rapid Amplification of cDNA End (RACE) was used to confirm that the intron was incorrectly predicted (data not shown). The two genes have open reading frames of 1545 bp (AeAS-CrA) and 1377 bp (AeAS-CrB), coding for proteins of 514 and 459 amino acids. Both receptors share 52% identity; but if the amino and carboxy terminal sequences are removed, the similarity increases to 73% (comparing only the sequence that comprises the seven transmembrane domains). The predicted amino acid sequences of these receptors exhibit some of the characteristic features of GPCRs, such as the seven transmembrane domains (TM1-TM7), N-linked glycosylation sites and the conserved motif "DRY" in the second intracellular loop between the TM3 and TM4 that is often involved in the activation of the G-coupled proteins (Fig. 1). AS-C receptor orthologs were searched in other species of insects. The following sequences were obtained from the databases and used for the alignments and phylogenetic analysis: *Culex pipiens* CPIJ010469 and CPIJ011191, *Anopheles gambiae* AGAP012268 and AGAP010486, *Apis mellifera* XP_396335, *Nasonia vitripennis* XM_001600604, *Bombyx mori* BGIBMGA006675 and *Tribolium castaneum* GLEAN_12842. All of these sequences were found to have close similarity with the two sequences of *D. melanogaster* CG13702 and CG7285. We also found receptor orthologs for several additional *Drosophila* sp. available in Flybase; the accession numbers for the receptors identified are: *D. ananassae* GF10817 and GF23626, *D. erecta* GG15739 and GG13659, *D. yakuba* GE19956 and GE22071, *D. sechellia* GM14937 and GM14973, *D. simulans* GD12341 and GD14751, *D. willistoni* GK11516 and GK11187, *D. pseudoobscura* GA12470 and GA23907, *D. grimshawi* GH17108 and GH14454, *D. mojavensis* GI11711 and GI13590 and *D. virilis* GJ11386 and GJ13930. In total, we found sequences for 32 orthologs of the 2 *D. melanogaster* AS-C receptors. A cladogram of the phylogenetic relationship of these sequences of the AS-C receptor family was generated (Fig. 2).

The intron presence-absence was also compared among the sequences studied. No introns are predicted among the Hymenoptera (*N. vitripennis* and *A. mellifera*) and Lepidoptera (*B. mori*). One intron located in the extracellular loop between TM6 and TM7 is predicted for the Coleoptera (*T. castaneum*). In the Diptera Nematocera, no introns are predicted for *A. gambiae*, and although there were predicted introns in Vectorbase for *A. aegypti* (AeAS-CrB) and *C. pipiens*, no introns were found in *Aedes* and by sequence homology in *C. pipiens*. In Diptera Brachycera, two introns were predicted in Flybase for *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba* and *D. erecta*. These five species have perfectly conserved positions for both introns in the two paralogs; intron "1" is located between the "TV" sequence and intron "2" between "QV", both located in the TM6 domain. The rest of the *Drosophila* species analyzed did not have any predicted introns in Flybase.

3.2. Functional expression and characterization of AS-C receptors in *A. aegypti*

Three different mammalian cell lines transiently expressing the putative receptors were used to functionally characterize the receptors. Activation of the receptors is coupled to the release of Ca^{2+} from intracellular stores, which can be measured using calcium-sensitive fluorescent dyes [8,6,35]. HEK293 cells expressing AeAS-CrA did not respond to AS-C; on the other hand, HEK293/G α 15 and HEK293T/G α 16gust44 cells expressing AS-CrA or AS-CrB receptors were robustly activated by AS-C in a dose-responsive manner (Fig. 3). The minimum concentrations of neuropeptide able to activate the receptors were in the low nanomolar range. EC₅₀ values differed moderately, being $27.7 \pm 7.2\ \text{nM}$ for AeAS-CrB and $89.0 \pm 18.5\ \text{nM}$ for AeAS-CrA. Thus, AeAS-CrB appears to have a ~ 3 -fold higher potency for AS-C than AeAS-CrA. HEK293T/G α 16gust44 cells had the most robust responses

A.aegypti_A	MDSIATES	TIVTTM	MV	PAVAPPVAG	LTFFPSDLLT	FIRQTASGSA	SSNLSYLLSN	ITRALSSPSN
A.aegypti_B		MDDNL	TYFL	TTG	GL.GENAS	IGENAL	NDTGGMPRG.	F.
A.gambiae_A								
A.gambiae_B		M	EEDNATSFLL	LGEYD	SLTDQNVSQ	LGNYLENSDP	NQT	FMG.
C.pipiens_A	MEQQWNGTVS	LE..NGTNGF	STASP.GVVA	.GMS..S.VG	G.P...E...	L...S.TV..	.G.....	F....T.TGT
C.pipiens_B			MDSNLSTYFL	GGG	EL.GENNS.	MLEGLESTL	EG.E.LFR.	F.
D.melanog_1		M.AD	SEANATNWN	TNES	LY.TELN	HR	W.SGSS	TIQPEES.YG
D.melanog_2		M.LT	SLI.PTEQLA	V.PNGTTLHQ	LESVE.ESYP	S.NG.QNETM	VTSVRPH.DH	RN.PTQNGS
A.aegypti_A	EFNLRRFP	LHPFFPLYND	TVFESCPSIQ	MPIGNLISMI	LYAVVGLIGL	FGNTLVIVV	LRFSKMQTVT	NMYILNLAIA
A.aegypti_B		...TD	..TTYTVF..C.V.V	M.....	...N....
A.gambiae_A			..V.V....	..L.AIV..
A.gambiae_B			..PTD	..TTYLFF..C.V.V	L.....	...N....
C.pipiens_A	D.....Y.Y	PYA...F...	S.Y.I.....AI...
C.pipiens_B			..LTD	..TTYTLF.VC.V..	M.....	...N....
D.melanog_1			..IATR	NSFAD.FTVV	..GF.CI...I.....V.
D.melanog_2	HY	..EY.	DDGPD.SYSY	NF.LK..T..	..L.CI...	M.....	..I.....
A.aegypti_A	DQCFLIGIPF	LIITMHLGEW	TFGNTMCKAY	MVSTISITQFT	SSIFLFIMS	DRYIAVCHPI	SSPRFRTPLV	SKIVSFFAWT
A.aegypti_B	..E.....	..A...MKR.	V..GA.....I..H.	...KY....	..RV..AL..L
A.gambiae_A	..E.....	..T.....	..A.....I..H.	...KY....	..V..AI..L
A.gambiae_B	..E.....	..A...MKR.	L..GA.....V....I..H.	...KY....	..R..LL..L
C.pipiens_A	..E.....	..T.....	..V.....I..H.	...KY....	..R..AI..L
C.pipiens_B	..E.....	..A...MKQ.	V..GA.....I..H.	...KY....	..RV..AL..L
D.melanog_1	..E.....	..LY..RICS.	R..EF.....S....L...Y..LHI	A.V..AI..S
D.melanog_2	..E.....	..LY..QV.N.	P...Y.....S....L...Y..F..	..L..A...M
A.aegypti_A	TSALIMLPVM	LYANTVHRDK	G	KISCNIW	PSENASNSGT	TFTLYSLILG	FAVPLSLILM	FYYLVIRKLR
A.aegypti_B	A.....I.	I.GDIIQLQP	N	SY..Q...	.ESHEHMP.YV...CF.MT	..C.....
A.gambiae_A	A.....	...IA.E.	D	M...M...	...TGA...SI..T...
A.gambiae_B	A.....I.	I..DVIEQKP	N	TY..Q.L.	.ETHGHL.P.YVI..CF.MT	..C.....
C.pipiens_A	A..M.....L	..S..IP.E.	D	..L.....I..T...
C.pipiens_B	A..I.....I.	I.GDIIIEVMP	N	YY..Q...	.ETHEHMQ.YVI..CF.MT	..C.....
D.melanog_1	..VL....I	..S..EQED	..I..Y..M.	..DAYKHH..	..I..TFF..	..T..CF..SS....PGT..
D.melanog_2	..V..L....I	..F.S..QSSN	..NV....E.	..DTQN.HTDS	..I....V...	..T..TF..V	..C.....HH..
A.aegypti_A	KEKKRSHRKV	TKLVLTIVTV	YVLCWLPYWI	SOVALINSPP	DICKSRLEI	TVFVLVSLCG	YSNSAMNPIL	YAFSLDNFKK
A.aegypti_B	RG.R.....I.....T..D	GS.ST..D.	IL.L..G...	..I...I...	..Y..E...
A.gambiae_A
A.gambiae_B	RG.R.....I.....T.DF	ET.ST..DL	IL.L..G...	..I...I...	..Y..E...
C.pipiens_AV.....
C.pipiens_B	RG.R.....I.....I..I...F	GT.S...D.	IL.L..G...	..I...I...	..Y..E...
D.melanog_1	..R.A....	..R.....S.	..I.....H..H.N.	AQRDL....	LI.L.LGA.VV....E..R.
D.melanog_2SA	..IF....H..S.A.	QR.A....L	A..LACG..S
A.aegypti_A	SFLKACTCTK	GKDINAQLQI	ENSFFPRFAR	NRGSEGRNST	KILHPGRQKV	GDQTHPNDTA	NNNTNNANA	NGSSRSCSGP
A.aegypti_BAA	RAEV...KL	..VM.KR	SRT.T..D	TTQLTT S..	QHRLLIDP.T	TATT.TT.AS	TVS.RNP.P.
A.gambiae_AA.	..E.....V.V.QSN
A.gambiae_BAA	RAEV...KL	..VM.KR	SRT.T..D	TTQLTTGS..	QHRLLVEP.T	TATT.TT.AS	CVS.RNP.P.
C.pipiens_AG.	A..N...PV	.G.N..N.P	AS..M.T.N
C.pipiens_BAA	RAEV...KL	..VM.KR	SRT.T..D	TTQLTT S..	QHRLLIDP.T	TATT.TT.AS	TVS.RNP.P.
D.melanog_1	..F..F..MN	KQ.....L	..P.V.TKQ	..KKRGGS	..R.LTSN	P..IP.LLPL	..AG.N.SSTT	TSSTTTAEKT
D.melanog_2	..M.....AA	R..V....LK.	..KG.Q S	ER.LG.NG.G	A..RGALTKK	KCLA.R.N..	PMATTTTTTT
A.aegypti_A	GGPISNTNTN	GNTTTTTMTT	TTMGLGGGSS	SSNVPIASK	ILDTS	SRPPVLHTDL		
A.aegypti_B	VVQ	RSLMNNN	HHGS.	GN.GKNCNND	RTADSANR.A	PNASNG.NS.	GELVQLS	
A.gambiae_A								
A.gambiae_B	PAQ	..QRNNN	H.LT VP	GTPACASSNG	GSNHSNNND	TNTTG		
C.pipiens_A	V..	..T ..T.	T...M....	...	A.....		
C.pipiens_B	VI.	RNPQNNNN	NNNHVN..T	GN.AKNCNNE	TQAAPAAGVT	KNNLN	N.	GELVQLS
D.melanog_1		..G.QKSC	NSN.KVTAPP	ENLIICLSEQ	QEAFCTTARR	GSGA.QQ...		
D.melanog_2	T	..G.DAV.	CLQPPVHQVP	AEIQ.GNPAT	VLVNAETNN	CK.....		

Fig. 1. Comparison of AS-C receptor sequences: multiple alignments of the deduced amino acid sequence for some of the AS-C receptors identified/predicted in Diptera. The seven transmembrane domains (TMs) are indicated by boxes. The conserved motif "DRY" is indicated by a box with dashed lines. Predicted N-glycosylation sites are underlined.

and were therefore used in subsequent experiments. We measured the responses of the transfected cells to two different *A. aegypti* peptides, AS-C and allatotropin (AT). Robust signals were detected when the cells expressing the receptors were exposed to AS-C, but no signal was detected when AT or a buffer control with no peptides were used (results not shown).

In accordance with the functional expression both AS-C receptors were localized at the cell surface in the transfected cells. This was assessed by expressing the AS-C receptors-YFP (Yellow Fluorescent Protein) fusion proteins (Fig. 4A) and

simultaneously marking the plasma membrane glycoproteins with biotin-conjugated concanavalin A and streptavidin-conjugated Alexa Fluor 633 (Fig. 4B). AS-C receptors located at the cell surface appear in yellow in the overlay (Fig. 4C).

3.3. Tissue distribution and developmental expression of AS-C receptor mRNA

Real time PCR was used to analyze the transcript tissue specificity of the two AeAS-C receptors in female and male

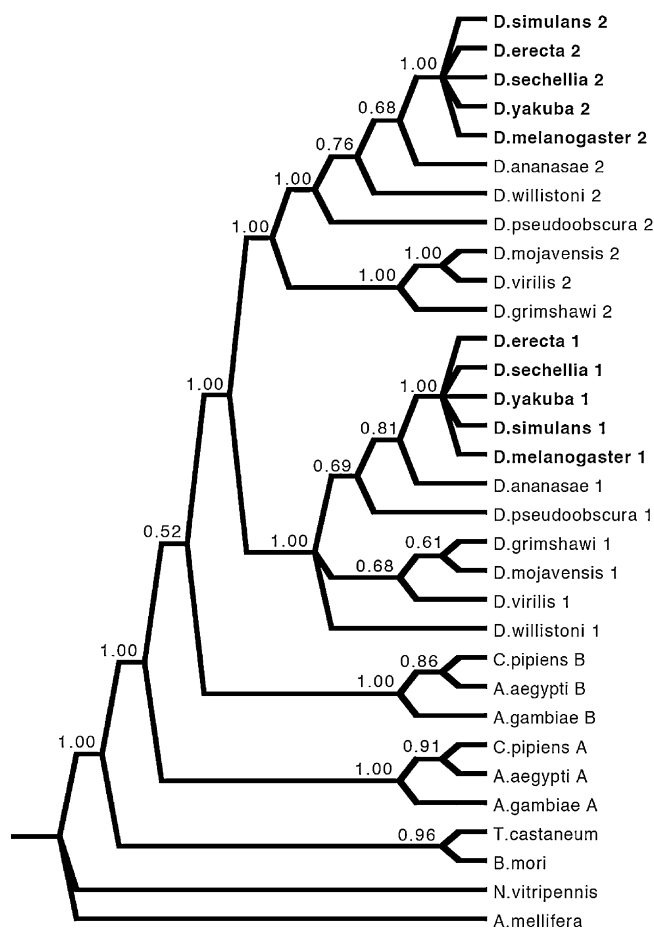


Fig. 2. Phylogenetic analysis of the AS-C receptor of different species of insects based on the primary amino acid sequences. Receptors in Diptera containing introns are in bold.

mosquitoes. In females, AeAS-CrA mRNA expression was higher in the abdominal ganglia, thoracic ganglia, brain and corpora allata (Fig. 5). Lower levels of mRNA transcripts were detected in the midgut, fat body and ovaries. It was also possible to detect low amounts of transcripts in the heart and Malpighian tubules (Fig. 5). High levels were also found in accessory glands and testes of males. Expression of AeAS-CrB mRNA was different from that of AeAS-CrA; very high levels were detected in abdominal ganglia, Malpighian tubules, the brain and thoracic ganglia; lower levels

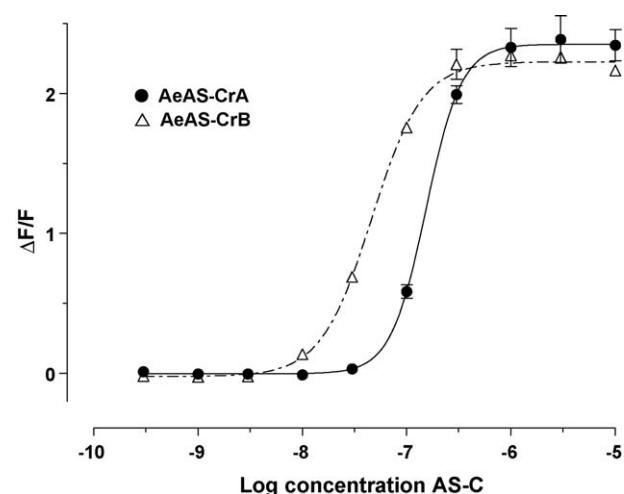


Fig. 3. Functional characterization of the AS-C receptors by FLIPR experiments. Dose-response curves of the effects of AS-C on the intracellular calcium concentration in cells expressing AeAS-CrA (filled circles) and on the AeAS-CrB (open triangles). Vertical scale: $\Delta F/F$ ratio; horizontal scale: logarithmic concentration of AS-C.

were measured in the heart (Fig. 5). In addition, the levels of the AS-C receptor transcripts were studied in the corpora allata during adult development. While the AeAS-CrB mRNA levels in the CA of sugar-fed females were constant from 0 to 72 h after emergence, the AeAS-CrA levels significantly increased at 72 h (Fig. 6A). AS-C receptor transcripts were also evaluated in heads of sugar-fed females during the first 4 days after adult eclosion. Levels for both receptors did not show significant changes (Fig. 6B). Analysis of the changes of receptor transcripts in abdominal and thoracic ganglia showed significant decreases of AeAS-CrA levels on day 4 (Fig. 6C and D).

3.4. Tissue distribution and developmental expression of allatostatin-C mRNA in the head

To further understand the pleiotropic roles of AS-C, we analyzed the tissue specificity for *A. aegypti* AS-C mRNA in females. The highest expression was in abdominal ganglia and the brain; lower levels of mRNA transcripts were also detected in corpora allata and thoracic ganglia (Fig. 7). We also analyzed the transcript levels of AS-C in the heads of sugar-fed adult female mosquitoes from eclosion to 7 days after emergence (Fig. 8A) and

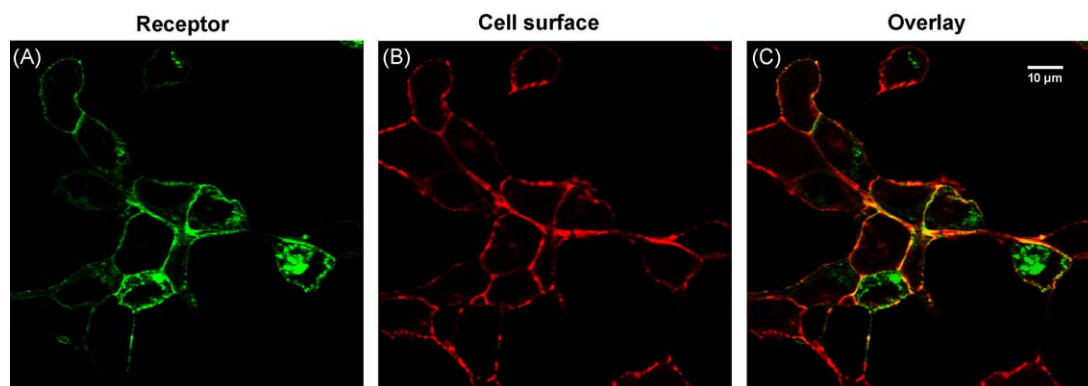


Fig. 4. Membrane localization of AeAS-CrA in HEK293T/Gα16gust44 cells: fluorescence images of HEK293T/Gα16gust44 cells transfected with AeAS-CrA-pEYFP-N1 DNA. The cell surface location of the AS-C receptor in transfected cells was recognized by expressing them fused to YFP (Yellow Fluorescent Protein) (panel A) and marking the plasma membrane glycoproteins with biotin-conjugated concanavalin A and streptavidin-conjugated Alexa Fluor 633 (panel B). AS-C receptors that are located at the cell surface appear in yellow in the overlay (panel C). Scale bars, 10 μm. Similar results were obtained in two independent transfection experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article)

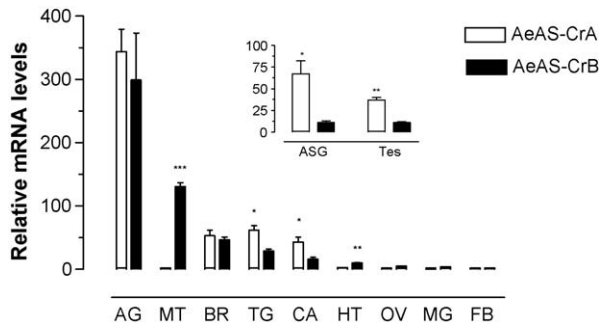


Fig. 5. Tissue specific expression of AS-C receptors mRNA. All tissues were dissected from 3-day-old sugar-fed females, except for testis (TE) and accessory glands (ASG) dissected from 3-day-old sugar-fed males. AG, abdominal ganglia; MT, Malpighian tubules; BR, brain; TG, thoracic ganglia; CA, corpora allata; HT, heart; OV, ovaries; MG, midgut; and FB, fat body. The inset shows the males tissues analyzed. AeAS-CrA (open bars) and AeAS-CrB (filled bars). Receptor mRNA is expressed as copy number of receptor mRNA/10,000 copies of rpl32 mRNA. Each RT-PCR data point is average of three independent biological replicates of 3–20 tissue samples. Asterisk above each bar indicates the values of AeAS-CrA or AeAS-CrB for each tissue are significantly different by unpaired *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$).

in heads of blood-fed females (Fig. 8B). In sugar-fed females transcripts were elevated at emergence and significantly increased during the first day and remained elevated during the first week. Blood-feeding did not result in significant changes in AS-C transcript levels (Fig. 8B).

4. Discussion

Each of the three families of insect allatostatins is associated with a unique GPCR family that includes vertebrate orthologs. The AS-A receptors are related to the vertebrate galanin receptors, the AS-B receptors to the bombesin receptors, and the AS-C receptors show similarity to the somatostatin/opioid receptors [5,19,34].

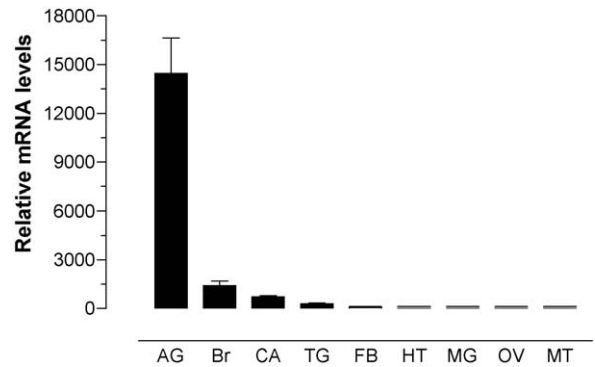


Fig. 7. Tissue specific expression of AS-C mRNA. All tissues were dissected from 3-day-old sugar-fed females. AG, abdominal ganglia; BR, brain; CA, corpora allata; TG, thoracic ganglia; FB, fat body; HT, heart; MG, midgut; OV, ovaries; and MT, Malpighian tubules. AS-C mRNA is expressed as copy number of AS-C mRNA/10,000 copies of rpl32 mRNA. Each RT-PCR data point is average of three independent biological replicates of 3–20 tissue samples.

Although human somatostatin receptors are significantly shorter (usually less than 400 amino acids), they share a high degree of sequence similarity with the AS-C receptors in insects, especially in the transmembrane regions TM1, TM2, TM3, and the intracellular loops where the receptors couple their heterotrimeric G-proteins. A highly conserved sequence in the TM7 (YANSCANPI/VLY), which is considered a mammalian somatostatin receptor signature [31], is still recognisable in the AS-C receptors (Fig. 1).

4.1. Phylogenetic relationship and evolution of allatostatin-C receptors

Neuropeptide GPCRs have been studied in the genomes of several insect species. Forty-four neuropeptide GPCRs have been described in *D. melanogaster*, with about two-thirds having

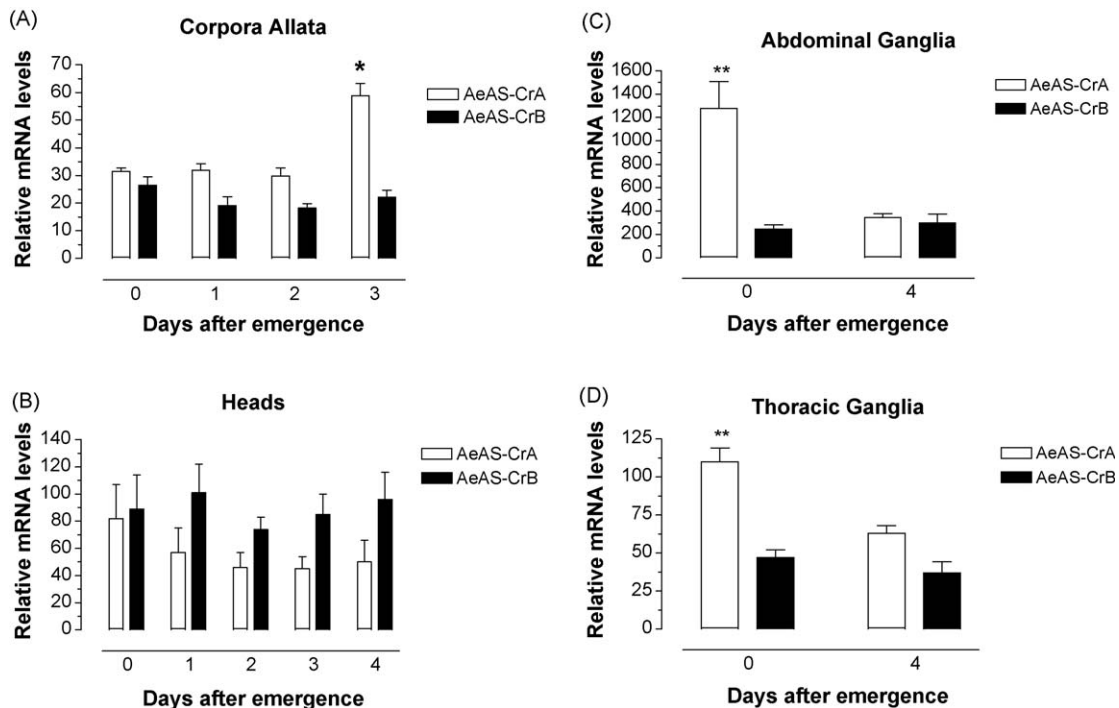


Fig. 6. Developmental expression of AS-C receptors mRNA. (A) Expression of AS-C receptors mRNA in corpora allata of sugar-fed females. (B) Expression of AS-C receptors mRNA in heads of sugar-fed females. (C) Expression of AS-C receptors mRNA in abdominal ganglia of sugar-fed females. (D) Expression of AS-C receptors mRNA in thoracic ganglia of sugar-fed females. AeAS-CrA (open bars) and AeAS-CrB (filled bars). Receptor mRNA is expressed as copy number of receptor mRNA/10,000 copies of rpl32 mRNA. Each RT-PCR data point is average of three independent biological replicates of 3–20 tissue samples. Asterisk above each bar indicates the values of AeAS-CrA or AeAS-CrB for each time is significantly different by unpaired *t*-test (* $P \leq 0.05$, ** $P \leq 0.01$).

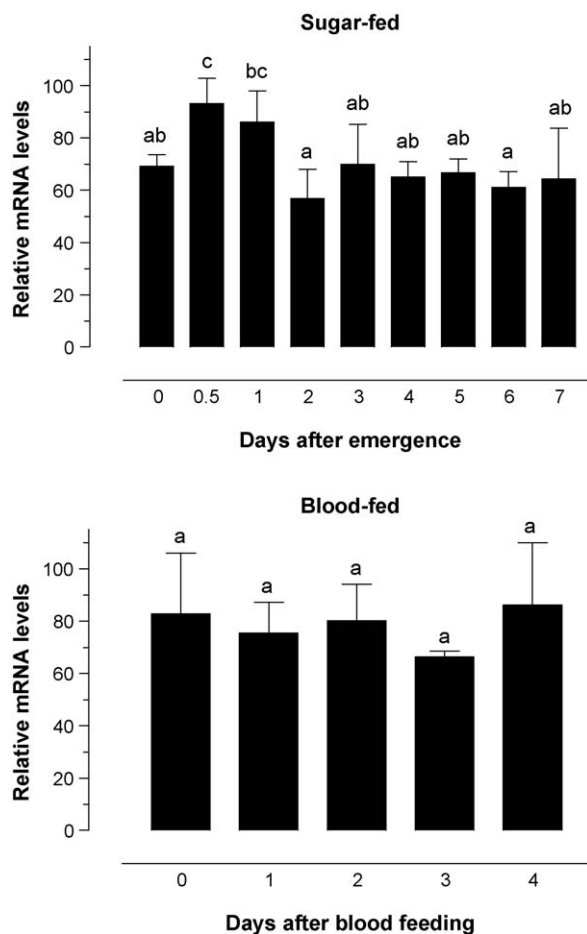


Fig. 8. Developmental expression of AS-C mRNA in heads of females. (A) Expression of AS-C mRNA in heads of sugar-fed females. (B) Expression of AS-C mRNA in heads of blood-fed females. AS-C mRNA were calculated as copy number of AS-C mRNA/10,000 copies of rpl32 mRNA and expressed as percentage of the maximum value. Each RT-PCR data point is average of three independent biological replicates of three heads. Values labeled with different letters denote significant differences (ANOVA $P \leq 0.05$).

identified ligands [12]. In *A. mellifera*, 35 neurohormone GPCRs were described, with 23 genes having orthologs with identified ligands in *D. melanogaster* [13]. In *T. castaneum* 48 neuropeptide GPCRs have been described, 29 of them having orthologs with known ligands in other insects [14]. Recently, a *B. mori* neuropeptide GPCR study revealed over 40 neuropeptide GPCRs, with many having orthologs with known ligands in *D. melanogaster* [36]. In mosquitoes, neuropeptide GPCRs have been identified in the genomes of *A. gambiae* [16] and *A. aegypti* [27]; the total number of neuropeptide receptors as well as the proportion with known ligands are very similar to those described for *D. melanogaster*.

Based on the sequences of *D. melanogaster* AS-C receptors, 32 AS-C receptor ortholog sequences were identified from 19 species of 5 different insect orders. The number of AS-C receptors varies in different insect species. Only one AS-C receptor was found in any species of the orders Coleoptera, Hymenoptera and Lepidoptera. All of the dipteran species analyzed in this study have two paralog AS-C receptors. It seems that gene duplication events have occurred at least two times independently in Diptera, once in the Culicidae (*A. aegypti*, *C. pipiens* and *A. gambiae*) and once in the Drosophilidae. One *Drosophila* subgroup includes *D. ananassae*, *D. pseudoobscura*, *D. willinstoni*, *D. mojavensis*, *D. virilis* and *D. grimshawi*; AS-C receptor genes in this group do not have introns in their sequences. The second subgroup in Drosophilidae includes *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba* and *D. erecta*

with two introns, each of them in exactly the same position among these five species in both paralogs. An analysis of the two paralog receptor intron sequences in the melanogaster group showed that introns 1 and 2 in the DROSTAR 1 group are different from introns 1 and 2 in the DROSTAR 2 group (results not shown). This suggests that the acquisition of these two introns in each paralog group occurred as independent events. Furthermore, each of these introns (1 and 2) were acquired independently at least two times for each paralog in the melanogaster group (a total of 4 independent events), once for each intron in the ancestor of *D. melanogaster*, *D. simulans* and *D. sechellia* and once in the ancestor of *D. yakuba* and *D. erecta*. This analysis is consistent with our ancestral state reconstruction in which the presences of the introns in the two DROSTAR groups were acquired independently. Also, each paralog of each species is more related with the orthologs of other species in the same group than with its own paralog (in the other DROSTAR group). The physiological reason for duplication of this gene in Diptera is still unclear. However, the possibility that these receptors took on new functions in Diptera coupled with the need for an independent regulation mechanism is one hypothesis. The differential tissue distribution of both receptors (discussed below) supports this.

4.2. Allatostatin-C receptor expression and pleiotropic effects of AS-C

Allatostatin-C is a pleiotropic neuropeptide that acts quickly and reversibly [10]. It has been proposed that during insect evolution AS-C functioned originally as myo- and/or neuroregulators and later was co-opted as a modulator of hormone synthesis [34]. There is a widespread distribution of allatoregulatory peptides in lepidopterans, which supports their pleiotropic functionalities. However, there is not always a strong correlation between localization and physiological requirements for these peptides, nor has a function always been assigned for a peptide in a particular tissue. Furthermore, the actions of these allatoregulatory peptides have been determined using *in vitro* bioassays whereas virtually nothing is known of their roles in the whole animal [3]. Comprehensive tissue expression studies of AS-C receptors should help to understand the physiological roles of the peptides and their target tissues. For AS-C this type of analysis has been limited to adults and larvae of *D. melanogaster* [18] and larvae of *B. mori* [36]. *In situ* hybridization experiments revealed expression of both *D. melanogaster* AS-C receptor genes within the optic lobes of adult flies, an area devoted to the processing of visual information. In *D. melanogaster* larvae, transcripts were detected only in a few cells of the nervous system. In contrast, immunolabeled peptide was found in the brain and CA of the larvae, presumably on axon terminals arising from the pars intercerebralis of the brain [18]. Recently, a quantitative tissue expression analysis of *B. mori* GPCRs was performed on two larval stages. The AS-C receptor was highly expressed in the corpora cardiaca–corpora allata complex and the fat body, with less expression in the epidermis and muscle tissue [36].

Here we report for the first time a detailed quantitative study of the expression of two AS-C paralog receptor transcripts in an adult insect. The tissue distribution of the two AS-C receptors differs in *A. aegypti*; the mRNA levels of AeAS-CrB in the Malpighian tubules were the highest detected, transcripts for AeAS-CrA were generally lower in this tissue. In addition, the transcript levels of both receptors were different in the thoracic and abdominal ganglia, CA and the testes of the male. If some of the pleiotropic effects in mosquitoes are mediated by the different receptor paralogs, we could expect to observe differential tissue expression of the two receptors implying that they are independently regulated. Expression patterns of both receptors differ in the CA. AeAS-CrB is expressed constantly at the same relatively low level with no

significant changes during the first 3 days of the adult life. Meanwhile the AeAS-CrA has lower expression during the first 2 days and had a twofold increase at the third day. This increase is statistically significant if means are compared ($P \leq 0.05$); but not statistically significant if the medians are analyzed (data not shown). The low level of expression and relatively high variability that we observed account for this disparity. This variability might be the result of the effect of the nutritional status of the mosquito and any additional environmental parameters on the activity of the CA [7]. In an effort to compensate for these low levels and variability, assays were performed with groups of several glands. It is extremely difficult to dissect CA of insects that are in exactly the same physiological condition. If we nevertheless assume that the increase we observed in the CA is physiologically relevant, then the increase in receptor levels match well temporally with the higher sensitivity of the CA to the peptide on the third day after emergence [23]. These results are also in agreement with the changes in JH synthesis in the CA as previously described [20]. The highest rates of JH synthesis were during the first 24 h after adult eclosion, after that JH synthesis is inhibited by factors present in the mosquito head [22]. These results suggest that only one of the receptors expressed in the CA may have an active role in the modulation of the JH synthesis. There are a limited number of studies reporting changes in the levels of CA allatostatin receptors and their relationship with JH synthesis. In *D. punctata* elevated expression of the AS-A receptor in the CA was found to correlate well with the decline of JH biosynthesis; in addition the decrease of receptor transcripts matched the loss of CA sensitivity to AS-A [25]. Further experiments are needed to clarify the relevance of the differential pattern of expression of AS-C receptors in the CA as well as the various functions in the other tissues.

Our FLIPR results indicate that both receptors responded in the low nanomolar concentration range comparable to the values already reported for *D. melanogaster* [18] and those recently published for *B. mori* [36]. These values match the biological concentration of AS-C measured in the head of mosquitoes, as well as the levels that activated the CA *in vitro* [23]. Although the EC₅₀ of both receptors are in the nanomolar range, there was a 3.2-fold difference between both paralogs; these dissimilar affinities for the ligand might be physiologically relevant, especially if the peptide is delivered by different means (axonal or humoral) in different tissues.

4.3. AS-C tissue distribution and role in JH synthesis

Prior research has shown that rates of JH biosynthesis closely reflect the levels of JH in mosquito implying that there is a quick and reversible modulation of synthesis in the CA. We previously described that by day 3 after eclosion factors from the brain cause the decrease of JH synthesis [22]. AS-C is one of these factors [23]. We also reported AS-C immunoreactivity in a few cells in the brain [15] and ELISA studies revealed an eightfold increase in head AS-C peptide levels on the third day of adult life when compared with levels at adult eclosion [23]. In contrast, we observed that the levels of the AS-C transcript measured by quantitative RT-PCR did not show major differences from eclosion to day 7. Similarly, after a blood meal, the levels of JH synthesis are among the lowest measured in the adult mosquito [20], but AS-C mRNA levels are relatively constant.

Conclusions about correlations of brain AS-A transcript levels and JH synthesis in different species of cockroaches are still unclear. In *D. punctata* there is an inverse relationship between JH production and AS-A mRNA expression [11]. In the brain of *Blattella germanica*, semi-quantitative studies of AS-A mRNA levels did not show a clear inverse relationship with JH synthesis [2]. Semi-quantitative studies of the AS-C levels in the brain of the moth

Spodoptera frugiperda indicated that peptide levels fluctuated in adult females during the reproductive cycle inversely with rates of JH synthesis [1].

In summary, transcription of the AS-C gene in the brain of mosquitoes might not have a key regulatory role; AS-C is likely stored in cells in the brain and it is released as needed. The release of AS-A within the CA of *D. punctata* has been compared using glands with intact nerves from the brain and those detached from the brain [24]; lower rates of JH synthesis by glands with intact nerves to the brain were most likely due to the release of small amounts of allatostatin within the CA. Similarly, in the termite *Reticulitermes flavipes*, the density of AS-A immunostained axons within the CA and the rate of JH synthesis by similar glands were negatively correlated; evidence that when AS-A was abundant in the glands, it was released *in vivo* to limit JH production [9].

5. Final conclusion

In the present work we describe the functional and molecular characterization of two *A. aegypti* allatostatin-C receptor paralogs (AeAS-CrA and AeAS-CrB) and for the first time we provide a detailed quantitative study of the expression of AS-C receptor transcripts in an adult insect. A phylogenetic analysis of AS-C receptor sequences from 19 species of 5 different insect orders indicated the duplication of this gene has only occurred in the order Diptera. The presence of two receptor paralogs in mosquitoes responding to AS-C stimulation in the nanomolar concentration range, but showing different tissue distribution suggest that they could mediate distinct pleiotropic effects. Transcriptional regulation of the AS-C receptors might not have a critical role in the changes of CA responsiveness to the peptide we previously described. Differences in CA sensitivity to AS-C might be mediated by post-transcriptional receptor modifications, but further studies are necessary to confirm this hypothesis.

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