Molecular cloning and characterization of the allatostatin receptor in the cockroach *Diploptera punctata*

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Abstract

Allatostatins (ASTs) are neuropeptides that inhibit the biosynthesis and release of juvenile hormone from the corpora allata (CA) of various insects including the cockroach *Diploptera punctata*. We hypothesized that a similar allatostatin receptor would exist in the cockroach *D. punctata* that may regulate the numerous physiological effects that this family of peptides exerts on a range of target tissues. Using polymerase chain reaction (PCR) strategies successful in isolating other insect allatostatin receptor sequences utilized primers designed to known mammalian somatostatin receptors and *Drosophila* allatostatin receptor (AlstR). Once an internal PCR fragment was proven to be consistent with the sequence of an allatostatin receptor (AstR) then the sequence was completed by rapid amplification of cDNA ends (RACE). The putative allatostatin-like receptor sequence encoding 425 amino acid residues was isolated from a cDNA library prepared from corpora allata of *D. punctata*.

We show that dsRNA targeting the allatostatin receptor gene of *D. punctata* injected into freshly moulted adult cockroaches produced a long-lasting reduction in the mRNA levels in midgut tissues. The effect lasted up to 6 days. Following dsRNA injection, the juvenile hormone (JH) titers in the corpora allata were clearly raised suggest that the putative inhibition of receptor RNA expression may increase JH production.

The receptor is expressed in brains, corpora allata, abdominal ganglion, midguts, ovaries, and testes. We have examined these same tissues with regard to changes in expression

levels of Dippu-AstR. JH biosynthesis peaks on day 5 post-emergence in mated females. In mated females, Dippu-AstR mRNA is expressed at the highest levels on day 6 post-emergence in brain and corpora allata and day 2 post-emergence in midgut. Dippu-AstR is likely responsible for the decline in JH biosynthesis after day 5 post-emergence. Virgin females midgut and CA Dippu-AstR mRNA expression dramatically elevated on days 6 and 7, respectively. Expression of Dippu-AstR was similar in the abdominal ganglia of mated or virgin females. Ovarian Dippu-AstR expression declines to low levels by day 4. Testes also express peaks of Dippu-AstR expression on days 4 and 7. A role for Dippu-AST in testes is yet unknown.

Co-authorship

The work described in the thesis could not have been accomplished without the collaboration of many talented researchers which I would like to acknowledge with co-authorship.

Chapter 2

Dr. Cam Donly	Completed the Dippu-AstR sequence using 5' rapid amplification of cDNA end
Jin Rui Zhang	Injected dsRNA into animals, carried out JH release assay, and oocyte measurement assay

Chapter 3

Jin Rui Zhang	provided the cockroach tissues (abdominal ganglion, corpora allata, midguts, brains, ovaries)

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List of Abbreviations

AMP	Ampicillin
AST	Allatostatin
AstR	Allatostatin receptor
AT	Allatotropin
bp	Base pair
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cDNA	Complementary DNA
CNS	Central nervous system
CA	Corpora allata
CC	Corpora cardiaca
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
Dippu-AST	*Diploptera punctata* allatostatin

Dippu-AstR	*Diploptera punctata* allatostatin receptor
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetracetic acid
GPCR	G protein-coupled receptor
IP_3	1,4,5-inositol triphosphate
JH	Juvenile hormone
JHB3	Juvenile hormone bisepoxide
LB	Luria-Bertani media
Mas-AST	*Manduca sexta* allatostatin
NBT	Nitro Blue Tetrazolium
NCC	Nervi corporis cardiaci
PAM	Peptidyl-glycine-α-mono-oxygenase
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
qPCR	Quantitative real-time PCR
RACE	Rapid amplification of cDNA ends
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNase	Ribonuclease

RNAi	RNA inhibition
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
Sec	Second
siRNA	Short interfering RNA
UV	Ultra violet

CHAPTER 1:

Introduction and literature reviews

1.1 Model organism: cockroach, *Diploptera punctata*

Diploptera punctata is the viviparous cockroach, in which the embryos make use of nutrient supplied by the mother during embryogenesis (Fig. 1.1). The brood sac of *D. punctata* is an insect integumentary gland which secretes milk containing protein and carbohydrate to feed the developing embryos. In *D. punctata*, mating occurs a few minutes after emergence (Roth and Willis 1951). Mating plays an important role in egg maturation. In virgins, the first batch of eggs takes several weeks or months to mature, whereas after mating only ten days are required for egg maturation (Roth and Willis 1951).

D. punctata has been widely used as a model species for the study of JH biosynthesis and its regulation because of its life history traits. For example, viviparity ensures well-timed cycles of JH biosynthesis in the adult female and high levels of hormone production (Stay 2000) make it very useful to the study of insect endocrinology. We have been studying the factors that inhibit the biosynthesis and release of juvenile hormone, allatostatins (ASTs), and focusing on the evolution and method of action of the allatostatins and their receptors.

1.2 Insect neuropeptides

Neuropeptides are a diverse class of molecules that regulate the physiology, development and behavior of an organism. Insect neuropeptides are produced in less than 1% of the cells of the central nervous system. They are important messenger molecules which influence almost all physiological processes. They can act as transmitters, modulators, and as classical hormones, and often exhibit pleiotropic functions when released into the haemolymph. Neuropeptides in insects usually occur in multiple forms and belong to distinct neuropeptide families. Mature peptides are made by processing a prohormone and several peptides may be made from a single prohormone. For example, the sequences of 14 ASTs were identified in the allatostatin prohormone of *P. americana* (Ding *et al.* 1995). At least 30 genes encode neuropeptides precursors in *Drosophila* (Adams *et al*. 2000; Hetru *et al.* 1991; Hewes and Taghert 2001; Rubin *et al*. 2000; Vanden Broeck 2001a). A number of unique sequences suggest that similar peptides may be functionally diverse.

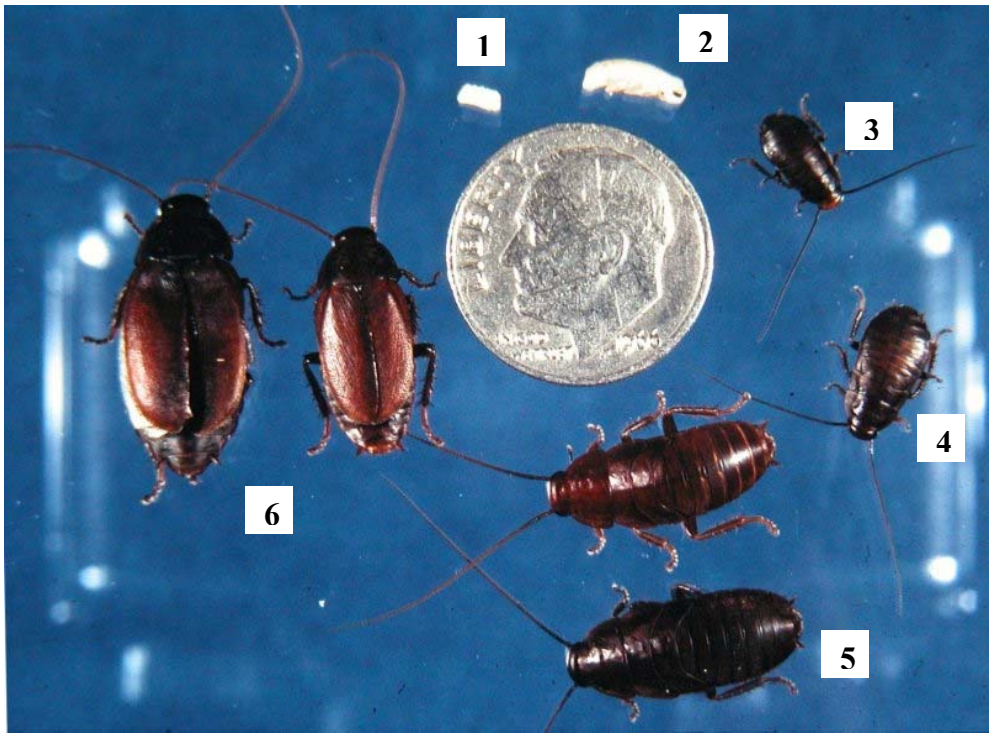

Fig. 1.1 Various developmental stages of the Cockroach *Diploptera punctata.* 1 = egg batch, 2 = first instar, 3 = second instar, 4 = third instar, 5 = fourth instar (top = male, bottom = female), 6 = adult (right = male, left = female). (figure is from Stephen Tobe Lab website: http://www.zoo.utoronto.ca/tobeweb/animals.shtml)

Major neurohaemal organs in insects are summarized in Fig. 1.2. The retrocerebral complex consists of two neurohaemal release sites, the corpora cardiaca (CC) and corpora allata (CA), having different cellular origins and different functions. Insects have an open circulatory system with a single aorta which usually transports haemolymph from the abdomen into the head. The retrocerebral complex is partially fused with the ventral wall of the aorta, thus enabling the rapid transport of released hormones into the hemolymph. The CC consists of an intrinsic neuroglandular part, synthesizing metabolic peptides (Gade *et al*. 1997a), and a neurohaemal part which stores and releases neuropeptides of the brain and suboesophageal ganglion into the dorsal aorta. Neurohaemal release sites are distributed throughout the insect body: along nerves, the aorta and at the ampullae of the antennal heart. In addition, the gut and reproductive organs such as oviduct, accessory glands, and even haemocytes (Skinner *et al*. 1997), are thought to release peptide hormones.

Neuropeptides are usually synthesized as large preproprotein precursors from which the active forms are cleaved and modified. The processing and modification steps yielding mature peptides are shown in Fig. 1.3. RNA is transcribed in the nucleus and is transported to the cytoplasm and translated into protein by a ribosome. Some mRNAs may undergo splicing following gene activation and transcription which lead to sequences derived from the gene. Processing starts at the rough endoplasmic reticulum (RER) during the translation. Peptides within prohormones are typically flanked by pairs of basic residues; Lys-Arg, Arg-Arg, Lys-Lys, or Arg-Lys, that serve as

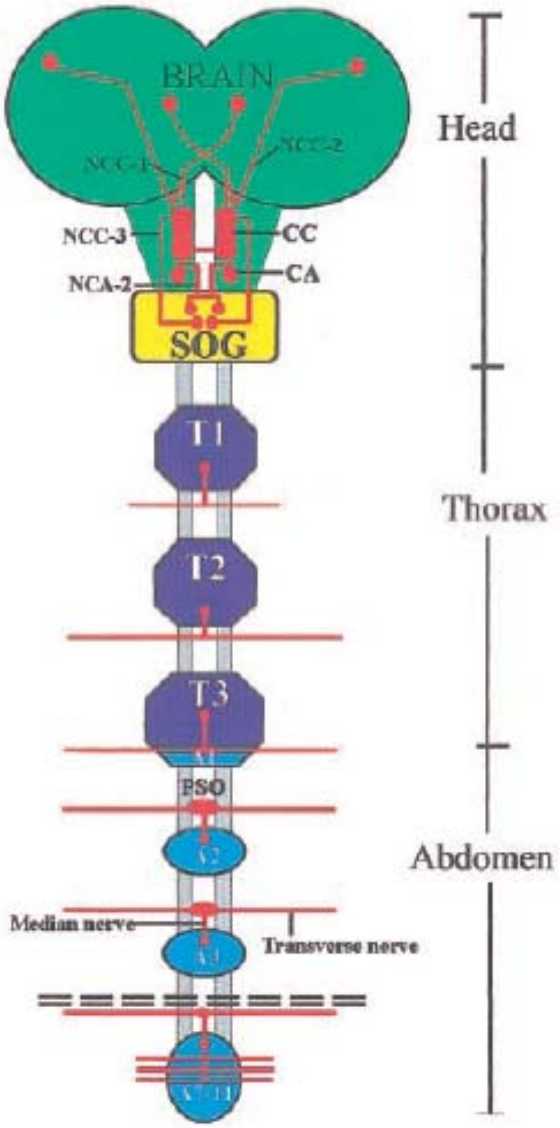

Fig. 1.2 Major neurohaemal release sites of the central nervous system of a cockroach. CC/CA Corpora cardiaca/allata; NCC/NCA nervus corporis cardiaci/corporis allati; SOG suboesophageal ganglion; T1, T2, T3 thoracic ganglia, A1-11 abdominal ganglia (figure is from Predel and Eckert, 2000)

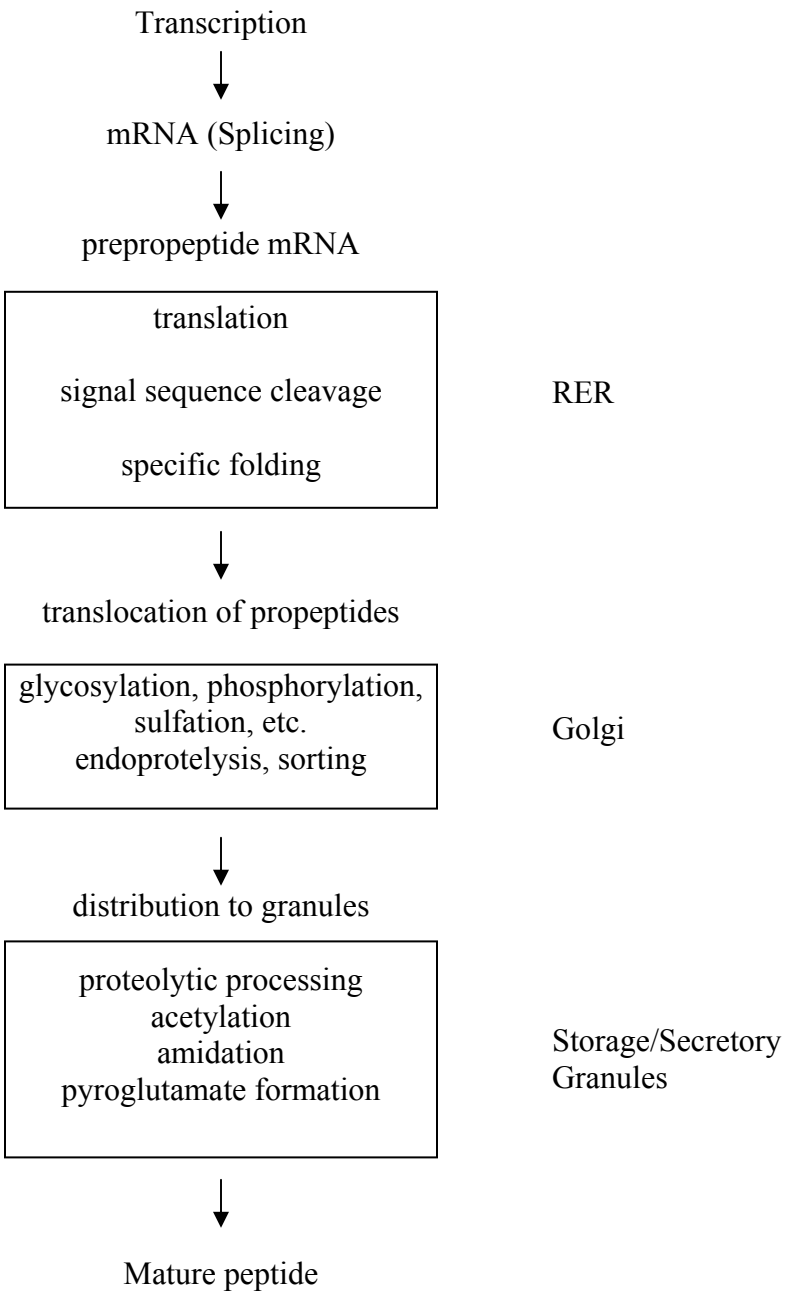

Fig. 1.3 Diagram showing post-translational events in neuropeptides maturation. RER: rough endoplasmic reticulum

recognition sites for specific endopeptidase cleavage (Fig 1.4.) (Docherty and Steiner 1982; Gainer *et al.* 1985). Trypsin-like endopeptidases then cleave at the carboxy side of basic amino acids, whereas carboxypeptidase-like enzymes will process from the carboxy end of the peptide. The propeptides may fold, with sulfide bond formation, and translocate to the golgi apparatus.

Intra-golgi sorting of propeptides occurs via a signal-based mechanism, leading to distribution into selected secretory/storage granules. Within the granules, propeptides undergo maturation in the form of endo- and exoproteolytic cleavages and trimming, and modifications of the C- and/or N- termini which may include acetylation, amidation or blocking through the formation of pyroglutamic acid residues. Post-translational processing regularly increases specificity and stability to the active peptide. Amidation is a common modification of neuropeptides, and is mediated by a specific enzyme known as peptidyl-glycine-α-mono-oxygenase (PAM) which removes a carboxy-terminal glycine, leaving the amide portion of the peptide bond. The amide group protects the neuropeptides from carboxy-terminal degradation. After peptide modification is completed, the matured peptides are stored and secreted from the cell by exocytosis in response to specific stimuli, and altimately contact their receptors on the target cell (Docherty and Steiner 1982).

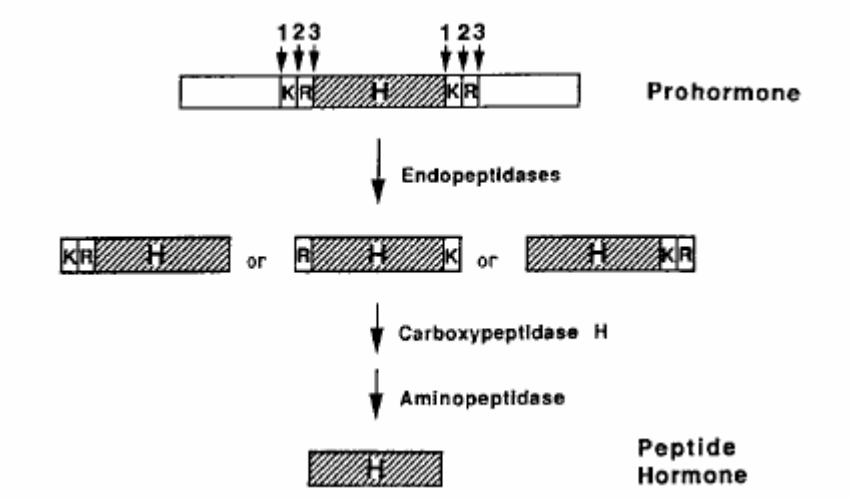

Fig. 1.4 A schematic illustration of proteolytic processing. Endoproteases cleave dibasic sites at positions 1, 2, or 3.

1.3 Allatostatins

Allatostatins (ASTs) are multifunctional peptides widely distributed in insects and other invertebrates. ASTs were first isolated by their action to inhibit JH biosynthesis by the CA *in vitro* in the cockroach *D. punctata* (Pratt *et al*. 1989, 1991; Woodhead *et al*. 1989, 1994). The actions of ASTs are not limited to modulation of JH biosynthesis. ASTs have been shown to have numerous functions including acting as inhibitors of muscle contraction in hindgut (Lange *et al.* 1995; Lange *et al.* 1993). Three types of peptides that effectively inhibit JH synthesis in different insect species have been isolated and characterized. These are the FGLamide, the $W(X)_6$ Wamide and the PISCF type.

1.3.1 FGLamide (A-Type) ASTs

FGLamide ASTs are characterized by a common C-terminal sequence Y/F-X-F-G-L/Iamide (where X = Ser, Gly, Asp, Ala, Asn), first isolated from brains of the cockroach *D. punctata* (Pratt *et al*. 1989; Pratt *et al.* 1991; Woodhead *et al.* 1989; Woodhead *et al*. 1994). The gene encoding AST peptide has been sequenced in *D. punctata* and five other species of cockroach (*P. americana, Supella longipalpa, Blaberus craniifer, Blattella germanica* and *Blatta orientalis*). Each precursor contains a family of 13 to 14 peptides that vary in their N-terminal sequences (Ding *et al*. 1995, Belles *et al*. 1999).

The AST preprohormone is characterized by a hydrophobic leader sequence and each putative peptide within the precursor is separated by an α-amidation signal (Gly) and cleavage sites (Lys-Arg) (Fig. 1.5) (Donly *et al.* 1993). After cleavage, the terminal glycine functions as the substrate for peptidylglycine α-amidating monooxygenase

(Eipper *et al.* 1992). AST molecules are C-terminally amidated and require amidation for activity (Pratt *et al.* 1991 b). The AST precursor peptides are grouped in the preprohormone into four regions (ASTs 1-4, 5-10, 11, 12-13) which are separated by three acidic spacer sequences. The function of the acidic spacers is unclear but they are thought to have some significance and may neutralize the prohormone as the number of acidic and basic amino acid residues are equal (Donly *et al.* 1993, Bendena *et al*. 1994).

This family of peptides occurs widely in other insect orders but has been shown to act as an AST only in cockroaches, crickets, and termites (Yagi *et al*. 2005). FGL-ASTs exhibit multiple inhibitory functions. Inhibition of visceral muscle contraction was demonstrated in the hindgut (Lange *et al.* 1993) and foregut (Duve *et al.* 1995) of cockroaches. Inhibition of the oviduct contraction was found in the locust *S. gregaria* (Veelaert *et al.* 1996). In the cockroach *B. germanica*, FGL-ASTs affected egg development by inhibiting vitellogenin production (Martin *et al.* 1996) and the release of vitellogenin from the periovarian fat body (Martin *et al.* 1998).

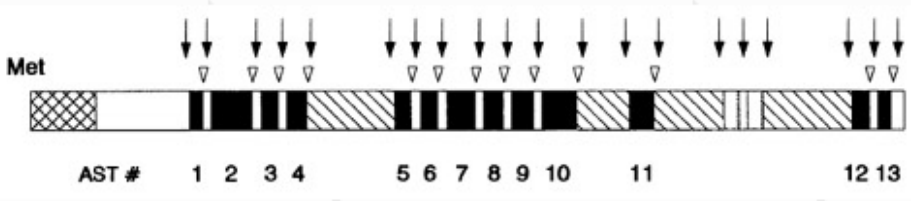

Table 1. *D. punctata* AST peptides deduced from cDNA

AST	Previous designation*	Primary structure
1		L Y D F G L $-NH_2$
2	ASB2	A Y S Y V S E Y K R L P V Y N F G L $-NH_2$
3		S K M Y G F G L $-NH_2$
4	VII	D G R M Y S F G L $-NH_2$
5	IV	D R L Y S F G L $-NH_2$
6		A R P Y S F G L $-NH_2$
7	I	A P S G A Q R L Y G F G L $-NH_2$
8	III	G G S L Y S F G L $-NH_2$
9	II	G D G R L Y A F G L $-NH_2$
10		P V N S G R S S G S R F N F G L $-NH_2$
11	VI	Y P Q E H R F S F G L $-NH_2$
12		P F N F G L $-NH_2$
13		I P M Y D F G I $-NH_2$

Fig. 1.5 Top: Diagram of the allatostatin polypeptide precursor from *D. punctata*. Black rectangles represent individual allatostatins; arrows are sites for endoproteolytic cleavage; white triangles are potential amidation sites. Below: *D. punctata* allatostatin amino acid sequences deduced from cDNA (figures are from Donly *et al*. 1993).

1.3.2 W(X_6) Wamide (B-Type) ASTs

The B-Type ASTs are C-terminally amidated peptides with tryptophan in the second and ninth positions, first isolated in the cricket *Gryllus bimaculatus* using HPLC (Lorenz *et al.* 1995). A partial sequence of the B-type preproAST was shown to encode four different peptides with the conserved W_2W_9 structure (Wang *et al*. 2004). The amino acid sequences of these four peptides are consistent with that of the peptides previously isolated by Lorenz *et al.* (1995). B-Type ASTs are active as ASTs in crickets but are less effective than the A-type ASTs.

1.3.3 PICF (C-Type) ASTs

The third family of ASTs is a single 15 amino acid non-amidated peptide, first isolated from the brain of the lepidopteran *Manduca sexta* and was called AST-C (Kramer *et al*. 1991). Genes encoding AST-C have been isolated from *Pseudaletia unipuncta* (Jansons *et al*. 1996), *Drosophila melanogaster* (Williamson *et al.* 2001, Price *et al*. 2002), and *Spodoptera frugiperda* (Abdel-latief, Meyering-Vos and Hoffmann 2003). AST-C inhibited JH biosynthesis by CA of *M. sexta* as well as other lepidopterans but were not effective as an AST in other orders of insects (Kramer *et al*. 1991). AST-C strongly inhibits JH biosynthesis *in vitro* by CA from *M. sexta* larvae and adult females as well as adult females of *Helicoverpa zea* (Kramer *et al*. 1991). Another study found that AST-C had no inhibitory activity of JH biosynthesis in *P. unipuncta* sixth instar larvae or newly-emerged adults but inhibited CA of 5-day-old adult females by 60% (Jansons *et al*. 1996).

1.4 Expression of allatostatins

1.4.1 Expression of FGLamide ASTs

Distribution and expression of ASTs has been examined using antibodies against specific AST peptides, and specific nucleic acid probes to detect AST mRNA. Cells that express ASTs have been detected in various tissues and have provided clues to AST functions.

Immunohistochemical localization with an antibody raised against *D. punctata* AST 7 (Dippu-AST7) indicated that AST immunoreactivity was localized in nerve fibers in the brain/retrocerebral complex of the cockroaches *D. punctata* (Stay *et al*. 1992), *P. americana* (Schildberger and Agricola 1992), and in the crickets *G. bimaculatus* and *A. domesticus* ((Neuhäuser *et al*. 1994; Witek *et al.* 1999). These results suggest that the ASTs are produced in lateral neurosecretory cells of the brain and are delivered to the CA through the nervi corporis cardiaci (NCC) II. Strong immunoreactivity was detected in four large anterior medial cells of the pars intercerebralis, but no immunoreactivity was detected in the NCC I from the medial neurosecretory cells. This result is in accordance with *in situ* hybridization using an AST probe on brains from day 5 mated female *D. punctata*, which shows that mRNA for the AST gene is strongly expressed by four medial cells in the pars intercerebralis of the protocerebrum (Bendena *et al.* 1994). These data suggest that ASTs may have interneuronal functions or serve as neuromodulators. Immunoreactive cells were also detected in the suboesophageal and abdominal ganglia of cockroaches. FGL-AST immunoreactivity could also be detected in the nerves of the antennal pulsatile organ and in hindgut muscles of the cockroaches *D. punctata* and *P. americana* (Lange *et al.* 1993; Woodhead *et al.* 1992) as well as in endocrine cells of the

midgut of *D. punctata* (Reichwald *et al.* 1994; Yu *et al.* 1995a). In addition, FGL-AST or AST-like immunoreactivity was demonstrated in the brain/retrocerebral complex, nerves, ganglia, or endocrine cells of the midgut from locusts, blowflies, moths and an earwig species, and in many non-arthropods (Hoffmann *et al.* 1999). ASTs are abundant in both the central and stomatogastric nervous systems of crustaceans (Dircksen *et al.* 1999; Skiebe 1999). The FGLamide peptides are strongly expressed in the CNS of the prawn *M. rosenbergii* and lower levels of expression can be observed in the gut ((Yin *et al.* 2006).

1.4.2 Expression of AST preprohormone (Dippu-AST)

AST preprohormone contains the sequence of the multiple members of ASTs. Previous work by Garside *et al*. (2002), using the quantitative competitive reverse transcriptase polymerase chain reaction (QC-RT-PCR) technique, reported the expression pattern of *D. punctata* preproAST mRNA in the oviducts and ovaries of mated female *D. punctata*. The highest levels of Dippu-AST mRNA expression were observed in lateral oviducts follow by the common oviducts, and the lowest levels can be seen in the ovaries throughout the first 13 days of the gonotrophic cycle. The same quantitative technique was also applied in the brain of mated female, virgin female and male of adult *D. punctata*. In mated female brain, Dippu-AST mRNA levels changed significantly during the first gonadotropic cycle. The levels of Dippu-AST mRNA in the virgin female brain also changed dramatically during the 11 days of the gonadotropic cycle. However, Dippu-AST in the brain of mated females is expressed at higher levels than in the brains of virgin females. No significant changes occur in male brains (Garside *et al*. 2003).

1.5 Juvenile hormones (JHs)

JH is a sesquiterpenoid that occurs in six forms (JH 0, JH I, JH II, JH III, JHB3, and Methyl farnesoate) (Fig. 1.6) that differ with respect to epoxidation and side chain length (Riddiford 1994; Baker 1990). The growth, development, metamorphosis and reproduction of insects are under control of JHs and ecdysteroids, or molting hormones, secreted by specific endocrine glands called the corpora allata (CA) and prothoracic glands (the molting glands), respectively (Gilbert *et al.* 2000; Stay 2000).

JH III is the most common form of JH in insects. JH III is the predominantly active juvenoid *in vivo* as was shown in *D. punctata* (Tobe *et al.* 1985) and in *P. americana* (Pratt *et al*. 1975). On the other hand, the form JHB3 (JH III bisepoxide) appears to be the most important JH in the Diptera, or flies. Certain species of crustaceans have been

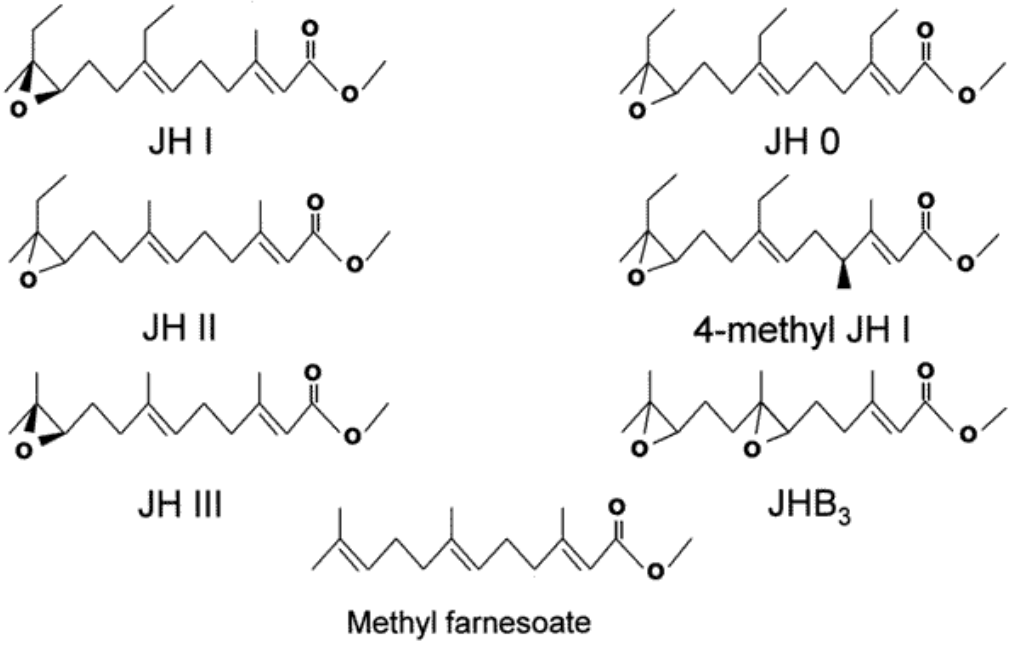

Fig. 1.6 The various forms of Juvenile hormone (JH). JH III is the major form of JH in cockroaches, whereas JHB3 is the apparent active form in Diptera. Methyl farnesoate is a precursor to JH III that appears to function as a hormone in crustacean (figure is from Kwok and Tobe, 2006).

shown to produce and secrete methyl farnesoate, JH III lacking the epoxide group, which is believed to play a role similar to that of JH in crustaceans.

JH biosynthesis by the CA is regulated by stimulatory modulators, allatotropins (ATs), and inhibitory modulators, ASTs, originating in the brain. The CA does not appear to store JH since little JH can be extracted and isolated from the glands (Tobe and Stay 1985). JH is released from the CA soon after its biosynthesis, as shown in *D. punctata* (Tobe *et al.* 1985), *Schistocerca gregaria* and *P. americana* (Feyereisen 1985). The release of JH from the CA into the hemolymph shows short term peaks with steep increases over a few hours.

The JH III biosynthetic pathway is considered to start with cytosolic acetyl-CoA as the substrate (Schooley and Baker 1985). The enzymes in the JH III biosynthetic pathway downstream from acetyl-CoA are cytoplasmic; therefore the mitochondrial acetyl-CoA generated from glucose and amino acid metabolism has to be transported to the cytosol in order to be utilized for JH III biosynthesis. It could be that the transfer of two-carbon units from the mitochondria to the cytoplasm is the target for AST action in the CA of *D. punctata* (Sutherland and Feyereisen 1996).

JH has two main functions. The absence of JH is required for metamorphosis of larvae to adult, but also in many insect species JH is an important regulator of reproductive cycles (Engelmann 1983). JH stimulates both the production of vitellogenin by the fat body and the uptake of vitellogenin by the ovaries (Engelmann 1983). In *D. punctata*, mating

releases inhibition of the CA and allows for a cycle of JH biosynthesis that is correlated with ovarian development (Stay and Tobe 1977). The lack of a cycle of JH biosynthesis in ovariectomized females indicates that the ovary is required to stimulate JH production (Stay and Tobe 1978). Vitellogenic ovaries stimulated JH biosynthesis within 24 hours, whereas pre-vitellogenic, late vitellogenic, and post-oviposition ovaries did not (Rankin and Stay 1984). These studies suggest that a factor released by the ovary at certain stages of development directly or indirectly stimulates JH biosynthesis by the CA.

Tobe (1980) and Johnson *et al.* (1985) described a relationship in the first gonadotrophic cycle by relating oocyte size to the rate of JH release. When female adults emerge and mate, basal oocytes are previtellogenic and JH biosynthesis is low. On days 2-3 the oocytes become vitellogenic and rates of JH biosynthesis begin to rise. Rates climb to a maximum on days 4-5 when oocytes are continuing to grow. On days 5 and 6, rates of JH biosynthesis decline rapidly, while the oocytes continue to grow and become chorionated. On day 7, oviposition occurs and the oocytes are retracted into the brood sac. JH biosynthesis is low (Fig. 1.7).

In mated females, a low JH titre is found in the first part of the first gonadotrophic cycle, with maximum titres on days 4-5 and then declining rapidly thereafter, to low titres after day 6. Virgin *D. punctata* do not mature oocytes at a rapid rate and do not show the cycle in JH biosynthesis described in mated females (Stay and Tobe, 1977). There are four larval stages in D. *punctata* and each of these exhibit distinct patterns of JH biosynthesis (Kikukawa and Tobe, 1986). First instars show low rates of JH biosynthesis for the first

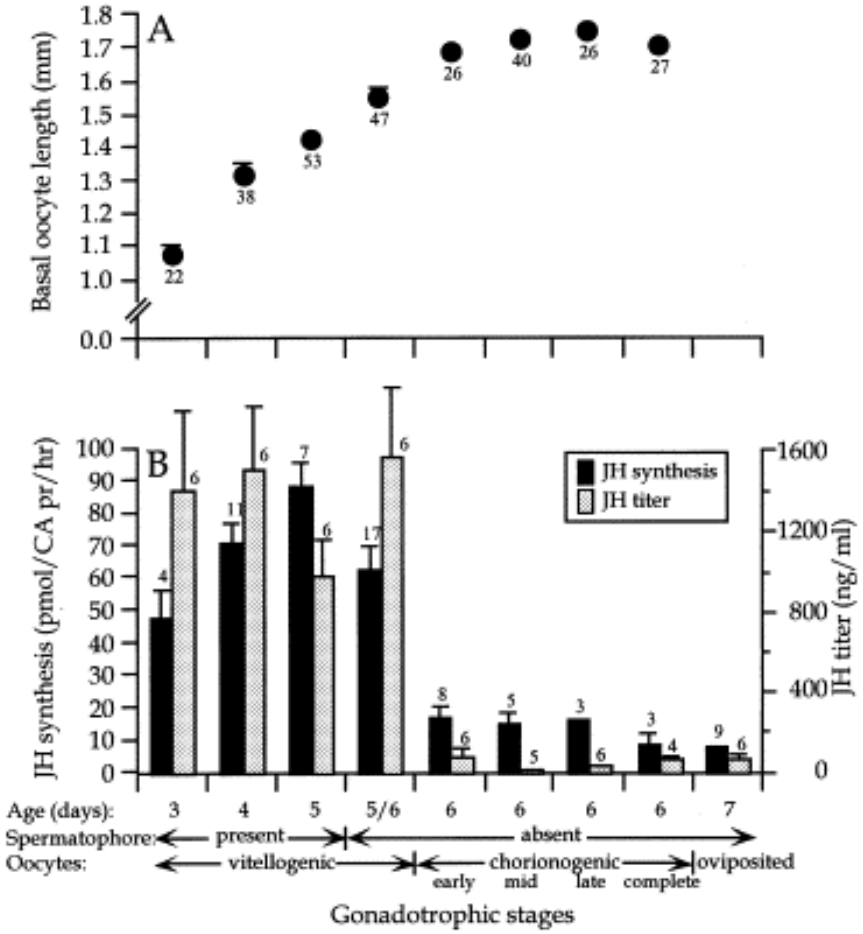

Fig. 1.7 Basal oocyte length (A), and JH synthesis by CA pairs in vitro and hemolymph JH titer (B) in mated females from day 3 to day 7 of the first gonadotrophic cycle. The X-axis shows the gonadotrophic stages. Data points are means±standard error of the mean (SEM). The number of individuals used to obtain measurements is given at each datum point or bar (Stoltzman *et al*., 2000).

six days, then an increased rate which remains high for the next three days, but sharply decreases thereafter. In second instars, rates of JH biosynthesis increase gradually from day 0-9, followed by reduced rates. In third instars, there is a high initial rate of production but within the first two days, the rate decreases sharply; rates then increase and remain high during the later half of the stadium. Fourth instars show high rates in newly ecdysed larvae but this level decreases sharply within the first 2 days. A small peak occurs on day 4, but thereafter, JH release declines and is virtually zero by day 10. JH production is undetectable from day 10-18 (Kikukawa and Tobe, 1986).

1.6 Target of cockroach ASTs in JH pathway

Many studies have shown that FGL-ASTs act before the final steps in JH biosynthesis. In the presence of farnesol, the tridecapeptide, AST1, showed no inhibitory activity on JH production suggesting that the target of the AST is located before the entrance of farnesol into the JH biosynthesis pathway (Pratt *et al.* 1989). Another study using AST1 and AST2 suggested that the targets for both ASTs occur before the entrance of mevalonate into the pathway (Pratt *et al.* 1991). Farnesoic acid, a JH III precursor, stimulated JH III biosynthesis both in the presence or absence of AST suggesting that AST must act before the farnesoic acid step (Wang *et al*. 1994).

1.7 JH biosynthesis: Mode of action

JHs are synthesized from the simple precursors acetate (JH III) and/or propionate (higher JH homologs) (Schooley and Baker 1985). Insects do not produce cholesterol; rather JH is the product of the pathway. JH III occurs in the more primitive insect orders (Tobe and

Stay 1985), whereas the higher homologs of JH III (JH I, JH II) occur in the more advanced orders. JH III is derived from acetate (C_2), whereas JH II is derived from both propionate (C3) and acetate (C2). JH I is derived from propionate (C3). The insect pathway for the production of farnesyl pyrophosphate is probably identical to that of vertebrates. Insects convert farnesyl pyrophosphate into JH III, whereas in vertebrates, it is converted into sterols.

1.7.1 cAMP and cGMP

In the CA of the cockroach, *D. punctata,* cAMP and cGMP undergo large changes during the gonadotrophic cycle. In virgin CA, the levels of both cAMP and cGMP remain relatively constant during the gonadotrophic cycle as does JH biosynthesis (Stay and Tobe 1977). However, in mated females, large changes in cAMP and cGMP can be correlated to changes in JH biosynthesis (Tobe 1990). This same study suggests that cAMP dependent kinases are involved in the inhibition of JH biosynthesis during pre- and postvitellogenesis and that cGMP-dependent kinases are involved in the inhibition of JH biosynthesis upon completion of vitellogenesis. The pattern of cAMP changes in mated females suggests that cAMP levels are low when JH biosynthesis rates are high.

Several studies have shown that the cAMP content of the CA of *D. punctata* may be correlated with the rates of JH biosynthesis. Forskolin is an adenylate cyclase activator used to study the role of cAMP by leading to cAMP accumulation in the CA (Daly 1984). Treatment of the CA with forskolin or with phosphodiesterase inhibitor, IBMX, as well

as with 8-Br-cAMP, an activator of cAMP dependent kinase, results in JH release inhibition (Meller *et al*.1985).

1.7.2 Octopamine

Octopamine is known to mediate neuropeptide hormone release in other insects (Orchard and Loughton 1981; Pannabecker and Orchard 1986). The presence of octopamine has been reported in the CA of the locusts *S. gregaria* (Evans 1985), *Locusta migratoria* (David and Lafon-Cazal 1979), and the cockroach *P. americana* (Evans 1978), suggesting that it may be involved in the control of JH biosynthesis. Treatment with octopamine shows a dose-dependent inhibition of JH biosynthesis in CA of the adult female *D. punctata* (Thompson *et al.* 1990). Although the elevation of either cAMP or cGMP within the CA is known to be associated with an inhibition of JH biosynthesis, treatment with high concentrations of octopamine results in an increase in the level of cAMP but not cGMP. Octopamine levels have not been measured in the haemolymph of *D. punctata*. Thompson *et al*. 1990 hypothesized that octopamine may be a natural neuromodulator of JH production by CA, regulating ion channels in CA cells themselves as well as release of the inhibitory neuropeptides, ASTs, from the terminals within the CA.

1.7.3 Allatostatins

Studies of the molecular action of allatostatins focused on their mechanisms of inhibition of JH biosynthesis. Two approaches have been taken to study the mode of action of FGL-ASTs on the CA. First, analyzing the biochemical steps involved in the *de novo*

biosynthesis of JH to determine which one(s) is/are affected by the incubation of the CA with the allatostatins (Sutherland and Feyereisen 1996) or binding assays for peptides to putative receptors in order to elucidate the second messenger responses and the ultimate biochemical target(s).

The mode of action of ASTs remains unclear. All existing data suggest the involvement of multiple intracellular signaling mechanisms. In *D. punctata*, ASTs most likely bind to specific membrane receptors of CA cells and stimulate signal transduction pathways (Cusson *et al*. 1991; Stay *et al*. 1994), resulting in the production of the intracellular second messengers which may then modulate enzymatic activity in the JH biosynthetic pathway (Tobe *et al.* 1994). Cells of the CA clearly contain many of the second messengers, including cAMP, cGMP, calcium, and protein kinases, as well as responding to the ASTs, suggesting that the cell membranes probably represent the site of integration of these various messages in the regulation of JH biosynthesis (Tobe *et al.* 1994). It is also possible that the ASTs exert effects on JH production, at least in part through the regulation of intracellular Ca2+ concentration and regulation of other ion channels (Tobe *et al.* 1994).

The second messenger system that has been studied in *D. punctata* is the 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG) pathway. The IP_3/DAG second messenger system is involved in the regulation of JH biosynthesis (Feyereisen and Farnsworth 1987). A subsequent study showed that treatment of CA cells with DAG kinase inhibitors (thereby elevating DAG), PKC activators, and PKC inhibitors resulted in an inhibition of

JH release, suggesting that DAG and PKC are involved in the decrease of JH biosynthesis and likely function as signal transducers for ASTs. Treatment of CA with both DAG kinase inhibitors and ASTs enhanced this effect, suggesting that DAG is an intermediate in the AST-induced inhibition of JH production (Rachinsky *et al.* 1994). Thapsigargin, the calcium-mobilizing drug, mimics the effects of IP_3 by releasing Ca2+ from intracellular stores without generation of IP_3 (Thastrup *et al.* 1990). Treatment of CA with thapsigargin results in a significant stimulation in JH biosynthesis and it was also able to reverse the effect of ASTs in high-activity mated CA but only at specific developmental times, suggesting that IP_3 may only be effective at certain physiological stages.

The synthetic ASTs from *D. punctata*, including Dip7, do not elicit an increase in cAMP or cGMP within CA, suggesting that these compounds may not act directly as second messengers of these peptides (Cusson *et al*. 1992; Stay *et al.* 1994; Tobe *et al*. 1994).

1.7.4 Calcium

Calcium plays a major role in the regulation of JH biosynthesis in the cockroach *D. punctata* (McQuiston and Tobe 1991; Thompson and Tobe 1986). Extracellular calcium at the optimum concentrations of 3-5 mM is required for the normal functioning of the glands. JH production is inhibited in the absence of extracellular calcium (Kikukawa *et al.* 1987). Whereas treatment of CA with the calcium-mobilizing drug, thapsigargin results in a significant stimulation in JH biosynthesis and can reverse the inhibitory effect of ASTs (Rachinsky *et al.* 1994), treatment of CA with forskolin, results in significant

inhibition of JH biosynthesis in females at the end of the reproductive cycle (Meller *et al.* 1985). Similary, CA treatment with phorbol esters, the activators of phospholipids/calcium-dependent PKC, results in inhibition of JH biosynthesis (Feyereisen and Farnsworth 1987). Treatment of CA with 8-bromo-cAMP also results in strong inhibition of JH biosynthesis in day 6 females (McQuiston and Tobe 1991). These data suggest that cAMP may modulates the excitability of cell membranes of the gland through regulation of calcium influx, Ca2+ currents might play an important role in the regulation of intracellular Ca2+ concentration and JH biosynthesis. Changes in the electrical properties of the CA may be important in the regulation of JH biosynthesis, possibly by altering Ca2+ influx. On the other hand, 8-Bromo-cGMP reduces the duration of action potentials in CA from day 1 tested animals (Tobe *et al*. 1994), suggesting that cGMP may also modulate calcium influx but at development times and in a fashion different from cAMP.

1.8 G-protein coupled receptors (GPCRs)

G-protein coupled receptors (GPCRs), also known as seven transmembrane receptors (7-TM), are the largest family of transmembrane receptors and represent a major class of drug targets. GPCRs are only found in eukaryotes such as yeast, plants, and animals. The NH_2-terminal and three extracellular loops may be involved in binding of larger agonists, whereas the COOH-terminal and the intracellular loops are involved in interactions with the guanine nucleotide binding proteins (G protein) and other cellular proteins. The ligands that bind and activate receptors include pheromones, hormones, and neurotransmitters, vary in size from small molecules to peptides to large proteins. GPCRs

which act as receptors for agonists that have yet to be identified are known as orphan receptors.

Insects are the largest animal group in the world, 75% of all species are insects. The genomes from the fruitfly, *D. melanogaster*, the mosquito, *A. gambiae*, (Diptera), and the silkworm, *B. mori*, (Lepidoptera), have been sequenced (Adams *et al.* 2000; Holt *et al.* 2002; Xia *et al.* 2004). The most recent insect genome completed is that of the honey bee *A. melliferi* (Robinson *et al*. 2006). More than 1,000 GPCRs have been identified in the *C. elegans* genome (Bargmann 1998). The completion of the *Drosophila* genome project has resulted in the identification of approximately 200 genes coding for GPCRs, including neurotransmitter and hormone receptors, and olfactory and putative taste receptors (Adams *et al.* 2000; Clyne *et al*. 2000; Rubin *et al*. 2000), 44 of which have been characterized as peptide GPCRs (Hewes and Taghert 2001). In the honey bee genome, about 240 GPCR genes have been identified (Robinson *et al*. 2006).

Insect GPCRs can be classified into four families: rhodopsin-like (family A), secretin receptor-like (family B), metabotropic glutamate receptor-like (family C), and atypical receptors (family D). All families have the same 7 TM topology, but differ by amino acid residues at certain characteristic positions. For example, family A receptors have a conserved Asp-Arg-Tyr (DRY) sequence motif just after the third transmembrane α helix, whereas this motif is lacking in members of the other GPCR families (Gether *et al*. 2002).

Ligands bind and activate receptors by signaling via G proteins. Signal transduction starts with an inactive G protein bound to the receptor in its inactive form. An inactive G protein exists as a heterotrimer, a molecule composed of three different protein subunits, α, β, and γ. Once the ligand is recognized, the conformation of the receptor changes and thus activates the G protein and causes one subunit (Gα) to dissociate from the other two G-protein subunits (Gβ and Gγ). The receptor can now either activate another G protein, or switch back to its inactive state (Wess, 1998).

The activated G protein subunits dissociate from the receptor and initiate signaling from many downstream effector proteins, including phosphodiesterase, adenylyl cyclases, phospholipases, and ion channels that permit the release of second messenger molecules such as cyclic AMP (cAMP), cyclic GMP (cGMP), inositol triphosphate (IP3), diacylglycerol (DAG), and calcium (Ca^{2+}) ions (Yang and Xia 2006).

The downstream signaling pathways activated by GPCR agonists are dependent on the class of heterotrimeric guanine nucleotide-binding (G) protein that is coupled to the cytoplasmic domains of the receptor. It has been shown that agonist binding to mammalian SST or opioid receptors leads to the activation of G-protein-gated inwardly rectifying potassium channels (GIRK) (Kreienkamp 1999). Birgul *et al*. (1999) demonstrated that AST is the ligand for the *Drosophila* receptor by coexpressing the receptor with GIRK in frog oocytes. The results showed that *Drosophila* AST receptor efficiently activated the inward potassium currents, but the activation of phospholipase C followed by activation of calcium induced chloride currents was not observed, suggesting that the Drosophila ASTR couples to a G protein of the G_i/G_0 family.

1.9 Allatostatin receptors (AstRs)

AST receptors belong to the rhodopsin-like GPCR family. Although insects possess a large variety of peptide transmitters and hormones (Gade *et al*. 1997b; Schoofs *et al.* 1993; Vanden Broeck 2001b), only a very few insect receptors have as yet been fully characterized. The existence of multiple ASTs and the multifunctional characters of ASTs might suggest the presence of individual receptors for each peptide. The identification of AST receptors is important for understanding the changing effects of ASTs on the production of JH and for detecting other target organs.

1.9.1 FGLamide receptors

The first AST receptors were identified in *D. melanogaster*. Using oligonucleotide probes screening the *Drosophila* genome project database, the *Drosophila* AST receptor (DAR-1) was cloned, yielding cDNA code for 394 amino acids (Lenz *et al.* 2000). This receptor was previously identified through a reverse physiological approach (Birgul *et al.* 1999). The Drosophila AST receptor, CG2872, belongs to the rhodopsin-like GPCR family and is closely related to the mammalian galanin receptors and to a lesser extent to somatostatin/opioid receptors (Birgul *et al*. 1999). However, FGLamide-ASTs do not show any sequence similarity to galanin, suggesting that the ligand specificity of the receptors probably evolved more rapidly than the ability to interact with specific intracellular components of the signal transduction pathways. *Drosophila* AST receptor mRNA is expressed in both heads and bodies of larvae, pupae, and adults but not in embryos.

A second *D. melanogaster* gene (DAR-2), CG10001, encodes a GPCR of 357 amino acids that is similar to *Drosophila* ASTR (Lenz *et al*. 2000). The DAR-2 gene has four exons and three introns. Two of these introns coincide with two introns in the DAR-1 gene, and have the same intron phasing, suggesting that the two receptor genes are evolutionary related. DAR-2 shares 47% overall sequence identity and 60% identity in the transmembrane regions of DAR-1.

Furthermore, FGLamide-AST receptor orthologs have been sequenced in other insect species. An AST receptor was cloned, expressed and functionally tested in the cockroach *P. americana* (PeramAstR) using degenerate primers based on known mammalian somatostatin receptors and *Drosophila* AST receptors. The receptor gene codes for a 423 amino acid GPCR, showing 60% amino acid identity in the transmembrane regions compared to DAR-1 and DAR-2 (Auerswald *et al.* 2001).

Another FGLamide AST receptor from *B. mori* (BAR) was also cloned, and functionally tested (Secher *et al.* 2001). BAR is a 361 amino acid GPCR, showing 60% amino acid overall sequence identity with DAR-1 and 48% identity with DAR-2. BAR gene has three exons and two introns. Two of these introns coincide with and have the same intron phasing as two introns in the DAR-1 and DAR-2 genes, suggesting that the three receptors are structurally and evolutionarily related. BAR mRNA was expressed in foregut, midgut, hindgut, and to a much lesser extent in the brain or in fifth instars (Secher *et al.* 2001).

Other FGLamide AST receptor orthologs in other insect species have been identified by genome sequencing in *Apis mellifera* (Robinson and Ben-Shahar 2002), *Anopheles gambiae* (Hill *et al.* 2002). FGLamide AST receptor orthologs have also been identified in other invertebrates such as *Caenorbabditis elegans* (Nathoo *et al*. 2001).

1.9.2 $W(X)_6$ Wamide receptors

The *D. melanogaster* cricket-type receptor gene (CG14484) has been identified using β-arrestin molecule tagging with a green fluorescent protein to interact with GPCRs. The gene appears to be a member of the bombesin family of peptide GPCRs, and responds specifically to AST-B neuropeptides (Johnson *et al*. 2003). A paralogous receptor, CG14593, has also been identified in the *Drosophila* sequence database, but has not been functionally tested (Johnson *et al*. 2003).

1.9.3 PISCF receptors

The two Manduca-type AST receptor genes in *Drosophila* (CG7285, and CG13702) were cloned, coding for GPCRs of 467 amino acids (Drostar 1) and 489 amino acids (Drostar 2), respectively. The receptors show 60% overall amino acid sequence identity, and 76% identity in the transmembrane regions. Though the two receptors show similarity to the somatostatin/opioid receptor, none of them was activated by any known mammalian agonists. However, two separate functional assays have shown that the two Drostars were activated only by the AST-C (Johnson *et al*. 2003; Kreienkamp *et al.* 2002).

1.10 Evidence of AST receptors in *D. punctata*

Several research groups have reported the presence of AST receptors in *D. punctata*. The CA is believed to be a primary target tissue for AST. Putative receptors for ASTs have been partially characterized using photoaffinity labeling showing the presence of two putative receptors (59 and 39 kDa) in the CA (Cusson *et al*. 1991; Cusson *et al.* 1992). Structure-activity studies by (Feyereisen *et al.* 1997) have also proposed a model for two types of AST receptors in the CA with two additive responses, as suggested from the biphasic inhibitory response of day 2 CA to some analogs of AST 2.

AST is expressed not only in CA but also in brain neurosecretory cells (Donly *et al*. 1993), and in endocrine cells of the midgut (Reichwald *et al*. 1994; Yu *et al.* 1995b). Immunocytochemical studies have shown a wide distribution of ASTs, including neurons of the brain and terminal abdominal ganglion (Stay *et al*. 1992)), the anterior hindgut (Lange *et al.* 1993), midgut (Yu *et al.* 1995b), antennal pulsatile organ muscle (Woodhead 1992), thereby suggesting that these tissues also possess AST receptors.

The putative receptors for *D. punctata* ASTs in brain and CA have been identified using a binding assay and photoaffinity labeling assay (Yu *et al.* 1995b). The result revealed the presence of a putative receptor (37 kDa) for AST 5 and AST 7. The two Kd values were obtained from the *in vitro* binding assay indicating the two possible putative receptor sites for AST 7. Though the putative receptor in brain may differ from that in the CA, there are likely to be structural similarities between them for any given ASTs.

A putative AST receptor in the midgut has been reported (Bowser and Tobe 2000). The results showed saturable and reversible binding sites for ASTs in the midgut which has lower affinity than those reported in the CA and brain (Yu *et al.* 1995b) suggesting that there are probably differences between the receptors in the CA and in the midgut. This suggestion was also confirmed by the enzyme activity assay in midgut of *D. punctata* using the AST analog in the range of concentrations known to be active in inhibiting JH biosynthesis (Fuse *et al.* 1999).

1.11 RNA interference (RNAi)

RNAi, originally discovered in *C. elegans*, is a gene silencing technique in which double-stranded RNAs (dsRNAs) are introduced into a cell. dsRNA activates a pathway that destroys the mRNA in a sequence-specific manner, thereby knocking out gene expression which can be used for loss-of-function studies (Fire *et al*. 1998).

RNAi is triggered by the introduction of dsRNA, typically longer than 200 bp (Fire *et al*. 1998). Many studies in plants, flies, and worms have revealed similar processes in which the dsRNA is cleaved into 21-23 bp small interfering RNAs (siRNAs) by an enzyme called Dicer (Bernstein *et al.* 2001; Hamilton and Baulcombe 1999), thus producing multiple molecules from the original single dsRNA. The siRNA-Dicer complex recruits additional components to form an RNA-induced Silencing Complex (RISC) in which the unwound siRNA base pairs with complementary mRNA, thus guiding the RNAi machinery to the target mRNA resulting in the effective cleavage and subsequent

degradation of the mRNA (Hammond *et al.* 2000; Pham *et al.* 2004; Zamore *et al.* 2000) (Fig. 1.8).

RNAi has been used extensively in various insect species including cockroaches. RNAi has first been used *in vivo* in the cockroach *P. americana* to analyze the function of a homeotic gene in relation to the control of axon pathfinding and synaptic target choice in neurons of the cercal sensory system (Marie *et al.* 2000). Martin *et al.* (2006) used RNAi to knock down the expression of the adult-specific vitellogenin gene in the cockroach *B. germanica*.

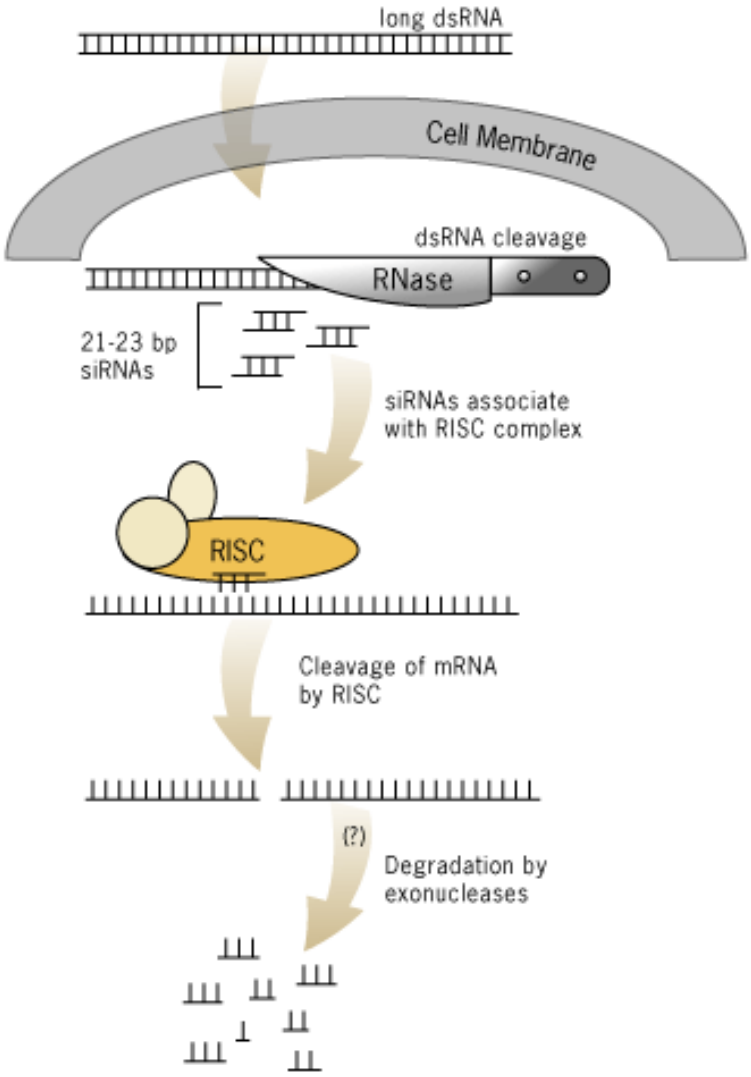

Fig. 1.8 Mechanism of RNAi action: RNAinterference (RNAi) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product, resulting in null phenotypes. dsRNA is cleaved into ~23 bp short interfering RNAs (siRNAs) by an RNase enzyme called Dicer, thus producing trigger molecules from the original single dsRNA. The siRNA-Dicer complex recruits additional components to form an RNA-induced Silencing Complex (RISC) in which the unwound siRNA base pairs with complementary mRNA, thus guiding the RNAi machinery to the target mRNA resulting in the effective cleavage and subsequent degradation of the mRNA (figure is from http://www.ambion.com/techlib/resources/RNAi/overview/index.html).

Experimental rationale

Due to multiplicity of cockroach ASTs, the wide distribution and expression in various tissues and multiple functions that ASTs exert we hypothesized that multiple, tissue-specific AST receptors would exist. To complete understanding of the regulation of the CA by neurosecretions, identification of the receptors for these peptides is important. To approach this problem I have screened cDNA libraries from *D. punctata* and have identified the putative AST receptor sequence. I demonstrated that the putative receptor is related in sequence to the known FGLamide-AST receptors. Using qPCR, I have shown that the FGLamide-AST receptor mRNA fluctuates in a manner expected for a regulator of JH biosynthesis during the reproductive cycle in both virgin and mated females.

In this work the following questions were asked:

1) What is the gene structure for the cockroach AST receptor

2) What are the expression levels of the gene in various tissues of adult *D. punctata*

3) How do changes in gene expression relate to animal endocrinology/physiology especially with regard to JH biosynthesis

CHAPTER 2:

Molecular Cloning and Characterization of an Allatostatin-like receptor in the Cockroach *Diploptera punctata*

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2.1 Abstract

Two *Drosophila* receptors (AlstR/ DAR-1 and DAR-2) with sequence similarity to mammalian galanin receptors have been previously identified. These receptors have been shown to form specific interactions with neuropeptides that resemble cockroach allatostatins (ASTs), which have a characteristic Tyr/Phe Xaa Phe Gly Leu-NH_2 carboxyl-terminus. We hypothesized that similar allatostatin receptors exist in the cockroach *Diploptera punctata* that may regulate the numerous effects that this family of peptides exerts on a range of target tissues. The polymerase chain reaction (PCR) with primer design based on the *Drosophila* allatostatin receptor (AlstR) was used to isolate a putative allatostatin-like receptor cDNA from a λZAP-cDNA library prepared from the corpora allata of the *D. punctata*. As an approach to testing the function of this receptor *in vivo*, the technique of double-stranded RNA (dsRNA) gene interference was tested. Initial experiments suggest that the putative inhibition of receptor RNA expression may increase JH production.

Keywords

Pest control, cloning, insect, G-protein coupled receptor, real-time PCR, RNA interference, juvenile hormone

2.2. Introduction

The cockroach, *Diploptera punctata,* exhibits precisely timed juvenile hormone (JH) biosynthesis in relation to reproduction and thus serves as a model organism for studies of the regulation of JH production. Allatostatins (ASTs) are insect neuropeptides that inhibit JH biosynthesis *in vitro*. Thirteen ASTs are potentially released from a precursor polypeptide in *Diploptera* [12]. Three unique AST structures are capable of inhibiting JH in differing insects. Cockroach-type AST was first identified in the cockroach *D. punctata* [49, 37, 42, 48] having a characteristic C-terminal sequence Tyr/Phe-Xaa-Phe-Gly-Leu-NH_2. The cockroach-type AST represents a large superfamily of peptides found throughout the invertebrates. However, despite having conserved sequence, the allatostatic function of these peptides appears to be less conserved, having been demonstrated only in cockroaches, crickets and termites [42, 50]. Cockroach-type ASTs appear to have a variety of functions including serving as neuromodulators, inhibitory regulators of muscle contraction and regulators of enzyme biosynthesis [4, 42]. In the cricket *Gryllus bimaculatus,* cockroach-type ASTs have only weak allatostatic activity; however, crickets also have four additional allatostatic nonapeptides with a common W_2W_9-NH_2 motif [30, 31]. These cricket-type allatostatic nonapeptides share amino acid sequence identity with *Locusta* myoinhibiting peptide that functions in muscle contraction [40]. Select Lepidoptera and Diptera appear to have a non-amidated 15 amino acid AST (moth-type) with C-terminal signature sequence PISCF-OH [23, 29]. In *Manduca* adults, this peptide serves as a potent inhibitor of JH biosynthesis, yet has limited allatostatic potency in the adult moths *Pseudaletia unipuncta* [20] or *Lacanobia oleracea* [13] . Similarly, the moth-type AST serves to inhibit JH biosynthesis in

mosquitoes [29] but has not yet been shown to have activity in the fruitfly, *Drosophila melanogaster* [38].

The NH_2-terminus of each cockroach-type AST within an insect is unique. This has led to speculation that the NH_2-terminus of the peptide may serve to modulate the affinity of binding of each peptide either with a single receptor or multiple receptors. Such variable binding affinity may regulate different functions or possibly control tissue-specific actions for these peptides. In *Diploptera,* each of the thirteen unique AST peptides has been shown to have a different rank order of effectiveness when tested in assays for inhibition of JH biosynthesis versus inhibition of muscle contraction [25].

Although much research has focused on insect neuropeptides and their functions, there has been relatively little work on the characterization of receptors for these neuropeptides. As a result of the sequencing of the *Drosophila* genome [1, 8, 19], many novel G-protein-coupled receptors (GPCRs) have been identified in *Drosophila* and orthologs identified in other insects. Four cockroach-type AST receptors (ASTRs) have been identified as mammalian galanin receptor orthologs, two from *Drosophila*, AlstR1/ DAR-1 [6, 27] and DAR-2 [27], one from the cockroach *Periplaneta americana* (PeramAstR) [3], and one receptor from the silkworm *Bombyx mori* (BAR) [41]. AlstR1/DAR1, DAR2 and BAR have been shown in functional expression assays to be activated by cockroach-type ASTs from *Drosophila* and *Diploptera* [6, 27, 28, 26]. Two somatostatin receptor orthologs (Drostar1 and 2) functionally interact with the moth-type

allatostatin [24, 21]. A receptor, CG14484, related to the vertebrate bombesin receptor, is the apparent receptor for the cricket-type ASTs [21].

RNA inhibition (RNAi) of specific gene expression *in vivo* has been demonstrated to be an effective technique with select genes in cockroaches. For example, RNAi has been used in the cockroach *P. americana* to analyze the function of the homeotic gene *engrailed* in relation to the control of axon path finding and synaptic target choice in neurons of the cercal sensory system [33]. An RNAi-induced reduction of expression of the RXR/USP gene allowed the last larval instars of *Blattella germanica* to progress correctly until the time of molting when development was arrested [34]. In addition, RNAi has been used in *Blattella* to significantly reduce preproallatostatin mRNA (70-80% reduction in brain) and peptide (53% reduction) [32]. In this latter study, reduction of allatostatin mRNA via RNAi was ineffective in increasing JH production.

In the present study, we have cloned and characterized the expression of a cDNA encoding a putative *D. punctata* AST receptor (Dippu-AstR) and have used the RNAi technique to inhibit its expression in injected insects.

2.3. Materials and methods

2.3.1 Insect rearing and tissue collection

Adult female *D. punctata* were obtained from a colony fed lab chow and water. Mating in *D. punctata* occurs immediately following female eclosion. Day 0 tissues were dissected following mating and prior to tanning of the cuticle. Cockroach females were isolated and used at the appropriate physiological ages. Corpora allata (CA) were carefully dissected and washed repeatedly in ice-cold cockroach saline (150 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM Hepes, 40 mM glucose). Tissues for RNA extraction were stored in RNAlater (Ambion, Inc.) at -20^{o}C until ready to use.

2.3.2 Cloning of the cockroach receptor

cDNA libraries made from RNA extracted from CA of *D. punctata* were used as the template in the PCR reaction with the degenerate oligonucleotide primers (ASTR forward: 5' AGGGAATTCGCNGTNGTNCAYCC 3', ASTR reverse: 5' GCCGGATCCAANGCRTANARDATNGGRTT 3') corresponding to the conserved region between mammalian somatostatin receptors and the *Drosophila* AST receptor (AlstR). PCR products of the expected size (500-700bp) were isolated and cloned into pCR4-TOPO vector (Invitrogen) and sequenced. A 550 bp DNA fragment was isolated and further characterized. To obtain a full-length cDNA, the rapid amplification of cDNA ends (RACE) technique was applied using the first-strand cDNA derived from *D. punctata* total RNA. The primer for 3'RACE was 5'-CAACAGACCTGATGGCTACAAC-3', primers for 5'RACE were 5'-GGTCTGCCACAGCGAGGTTGATTA-3' and 5'-CACCTTCTGGATGAGTTGGAG

AGA-3'. RACE reaction products were cloned into pGEM-Teasy (Promega) and sequenced. Based on this sequence information, primers were designed that recognize the sequence surrounding the ATG start codon and the stop codon of the novel receptor cDNA and these primers were then used to amplify a full length cDNA from *D. punctata*. The PCR product of 1275 bp was cloned into pGEM-Teasy (Progmega), and several independent clones were sequenced to verify the absence of PCR-induced errors in the final sequence.

2.3.3 Phylogenetic analysis

Evolutionary tree analyses were carried out using ClustalX program and njplot (ftp://ftp.ebi.ac.uk/pub/software/dos/clustalx). The protein sequences were obtained from GenBank™.

2.3.4 Synthesis of double-stranded RNA (dsRNA) and injection

To prepare the dsRNA targeted to Dippu-AstR (dsDippu-AstR), a 560-bp fragment was subcloned into the pGEM-Teasy vector. *In vitro* transcription using T7 and T3 RNA polymerase was performed to produce sense and antisense RNA strands for the fragment. Equimolar amounts of sense and antisense RNAs were mixed in annealing buffer to a final concentration of 0.50 µM each. The mixture was heated at 100°C for 1 min and allowed to cool slowly to room temperature for 18 hours. The reaction mix was purified and concentrated by phenol: chloroform extraction and isopropanol precipitation. RNA precipitates were dissolved in injection buffer (20 mM Tris, pH 7.8, 150 mM NaCl) to a final concentration of 5 µM. Up to 10 µl of 0.1 µg/µl or 0.5 µg/µl dsDippu-ASTR RNA

were injected into mated female cockroaches at the different stages through the membrane between the coxa and femur of the third pair of legs of the cockroaches. Control dsRNA consisted of a non-coding sequence from the pGEM-Teasy vector (dscontrol).

2.3.5 Quantification of juvenile hormone production and measurement of basal oocyte growth

CA were dissected and JH production determined using the radiochemical assay [15]. The incubation medium was 50 µl of TC199 media (GIBCO) without methionine with 2% Ficoll and [L-methyl-^{14}C] methionine specific activity 2.07 GBq/mmol, 50 µM final concentration. The length of the basal follicle (oocyte including follicle cells), was measured. Assays were then performed on individual animals as described in the figure legends.

2.3.6 Semi-quantitative RT-PCR analysis of Dippu-AstR from various adult tissues

The one-step RT-PCR kit (Qiagen) was used for one-step RT-PCR. Total RNA was extracted from brain, gut, testes and ovaries of adult *D. punctata*. First-strand cDNA was synthesized from 1 µg of total RNA from each tissue using the supplied reverse transcriptase. Primers were designed based on the sequence of the putative allatostatin-like receptor (forward primer 5'-ATGACGCTGGTGTTAGTGATGT-3'; reverse primer 5'-CAACAGACCTGATGGCTACAAC-3'). In addition, as a positive control, primers were designed to amplify a 415-bp fragment of *D. punctata* 16S rRNA (forward primer,

5’-TTACGCTGTTATCCCTAAGG-3’; reverse primer, 5’-CGCCTGTTTAACAAAAAC -3’).

2.3.7 Quantitative real-time PCR analysis of Dippu-AstR

Dippu-AstR and 16S rRNA were analyzed using standard real-time quantitative RT-PCR. Dippu-AstR primers were designed to amplify a 150 bp fragment (forward primer, 5’-ATCCTGGTGCTGAAGAGTGCGGACC-3’; reverse primer, 5’-GCGGAATGCTTTG CGGAAGTGG-3’). The housekeeping gene, 16S rRNA, served as control for the integrity of the RNA and the efficiency of the RT-PCR. The real-time RT-PCR reactions were performed using a mastermix (Eurogentec kit). All reactions were performed in triplicate using a total of 100 ng of mRNA per reaction. The assays were performed on an ABI 7500 system. The conditions were as follows: 1 cycle (95^{o}C for 15 min), 45 cycles (95^{o}C for 15 sec, 55^{o}C for 30 sec, and 72^{o}C for 32 sec). No-template controls (NTC), containing water instead of template mRNA, were also run under the same conditions for each gene. The formula $2^{\Delta CT}$ was used to calculate the fold change.

2.4. Results

2.4.1 Cloning of the *D. punctata* AST receptor

Using PCR with degenerate primers and a *D. punctata* CA cDNA library as template, we were able to amplify a 550 bp fragment of cDNA coding for transmembrane domain 5 to domain 7 of the seven transmembrane regions of a *D. punctata* AST receptor. The 5' and 3' ends of the cDNA were completed by using 5' and 3' RACE. The receptor mRNA sequence is 1722 nucleotides with a 5' untranslated region of 66 nucleotides and 3' untranslated region of 381 nucleotides that includes a polyadenylation signal and a poly(A^+) tail. Based on Kozak's rules the second ATG is in the correct context to create an open reading frame that encodes a protein of 425 amino acid residues. There are two stop codons prior to the initiating ATG (Fig 2.1). Hydrophobicity analysis, analyzed using Vector NTI software (Invitrogen), of the deduced amino acid sequence displayed seven hydrophobic domains typical of G-protein coupled receptors (Fig 2.1). The N-linked glycosylation signature sequence AsnXaaThr/Ser typical of GPCRs occurs for seven Asn residues, three (Asn28, Asn32 , Asn44) in the extracellular NH_2-terminus prior to the first transmembrane domain (TMD), one between TMD VI and VII (Asn326) and three in the intracellular carboxyl-terminus (Asn 404, Asn410, Asn 418). Second messenger-dependent kinase signatures required for receptor desensitization were also noted. Protein kinase C phosphorylation sites that may be utilized are found in the first intracellular loop (Ser104, Thr 105) between TMDI and TMDII and the third intracellular loop (Thr293) among TMD V and VI. Potential protein kinase A phosphorylation sites are found in the second (Ser 179) and third intracellular loops (Ser285) and in four positions (Ser354, Thr375, Ser383, Thr384) in the intracellular carboyxl-terminus.

2.4.2 Comparison of the *D. punctata* AST receptor with other receptors

Amino acid sequence alignments with other known insect ASTRs showed that the novel *D. punctata* receptor is closely related to the PeramAstR, showing 85% overall identity, 60% overall identity to BAR, 52% overall identity to DAR-1, and 44% overall identity to DAR-2 (Fig. 2.2). Like all other insect ASTRs, Dippu-AstR is related to the mammalian galanin receptors (32% overall identity with the rat galanin receptor type-1), somatostatin receptors (27% with the rat somatostatin receptor type-2), and opioid receptors (27% with the rat μ opioid receptors) [36, 22, 44]. N-linked glycosylation sites are conserved between the two cockroach AST receptors (Fig 2.2). The second potential glycosylation site is also found to be conserved in DAR-2, and occurs at nearly the same position as in DAR-1. In this alignment the third cockroach glycosylation site aligns with that of BAR. The DRY motif that controls GPCR activation and selectivity/promiscuity in mammalian GPCRs [9] is found as DRF flanking the carboxyl-side of transmembrane domain III in all insect ASTRs (Fig 2.2). Similarly, a palmitoylation site following transmembrane domain VII, that may anchor a portion of the cytoplasmic C-terminus to the lipid membrane, is conserved in all insect ASTR sequences. The conservation of transmembrane domain size and spacing as well as the conservation in post-translational modification motifs confirm the structural similarities between all the insect AST receptors. This finding is confirmed by the generation of a phylogenetic tree that illustrates the evolutionary distance between the ASTRs. The cockroach ASTRs share a closer relationship to each other than to other insect ASTRs (Fig. 2.3).

2.4.3 Double-stranded RNA interference in cockroaches

The effect of dsDippu-AstR RNA on JH release by CA from mated female cockroaches was tested by injection with injection buffer containing dsDippu-AstR RNA or injection buffer (control). The injection of dsDippu-AstR RNA after 24 hours led to a significant increase (basal level 55.5 $pmol.hr^{-1}$ per CA pair) in mean JH release (Fig 2.4, solid bar, right side) compared to mean JH release of the control buffer injection (basal level 39.5 $pmol.hr^{-1}$ per pair) (Fig 2.4, solid bar, left side). In control injected animals, incubating CA with Dippu-AST7 led to a 63% inhibition of JH release (Fig 2.4, checked bar, left). Dippu-AST7 had significant less of an inhibitory effect (46%) on JH release from CA paired glands in animals injected with dsDippu-AstR RNA (Fig 2.4, checked bar, right).

The effect of dsDippu-AstR RNA on JH release was also measured during the first gonadotrophic cycle in mated females. With two dsDippu-AstR RNA injections on day 0 and day 1 post-mating, significant increases in JH release followed on days 2, 3 and 6 relative to control injected animals (Fig. 2.5). To determine the time and concentration required to obtain a significant reduction in Dippu-AstR mRNA levels, a time-course study following treatment of 1, 2.5, and 5 µg of dsDippu-AstR was conducted. Midguts were dissected out on days 2, 3, and 6 after treatment. Tissues were then processed for RT-PCR analysis. Reductions in midgut mRNA for Dippu-AstR were not detected 2 day after treatment. An apparent reduction in mRNA levels was observed at 26.6 ± 3.3% on day 3 ($n=3$, $p<0.05$; student's t-test) when 1 µg of dsRNA was injected. Expression was further reduced to 35.6 ± 2.7%, and 51.8 ± 13.2% after 2.5 and 5 µg of dsRNA treatment, respectively ($n=3$, $p<0.05$; student's t-test). The reduced expression lasted at least until

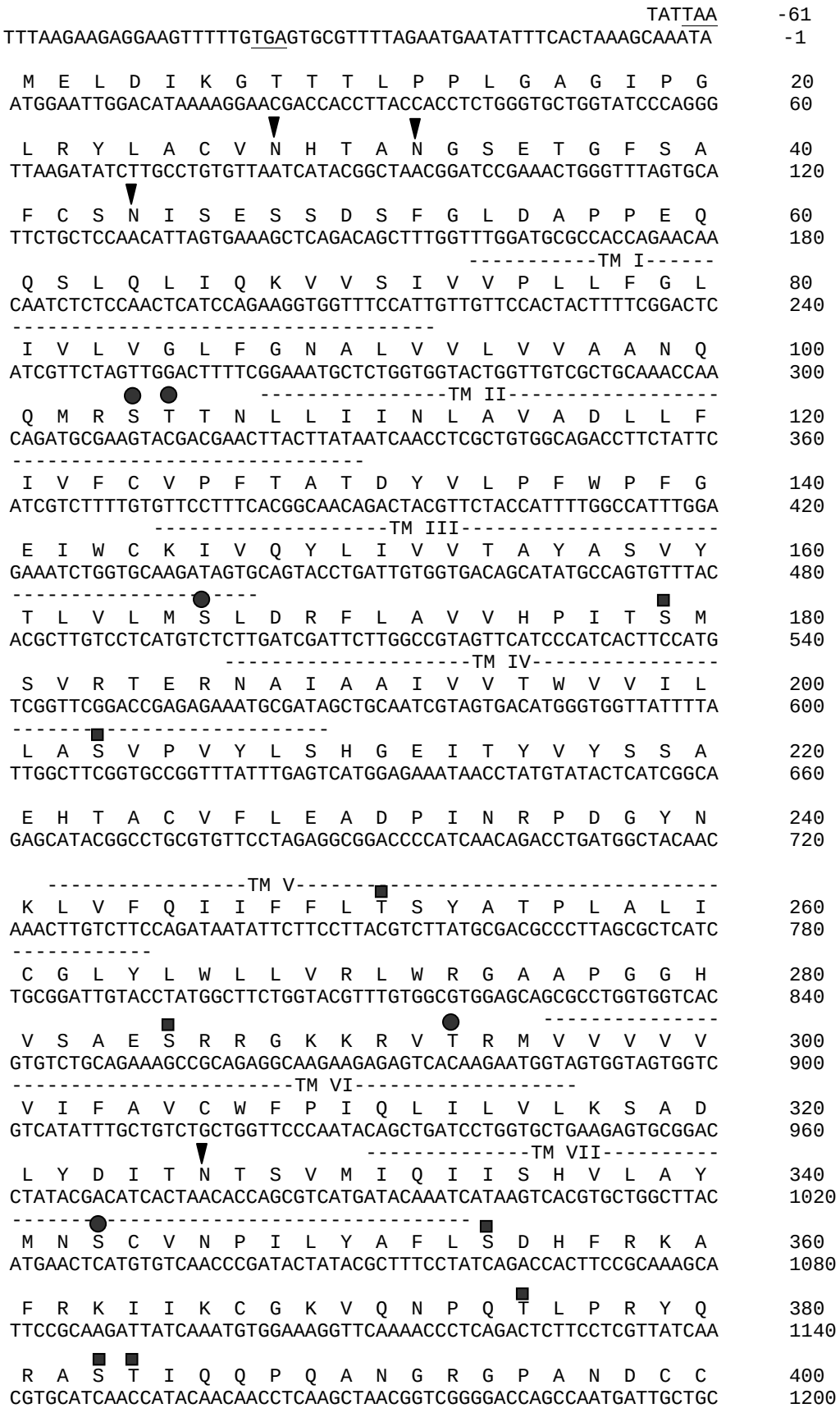
day 6, where a reduction to 32 ± 10.1%, 44 ± 17.9%, and 63.2 ± 7.5% was still observed after treatment of 1, 2.5, and 5 µg of dsRNA, respectively ($n=3$, $p<0.05$; student's t-test) (Fig. 2.6).

Three concentrations (1, 2.5 and 5 µg) of dsDippu-AstR RNA were tested for their effect on JH release on day 2, day 3 and day 6 animals (Fig 2.7) Animals injected with dsDippu-AstR RNA on day 1 and assayed on day 2 showed no significant increased from control with all doses of dsDippu-AstR RNA. In fact, the injection of 2.5 µg dsDippu-AstR RNA had a significant decrease in JH release on day 2. When assayed on day 3, all three doses of dsDippu-AstR RNA gave rise to a significant but consistent (mean 1.65 fold) increase in JH release. This stimulation of JH release further increased in animals assayed on day 6, with 1µg or 5µg doses of dsDippu-AstR RNA resulting in a 3.5 fold stimulation of JH release (Fig 2.7). These significant increases in JH release suggest that Dippu-AstR participates in regulating JH levels during the stimulation phase of JH biosynthesis as well as after peak JH biosynthesis has been achieved.

Increased JH biosynthesis is required to stimulate the growth of oocytes of mated females. The mean lengths of basal oocytes were measured in animals injected with dsDippu-AstR RNA or injection buffer (Fig. 2.8). The mean length of basal oocytes from treated animals at 3 days after mating was significantly greater than that of injected control animals. However, the injection of dsDippu-AstR RNA, under the conditions of injection tested, did not produce any significant effect on oocyte growth on the other days of the gonadotrophic cycle (Fig. 2.8).

2.4.4 Analysis of Dippu-AST receptor mRNA expression

Total RNA samples from brain, gut, testes, and ovaries from adult *D. punctata* were analyzed by semi-quantitative RT-PCR. RT-PCR amplification yielded a unique product of 350 bp in all of the tissues compared to levels of 16S rRNA amplified. The results showed that the Dippu-AstR gene was highly expressed in brain, and to a lesser extent in the gut, testes, and ovaries (Fig. 2.9A). These data were confirmed using quantitative real-time PCR (Fig. 2.9B). Expression of Dippu-AstR is therefore consistent with a role as a gut/brain receptor.


TATTAA -61
TTTAAGAAGAGGAAGTTTTTGTGAGTGCGTTTTAGAATGAATATTTCACTAAAGCAAATA -1
M E L D I K G T T T L P P L G A G I P G 20
ATGGAATTGGACATAAAAGGAACGACCACCTTACCACCTCTGGGTGCTGGTATCCCAGGG 60
L R Y L A C V N H T A N G S E T G F S A 40
TTAAGATATCTTGCCTGTGTTAATCATACGGCTAACGGATCCGAAACTGGGTTTAGTGCA 120
F C S N I S E S S D S F G L D A P P E Q 60
TTCTGCTCCAACATTAGTGAAAGCTCAGACAGCTTTGGTTTGGATGCGCCACCAGAACAA 180
TM I
Q S L Q L I Q K V V S I V V P L L F G L 80
CAATCTCTCCAACTCATCCAGAAGGTGGTTTCCATTGTTGTTCCACTACTTTTCGGACTC 240
I V L V G L F G N A L V V L V V A A N Q 100
ATCGTTCTAGTTGGACTTTTCGGAAATGCTCTGGTGGTACTGGTTGTCGCTGCAAACCAA 300
TM II
Q M R S T T N L L I I N L A V A D L L F 120
CAGATGCGAAGTACGACGAACTTACTTATAATCAACCTCGCTGTGGCAGACCTTCTATTC 360
I V F C V P F T A T D Y V L P F W P F G 140
ATCGTCTTTTGTGTTCCTTTCACGGCAACAGACTACGTTCTACCATTTTGGCCATTTGGA 420
TM III
E I W C K I V Q Y L I V V T A Y A S V Y 160
GAAATCTGGTGCAAGATAGTGCAGTACCTGATTGTGGTGACAGCATATGCCAGTGTTTAC 480
T L V L M S L D R F L A V V H P I T S M 180
ACGCTTGTCCTCATGTCTCTTGATCGATTCTTGGCCGTAGTTCATCCCATCACTTCCATG 540
TM IV
S V R T E R N A I A A I V V T W V V I L 200
TCGGTTCGGACCGAGAGAAATGCGATAGCTGCAATCGTAGTGACATGGGTGGTTATTTTA 600
L A S V P V Y L S H G E I T Y V Y S S A 220
TTGGCTTCGGTGCCGGTTTATTTGAGTCATGGAGAAATAACCTATGTATACTCATCGGCA 660
E H T A C V F L E A D P I N R P D G Y N 240
GAGCATACGGCCTGCGTGTTCCTAGAGGCGGACCCCATCAACAGACCTGATGGCTACAAC 720
TM V
K L V F Q I I F F L T S Y A T P L A L I 260
AAACTTGTCTTCCAGATAATATTCTTCCTTACGTCTTATGCGACGCCCTTAGCGCTCATC 780
C G L Y L W L L V R L W R G A A P G G H 280
TGCGGATTGTACCTATGGCTTCTGGTACGTTTGTGGCGTGGAGCAGCGCCTGGTGGTCAC 840
V S A E S R R G K K R V T R M V V V V V 300
GTGTCTGCAGAAAGCCGCAGAGGCAAGAAGAGAGTCACAAGAATGGTAGTGGTAGTGGTC 900
TM VI
V I F A V C W F P I Q L I L V L K S A D 320
GTCATATTTGCTGTCTGCTGGTTCCCAATACAGCTGATCCTGGTGCTGAAGAGTGCGGAC 960
TM VII
L Y D I T N T S V M I Q I I S H V L A Y 340
CTATACGACATCACTAACACCAGCGTCATGATACAAATCATAAGTCACGTGCTGGCTTAC 1020
M N S C V N P I L Y A F L S D H F R K A 360
ATGAACTCATGTGTCAACCCGATACTATACGCTTTCCTATCAGACCACTTCCGCAAAGCA 1080
F R K I I K C G K V Q N P Q T L P R Y Q 380
TTCCGCAAGATTATCAAATGTGGAAAGGTTCAAAACCCTCAGACTCTTCCTCGTTATCAA 1140
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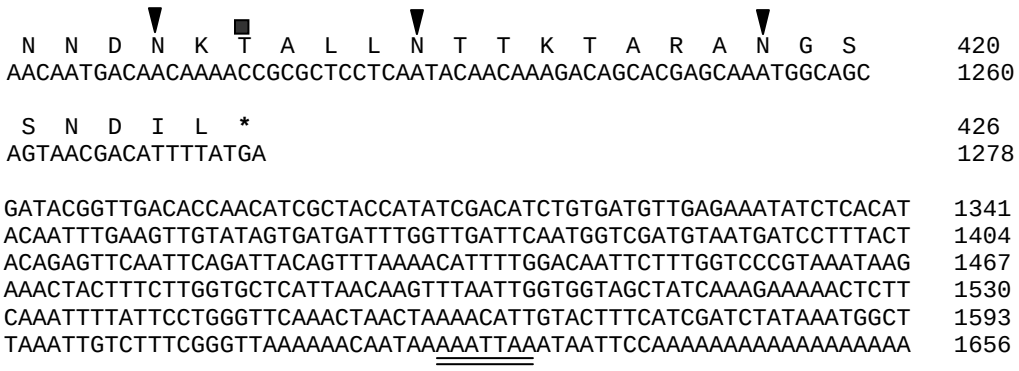


Fig. 2.1 cDNA and deduced amino acid sequences of the *D. punctata* AST receptor (Dippu-AstR). Nucleotides are from 5'- to 3'-end, and the amino acid residues start with the second ATG codon in the open reading frame. The seven membrane spanning domains are indicated by TM I – TM VII. The translation termination codon is indicated by an *asterisk*. In-frame stop codons in the 5'-noncoding region are *underlined*. The putative polyadenylation site in the 3'-noncoding region is *underlined twice*. The putative glycosylation sites, having the N-*X*-S or N-*X*-T consensus sequence, are indicated by *triangles*. *Squares* represent putative protein kinase C phosphorylation sites. *Circles* represent putative protein kinase A phosphorylation sites.

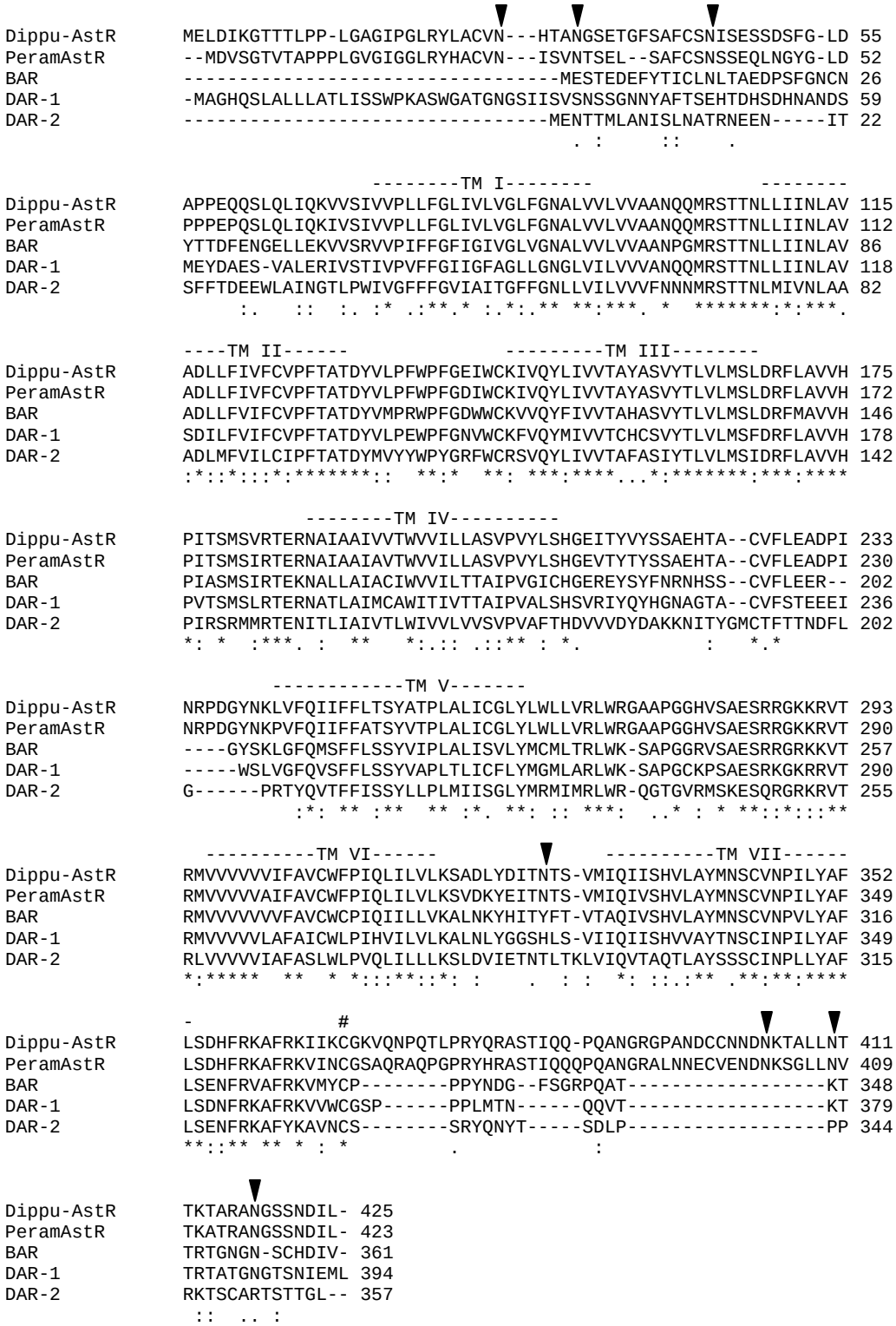

Dippu-AstR MELDIKGTTTLPP-LGAGIPGLRYLACVN---HTANGSETGFSAFCSNISESSDSFG-LD 55
PeramAstR --MDVSGTVTAPPPLGVGIGGLRYHACVN---ISVNTSEL--SAFCSNSSEQLNGYG-LD 52
BAR ----------MESTEDEFYTICLNLTAEDPSFGNCN 26
DAR-1 -MAGHQSLALLLATLISSWPKASWGATGNGSIISVSNSSGNNYAFTSEHTDHSDHNANDS 59
DAR-2 ----------MENTTMLANISLNATRNEEN-----IT 22
--------TM I--------
Dippu-AstR APPEQQSLQLIQKVVSIVVPLLFGLIVLVGLFGNALVVLVVAANQQMRSTTNLLIINLAV 115
PeramAstR PPPEPQSLQLIQKIVSIVVPLLFGLIVLVGLFGNALVVLVVAANQQMRSTTNLLIINLAV 112
BAR YTTDFENGELLEKVVSRVVPIFFGFIGIVGLVGNALVVLVVAANPGMRSTTNLLIINLAV 86
DAR-1 MEYDAES-VALERIVSTIVPVFFGIIGFAGLLGNGLVILVVVANQQMRSTTNLLIINLAV 118
DAR-2 SFFTDEEWLAINGTLPWIVGFFFGVIAITGFFGNLLVILVVVFNNNMRSTTNLMIVNLAA 82
----TM II------
---------TM III--------
Dippu-AstR ADLLFIVFCVPFTATDYVLPFWPFGEIWCKIVQYLIVVTAYASVYTLVLMSLDRFLAVVH 175
PeramAstR ADLLFIVFCVPFTATDYVLPFWPFGDIWCKIVQYLIVVTAYASVYTLVLMSLDRFLAVVH 172
BAR ADLLFVIFCVPFTATDYVMPRWPFGDWWCKVVQYFIVVTAHASVYTLVLMSLDRFMAVVH 146
DAR-1 SDILFVIFCVPFTATDYVLPEWPFGNVWCKFVQYMIVVTCHCSVYTLVLMSFDRFLAVVH 178
DAR-2 ADLMFVILCIPFTATDYMVYYWPYGRFWCRSVQYLIVVTAFASIYTLVLMSIDRFLAVVH 142
--------TM IV----------
Dippu-AstR PITSMSVRTERNAIAAIVVTWVVILLASVPVYLSHGEITYVYSSAEHTA--CVFLEADPI 233
PeramAstR PITSMSIRTERNAIAAIAVTWVVILLASVPVYLSHGEVTYTYSSAEHTA--CVFLEADPI 230
BAR PIASMSIRTEKNALLAIACIWVVILTTAIPVGICHGEREYSYFNRNHSS--CVFLEER-- 202
DAR-1 PVTSMSLRTERNATLAIMCAWITIVTTAIPVALSHSVRIYQYHGNAGTA--CVFSTEEEI 236
DAR-2 PIRSRMMRTENITLIAIVTLWIVVLVVSVPVAFTHDVVVDYDAKKNITYGMCTFTTNDFL 202
------------TM V-------
Dippu-AstR NRPDGYNKLVFQIIFFLTSYATPLALICGLYLWLLVRLWRGAAPGGHVSAESRRGKKRVT 293
PeramAstR NRPDGYNKPVFQIIFFATSYVTPLALICGLYLWLLVRLWRGAAPGGHVSAESRRGKKRVT 290
BAR ----GYSKLGFQMSFFLSSYVIPLALISVLYMCMLTRLWK-SAPGGRVSAESRRGRKKVT 257
DAR-1 -----WSLVGFQVSFFLSSYVAPLTLICFLYMGMLARLWK-SAPGCKPSAESRKGKRRVT 290
DAR-2 G------PRTYQVTFFISSYLLPLMIISGLYMRMIMRLWR-QGTGVRMSKESQRGRKRVT 255
----------TM VI------
----------TM VII------
Dippu-AstR RMVVVVVVIFAVCWFPIQLILVLKSADLYDITNTS-VMIQIISHVLAYMNSCVNPILYAF 352
PeramAstR RMVVVVVAIFAVCWFPIQLILVLKSVDKYEITNTS-VMIQIVSHVLAYMNSCVNPILYAF 349
BAR RMVVVVVVVFAVCWCPIQIILLVKALNKYHITYFT-VTAQIVSHVLAYMNSCVNPVLYAF 316
DAR-1 RMVVVVVLAFAICWLPIHVILVLKALNLYGGSHLS-VIIQIISHVVAYTNSCINPILYAF 349
DAR-2 RLVVVVVIAFASLWLPVQLILLLKSLDVIETNTLTKLVIQVTAQTLAYSSSCINPLLYAF 315
#
Dippu-AstR LSDHFRKAFRKIIKCGKVQNPQTLPRYQRASTIQQ-PQANGRGPANDCCNNDNKTALLNT 411
PeramAstR LSDHFRKAFRKVINCGSAQRAQPGPRYHRASTIQQQPQANGRALNNECVENDNKSGLLNV 409
BAR LSENFRVAFRKVMYCP--------PPYNDG--FSGRPQAT-----------------KT 348
DAR-1 LSDNFRKAFRKVVWCGSP------PPLMTN------QQVT-----------------KT 379
DAR-2 LSENFRKAFYKAVNCS--------SRYQNYT-----SDLP-----------------PP 344
Dippu-AstR TKTARANGSSNDIL- 425
PeramAstR TKATRANGSSNDIL- 423
BAR TRTGNGN-SCHDIV- 361
DAR-1 TRTATGNGTSNIEML 394
DAR-2 RKTSCARTSTTGL-- 357

Fig. 2.2 Amino acid sequence comparison of the *D. punctata* AST receptor (Dippu-AstR) and four other insect AST receptors (PeramAstR, BAR, DAR-1, DAR-2). The seven membrane spanning domains are indicated by TM I – TM VII. Dashed lines represent spaces introduced to optimize alignment. The positions of the amino acid residues are given at the right. The putative glycosylation sites, having the N-*X*-S or N-*X*-T consensus sequence, are indicated by *triangles*. The palmitoylation site is indicated by #. Below the sequence, an * represents identical residues in all sequences in the alignment. A colon (:) indicates conserved substitutions. A single dot (.) indicates semi-conserved substitutions.

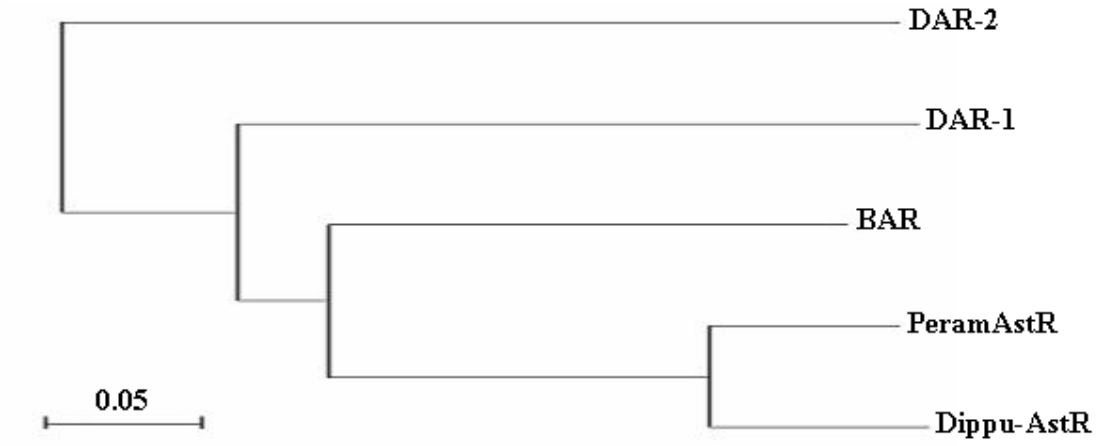


Fig. 2.3 Phylogenetic analysis based on the primary amino acid sequences of Dippu-AstR, PeramAstR, DAR-1, DAR-2, and BAR showing the evolutionary relationship between insect AST receptors. The length of the horizontal lines indicates reciprocally the sequence similarities. The *bar* represents a mutation rate of 5 substitutions per 100 residues.

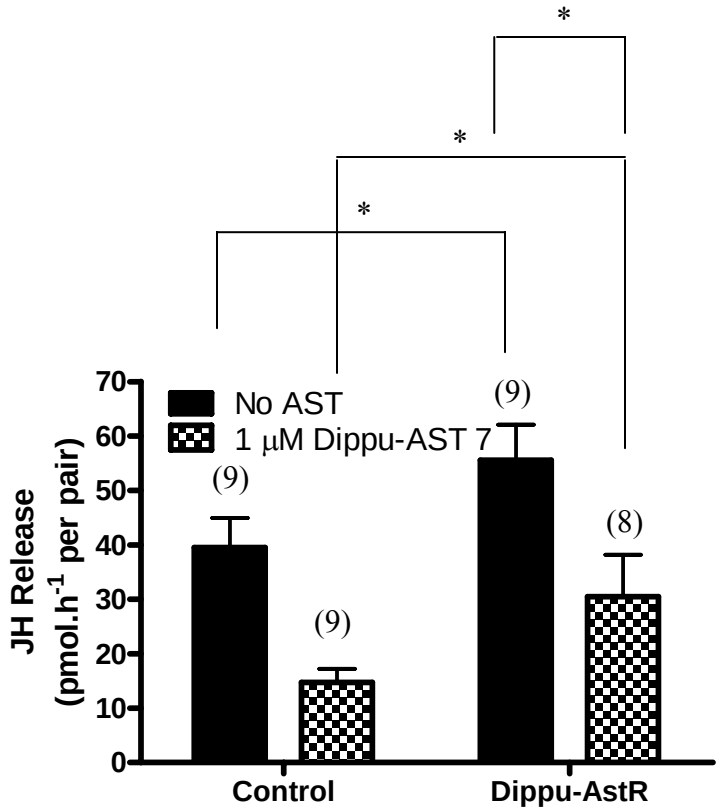


Fig. 2.4 Rates of JH biosynthesis of corpora allata from animals injected with buffer only (control group: untreated (black bar graph) and treated with 1 μM Dippu-AST7 (checkered bar graph)), and animals injected with dsDippu-AstR (Dippu-AstR group: untreated (black bar graph) and treated with 1 μM Dippu-AST7 (checkered bar graph)). 0.5 μg of dsDippu-AstR was injected twice into day 0 and day 1 animals. CA were isolated from individual animals and each assayed for JH release. The control therefore represents the mean of 9 individual animals. This data represents one experiment. Measurements were made after 24 hrs of the second injection of dsDippu-AstR. Each bar represents mean ± SEM for the number of individual measurements indicated at the top of error bars. Asterisks indicate significant differences between control group and Dippu-AstR injected group of animals as determined by t-test (*$P<0.05$).

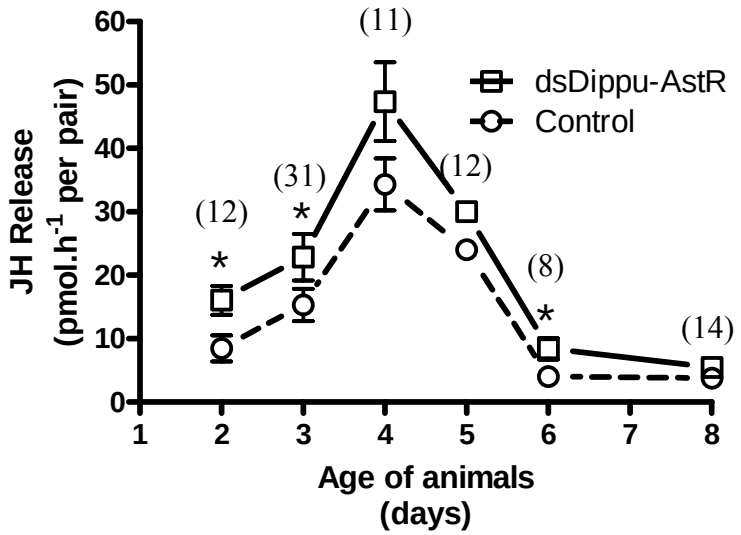
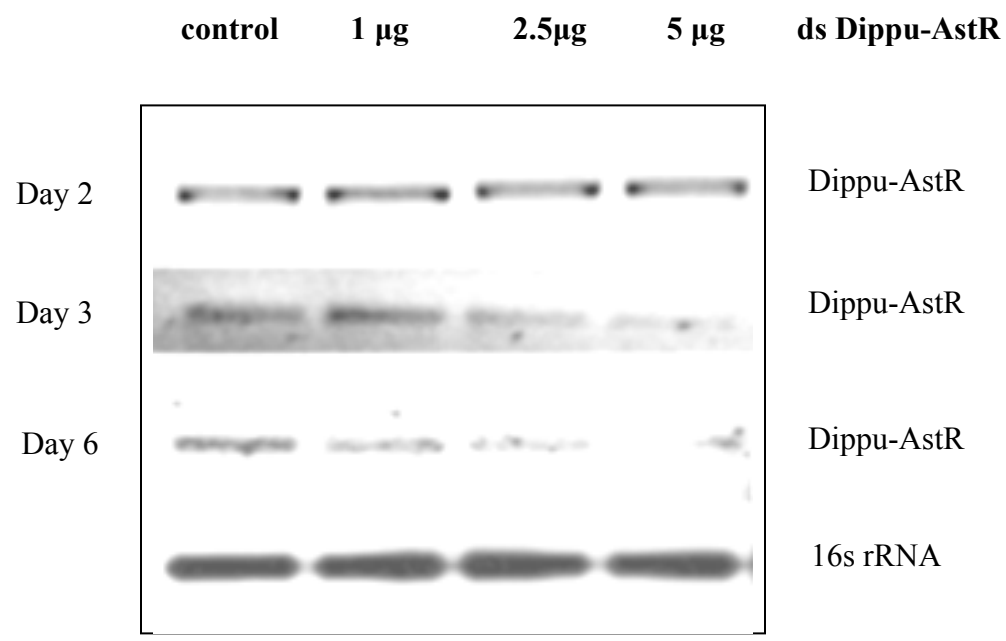


Fig. 2.5 Rates of JH biosynthesis of corpora allata from animals injected with buffer only (control) and animals injected with dsDippu-AstR during the first gonadotrophic cycle of mated female *D. punctata*. 0.5 μg of dsDippu-AstR was injected twice, on day 0 and day 1 animals. Measurements were made after 24 hrs of the second injection of dsDippu-AstR. Measurement on day 2 represents animals assayed 24 hours after the day 1 injection. Animals assayed on day 3 were received the second injection on day 2. Subsequent days were assayed in the same manner. This data represents one experiment. Each point represents mean ± SEM for the number of individual measurements indicated at the top of error bars. Asterisks indicate significant differences between control and Dippu-AstR injected group of animals as determined by t-test (*$P<0.05$).


control
1 µg
2.5µg
5 µg
ds Dippu-AstR
Day 2
Day 3
Day 6
Dippu-AstR
Dippu-AstR
Dippu-AstR
16s rRNA

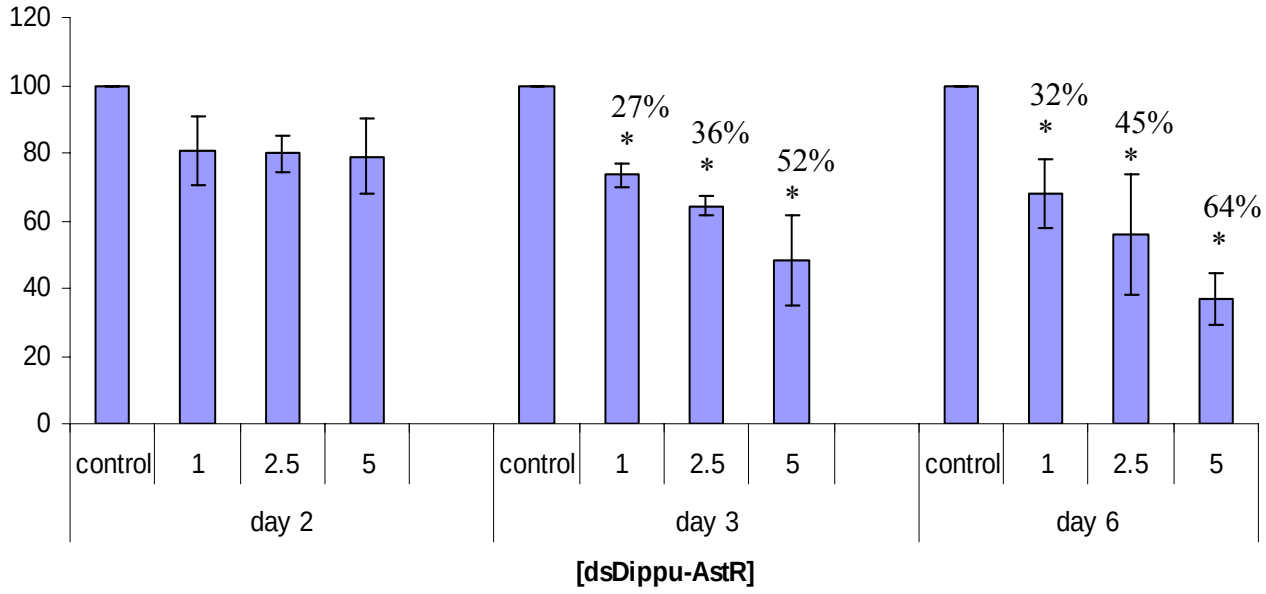

120
100
80
60
40
20
0
27%
*
36%
*
52%
*
32%
*
45%
*
64%
*
control
1
2.5
5
control
1
2.5
5
control
1
2.5
5
day 2
day 3
day 6
[dsDippu-AstR]

Fig. 2.6 Effects of dsRNA treatment on Dippu-AstR mRNA in midgut tissues of adult females *D. punctata*. (A)Dippu-AstR mRNAs from control (animals injected with buffer only) and dsDippu-AstR treated animals were analyzed using RT-PCR. 16s rRNA levels were used as a reference. The data shown are representative of three separate experiments. (B) Quantitative densitometric analysis of the expression of Dippu-AstR mRNA. The data shown are the relative ratios (Dippu-AstR/16s rRNA) measured by densitometry. Data are the mean ± S.E.M. of three independent experiments. In each set of experiments the normalized data for the controls were set to 100%. Values that differed significantly from controls are indicated with an asterisk using ANOVA (*$P<0.05$).

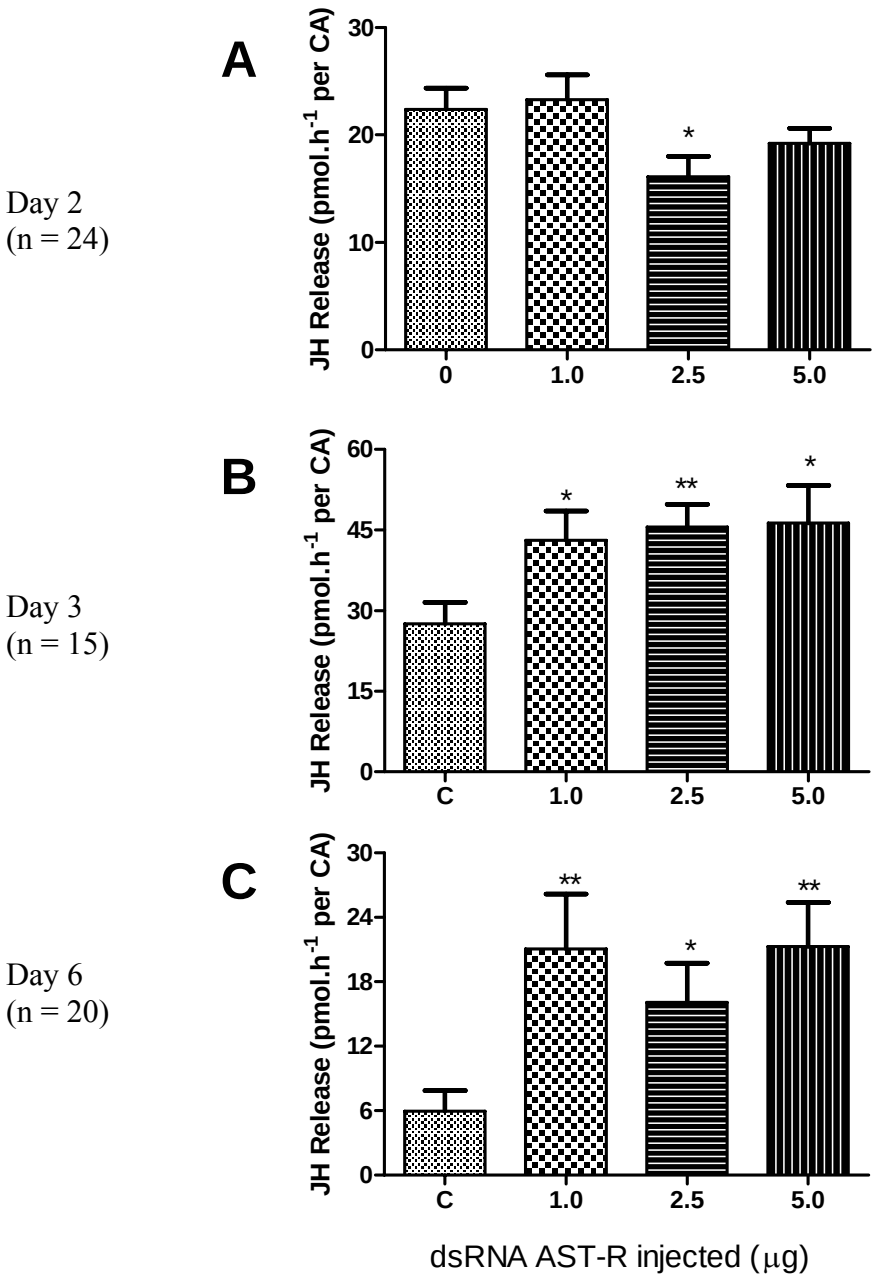


Fig. 2.7 Rates of JH biosynthesis of corpora allata from animals injected with buffer only (control, c) and animals injected with different concentration of dsDippu-AstR (1.0, 2.5, and 5.0 μg). dsDippu-AstR was injected on day 0 animals. Measurements were made on day 2, day 3, and day 6. Each bar represents mean ± SEM for the number of individual measurements (n). Values that differed significantly from controls are indicated with an asterisk using ANOVA (*P<0.05; **P<0.01). This experiment was done once with the number of CA's assayed indicated.

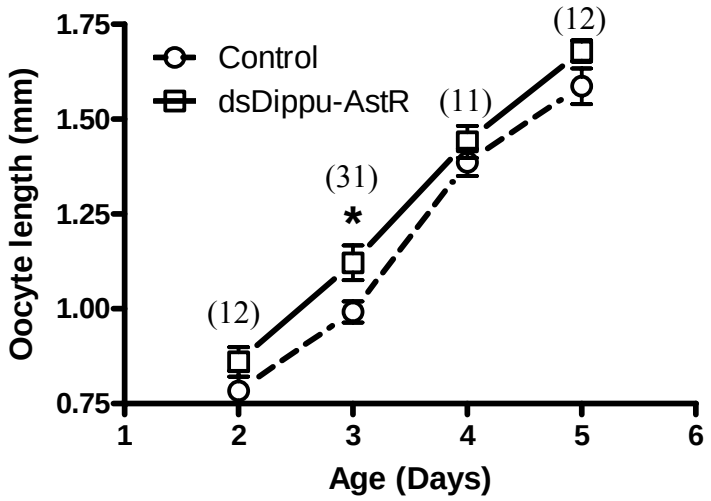
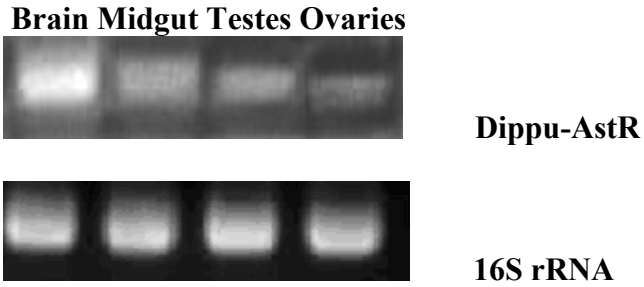


Fig. 2.8 Length of basal oocytes from animals injected with buffer only (control) and animals injected with dsDippu-AstR. Measurements were made as per Fig. 2.5. 0.5 μg of dsDippu-AstR was injected twice on day 0 and day 1 animals. Measurements were made after 24 hrs of the second injection of dsDippu-AstR. Each point represents the mean ± SEM for the number of individual measurements indicated at the top of error bars. Asterisks indicate significant differences between control group and Dippu-AstR injected group of animals as determined by t-test (*$P<0.05$).

A.



B.

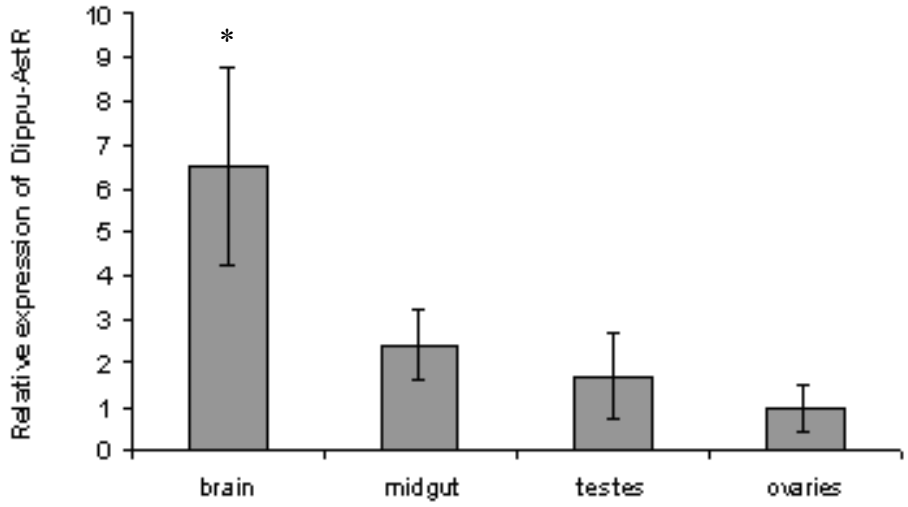


Fig. 2.9 Quantification of Dippu-AstR mRNA levels in brain, midgut, testes, and ovaries of adult *D. punctata*. A) mRNA levels of Dippu-AstR were analyzed using RT-PCR on 1% agarose gel. 16S rRNA levels were used as a reference. Result shown is from one of two experiments. B) Relative expression of Dippu-AstR mRNA using qPCR analysis. RNA extractions were prepared in triplicate (40-50 brains, 10-15 midguts, 30-40 testes, and 20-30 ovaries were required in each RNA extraction). Real time PCR measurement for each sample was done in duplicate. The average of duplicate real time PCR measurements was used to calculate the mean ± SEM for each tissue.16S rRNA served as an internal standard of a housekeeping gene transcript. Asterisks indicate significant differences among the tissues (brain, midgut, testes and ovaries). Statistical analyses were performed using one-way ANOVA (*$P < 0.05$).

2.5. Discussion

The sequence of Dippu-AstR reveals that it is a typical GPCR of 425 aa that most closely resembles PeramAstR. Conservation of the insect AST receptors may not be surprising as most of the AST peptides identified in insects share strong amino acid identity to those found in *D. punctata* [4]. In *Periplaneta,* five of the peptides are identical and the remaining peptides altered by minor conservative substitutions between these two cockroach species [11]. *Periplaneta*, the more primitive oviparous species, displays continuous and overlapping ovarian cycles that are accompanied by on/off switching of JH biosynthesis by cells of the CA [45, 46]. In contrast, *Diploptera* is a viviparous species that incubates embryos in a brood sac during pregnancy. During this period, JH is significantly reduced and basal oocyte development is minimal [43]. If evolutionary alterations in AST precursors, the processed peptides or their respective receptor is linked to the unique reproductive mode in each species such alterations are not obvious. The structural and predicted post-translational modification similarities between AST receptors of cockroaches and insects that do not use this AST family to regulate JH biosynthesis is suggestive of spatial and temporal regulation accounting for the differences in receptor action.

RNA interference (RNAi) has become a powerful tool to suppress gene expression in both invertebrates and vertebrates. RNAi methodology has proven to be an effective means to knock-down AST expression in brain and midgut of *B. germanica* [32]. Although AST mRNA levels were significantly reduced, JH biosynthesis did not increase in any of the treated animals [32]. These results suggest that minimal amounts of AST

peptide may be sufficient to produce inhibition. Conversely, dsRNA targeting of cockroach-type AST gene expression in *G. bimaculatus* and *Spodoptera frugiperda* reduced RNA levels for up to 7 days with resultant increases in JH titers in the hemolymph [35]. This result is surprising since the cockroach-type ASTs have not been shown to inhibit JH biosynthesis by moth CA *in vitro.* In the present study, we used RNAi to reduce Dippu-AST receptor expression and investigated the effects of receptor expression on rates of JH biosynthesis and basal oocyte growth. In a previous study [10], sensitivity of CA to ASTs declines following adult emergence, then increases, and reaches a maximum on days 6-7 as JH biosynthesis declines. Our results indicate that treatment with dsDippu-AstR RNA resulted in a significant increase in JH release in CA of 2, 3 and 6 day old cockroaches. The dose of dsDippu-AstR RNA injected appeared to be saturating as injection of 1µg was as effective as injecting 5µg of RNA. In RNAi dose-response assays, the most dramatic increase (approximately 3.5 fold) in JH biosynthesis was noted on day 6. As JH biosynthesis reaches a maximum on day 5 and dramatically decreases on day 6, the Dippu-AstR may be associated with the decline in JH production. If Dippu-AstR is reduced by dsRNA treatment in CA as it is in midgut (Fig. 2.6) then these comparatively high rates of JH release may be associated with the reduction in expression of Dippu-AstR. Although expression is reduced, the receptor itself has not been eliminated, since the addition of Dippu-AST7 to CA from animals that had been treated with dsDippu-AstR RNA resulted in a decrease in JH release. Thus, although reduction of Dippu-AstR mRNA levels has led to diminished levels of receptor, sufficient receptor protein persists to allow Dippu-AST action. The growth of basal oocytes during the first gonadotrophic cycle in mated *Diploptera* adult females is

dependent on controlled levels of circulating JH. Increasing the levels of JH release through reduction of Dippu-AstR led to a significant effect on growth of basal oocytes on only one of the days (day 3) tested (Fig. 2.8). Although JH was increased, the increase may not have crossed a threshold level that may have been necessary to result in a dramatic difference in oocyte growth.

Whereas DAR-1 and DAR-2 are mainly expressed in the gut, BAR is expressed in both gut and brain and therefore is considered to be a gut-brain receptor [41]. Using two different methods (semi-quantitative RT-PCR, and quantitative real-time PCR), the *D. punctata* receptor is expressed mainly in brain and to a lesser extent in gut and testes, with very little expression in ovaries. The pattern of Dippu-AstR expression observed in these tissues is similar to that of the preproAST mRNA in *B. germanica,* in which the expression of preproAST mRNA is most abundant in brain and to a lesser extent in midgut. Expression was not found in the ovaries [2]. The pattern of Dippu-AstR expression is consistent with expectation relative to the patterns of Dippu-AST mRNA expression in *D. punctata* [17, 18, 51]. However, the ASTs may act at a distance from the tissue of mRNA expression in their capacity as neurohormones. The concentration of receptor may also differ in a tissue-specific manner as different tissue types may express ASTs at differing levels. As an example, expression levels of Dippu-AST mRNA in the ovary are relatively low compared to common and lateral oviducts [18]. Additionally, Dippu-ASTs appear to have a gradient of expression that is more concentrated in the anterior midgut relative to the posterior [51]. Using a photoaffinity label, putative receptors that bind Dippu-ASTs in *D. punctata* have previously been partially

characterized [10, 51]. Feyereisen *et al.* [14] have proposed a model for two types of AST receptors in the CA. As well, Bowser and Tobe [7] have reported a putative AST receptor in the midgut which shows a lower binding affinity than those reported in the CA and brain, suggesting that there are subtle differences between the receptors in the CA and in the midgut [51]. This suggestion was further supported by the results of enzyme activity assays in midgut of *D. punctata* using an AST analog in a range of concentrations known to be active in inhibiting JH biosynthesis [16]. Potentially there is yet another Dippu-AST receptor.

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2.6. References

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CHAPTER 3:

Quantification of Allatostatin Receptor mRNA Levels in the Cockroach, *Diploptera punctata*, using Real-time PCR

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3.1. Abstract

The cockroach allatostatin receptor (Dippu-AstR) is a 425 amino acid G-protein coupled receptor that is related to the mammalian galanin receptor. Using relative standard curve real-time PCR analysis, changes in Dippu-AstR mRNA expression levels were examined in tissues of adult mated and virgin female *D. punctata*. Tissues were chosen that were either known targets of allatostatin (Dippu-AST) action or sites of Dippu-AST localization. Tissues examined included brain, corpora allata, gut, ovaries, testes and abdominal ganglia. Dippu-AstR was expressed in all tissues examined for seven days after adult emergence. Juvenile hormone (JH) biosynthesis is known to peak on day 5 post-emergence in mated females. In mated females, Dippu-AstR mRNA was at the highest levels on day 6 post-emergence in brain and corpora allata and day 2 post-emergence in midgut. Dippu-AstR expression was found to correlate with the decline in JH biosynthesis noted on day 5 post-emergence and early inhibition of feeding. Dippu-AstR mRNA expression in virgin female midgut and CA was dramatically elevated on days 6 and 7, respectively. Expression of Dippu-AstR mRNA was found to be similar in the abdominal ganglia of mated or virgin females. Ovarian Dippu-AstR expression declined to low levels by day 4. Testes exhibited maximal Dippu-AstR mRNA expression on days 4 and 7 of adult life. A role for Dippu-AST in testes of *Diploptera* is unknown.

3.2. Introduction

F/YFGLamide allatostatins (A-type ASTs) are a super family of invertebrate neuropeptides that were originally defined by their action as inhibitors of juvenile hormone (JH) biosynthesis (Bendena et al., 1999; Stay et al., 2007; Tobe et al., 2006). The ASTs act on a G-protein coupled receptor(s) (GPCR(s)) in cells of the corpora allata (CA) to inhibit JH biosynthesis in select insects that include cockroaches (Belles et al., 1994; Ding et al., 1995; Woodhead et al., 1989; Woodhead et al., 1994), crickets (Lorenz et al., 1995) and termites (Yagi et al., 2005). In the cockroach, *Diploptera punctata, in situ* hybridization analysis has demonstrated that ASTs are expressed in many diverse cell types. This accounts for the extensive distribution of the ASTs as shown by immuno histochemistry (Bendena et al., 1999). In the brain, AST expession appears abundant. Approximately 35-40 lateral neurosecretory cells of the pars lateralis (in each brain hemisphere) express ASTs and extend axons in the nervi corporus cardiaci (NCC) II to the corpora cardiaca and to the CA (Stay et al., 1992). Two pairs of cells in the pars intercerebralis express ASTs and function as interneurons sending out axons in each hemisphere that arborize adjacent to the lateral neurosecretory cells. AST containing neurons are found in the optic, central and antennal brain lobes as well as in cells between the optic lobes, between the deutocerebrum and protocerebrum as well as the tritocerebrum. AST immunoreactivity is detected in the NCCIII that extends through the tritocerebrum to arborize with the pulsatile organ muscle (Lange et al., 1993; Stay et al., 1992; Woodhead et al., 1992). In tissues apart from the brain, AST immunoreactive neurons of the ventral ganglia and terminal abdominal ganglia (TAG) extend to the proctodeal nerves that innervate rectal dilator muscles, muscles of the rectum, anterior

hindgut and midgut (Lange et al., 1993; Yu et al., 1995). All thirteen *Diploptera* ASTs have been shown to inhibit both spontaneous and proctolin induced hindgut contractions (Lange et al., 1995). The TAG contains approximately 45 pairs of dorsal and ventral AST immunoreactive cells and four large ventral and 2 large dorsal AST containing unpaired medial neurons. AST immunoreactivity extends from the TAG via ventral nerve 7 to the common and lateral oviducts and through nerve 8 to the bursa copulatrix (Garside et al., 2002). ASTs have no effect on contraction of ovarian muscle (Lange et al., 1995). AST immunoreactivity is also located in open-type midgut endocrine cells (Reichwald et al., 1994; Yu et al., 1995). The highest concentration of these endocrine cells is found at the anterior portion of the midgut and these cells may contribute to the release of ASTs into the hemolymph during starvation (Yu et al., 1995). ASTs also have action on stimulating gut luminal carbohydrate metabolizing enzymes invertase and α-amylase (Fuse et al., 1999). Contribution to the humoral AST concentration is also made from a population of circulating granulated hemocytes which comprise approximately 5% of the total hemocytes (Skinner et al., 1997). As ASTs have been found to innervate the pulsatile organ muscle (Lange et al., 1993) it may be that humoral ASTs are pumped into the antennae to serve a sensory function or cross the perineum to function with actions on the brain.

Although much is known about the sites of expression and distribution of *Diploptera* ASTs, very little is known about the tissues that express the corresponding AST receptor (AstR). Known insect AstRs are rhodopsin-like G-protein coupled receptors (GPCRs) that are orthologs of the mammalian galanin receptor (Birgul et al., 1999). To date, five

insect AstRs have been sequenced. Two from *Drosophila melanogaster,* Alst R1/Dar1(Birgul et al., 1999; Lenz et al., 2000a) and Dar2 (Lenz et al., 2000b), one in each of the cockroaches *Periplaneta americana,* Peram-AstR (Auerswald et al., 2001) and *D. punctata*, Dippu-AstR (Lungchukiet et al., 2007) and one from *Bombyx mori,* Bommo-AstR (Secher et al., 2001). A partial AstR sequence was also identified in the stick insect *Carausius morosus* (Auerswald et al., 2001). In this paper, we quantified the level of expression of Dippu-AstR mRNA levels using quantitative real-time RT-PCR (qRT-PCR). As much is known about the fluctuating levels of ASTs in tissues during the first seven days post-adult emergence we have examined these same tissues with regard to changes in expression levels of Dippu-AstR.

3.3. Materials and methods

3.3.1 Insect rearing and tissue collection

D. punctata were maintained in a colony fed lab chow and water. Freshly molted virgin females adults were isolated and used to stage the appropriate physiological ages. Tissues were carefully dissected and washed repeatedly in ice-cold cockroach saline (150 mM NaCl, 12 mM KCl, 10 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM Hepes, 40 mM glucose). Fat body, trachea and the neural sheath were removed. Tissues were stored in RNAlater (Ambion) at 4^{o}C until sufficient tissue collected. Mating in *D. punctata* occurs immediately following female eclosion. Day 0 tissues were dissected following mating and prior to tanning of the cuticle (0-3 h).

3.3.2 RNA extraction and first strand cDNA synthesis

Total RNA was isolated from tissues using RNeasy mini kit (Qiagen). RNA was resuspended in Rnase-free water and the concentration determined spectrophotometrically. RNA (400-500 ng) was reverse transcribed with reverse transcriptase using the high capacity reverse transcription cDNA kit (Applied Biosystems). The reaction was performed at 25^{o}C for 10 min, 48^{o}C for 30 min, followed by heating at 95^{o}C for 5 min. Two concentrations (100 ng and 1 ng) of cDNA template was used in the real-time RT-PCR experiments for measuring expression level for Dippu-AstR and 16S rRNA, respectively.

3.3.3 Polymerase chain reaction (PCR) optimization for real-time PCR analysis

Target genes	Primer sequence	Product size (bp)
Dippu-AstR	F: 5'-ATCCTGGTGCTGAAGAGTGCGGACC–3'	150
	R: 5'-GCGGAATGCTTTGCGGAAGTGG–3'	
Dippu-AST	F: 5'- TTGGGCAAACGAGCACCATC-3'	160
	R: 5'- CAGAGGACCTTCCAGAGTTGACTG-3'	
16s rRNA	F: 5'- TTACGCTGTTATCCCTAAGGTAA-3'	150
	R: 5'- GTTGGACTACTGTCTTAATT-3'	

PCR reactions (25 µl) consisted of 1 x reaction buffer, 0.2 mM dNTPs, 0.4 mM of each primer, 0.5 U Taq-polymerase, and 100 ng cDNA (Applied Biosystems). After initial DNA-denaturation (5 min at 95^{o}C) PCR was as follows: 35 cycles for 60 sec at 94^{o}C, 30 sec at 55^{o}C for Dippu-AstR and Dippu-AST or 53^{o}C for 16S rRNA followed by 30sec at 72^{o}C. Samples were finally incubated for 10 min at 72^{o}C. Ten µl of each sample was loaded on a 1% (w/v) agarose gel and visualized by staining with ethidium bromide after electrophoresis.

3.3.4 Generation of plasmid cDNA standards

Partial cDNAs for Dippu-AstR, Dippu-AST and 16S rRNA containing the amplicon region for the real-time PCR was generated and inserted into pGEM-Teasy vector. For each assay, a standard curve was obtained by analyzing a dilution series of plasmid cDNA standards for Dippu-AstR, Dippu-AST and 16srRNA, 10-fold dilutions (from $1x10^{8}$ to 10^{2} specific copies) in sterile water. The concentration of plasmid cDNA for each target mRNA species was estimated by measuring the A_{260} in triplicate, using a spectrophotometer. The copy number was calculated using the following formula:

$$\text{Copies / ml} = \frac{6.023 \times 10^{23} \times C \times A_{260}}{MW_t}$$

Where C = 50 µg/ml for double-stranded (ds) DNA and MW_t = cDNA molecular weight (base pairs x 6·5 x 10^{2} Daltons).

After completion of the PCR amplification, the threshold cycle (C_T) values generated from the amplification of plasmid cDNA samples were plotted against the log of cDNA

copy numbers to create a linear standard curve. The real-time PCR analysis of the same series of cDNA standard dilutions was repeated one week later.

3.3.5 qRT-PCR assays

qPCR of Dippu-AstR, Dippu-AST and 16S rRNA was performed on ABI 7500 sequence detector system. All PCR reactions were performed in duplicate for each cDNA sample in a final volume of 25 µl. The reaction master mixes were prepared using 12.5 µl of 2 x reaction buffer (qPCR master mix plus for SYBR Green I low ROX, Eurogentec), 10 µl diluted cDNA corresponding to 100 ng of total RNA for Dippu-AstR and Dippu-ASt; and 1 ng for 16S rRNA, 1.75 µl for each of the 10 µM Dippu-AstR forward and reverse primers, 2.5 µl for each of the 10 µM 16S rRNA forward and reverse primers. Cycling conditions include initial denaturation (15 min at 95°C), amplification, and quantification program repeated 45 times (15 sec at 95°C, 30 sec at 55°C for Dippu-AstR, and Dippu-AST; and 53°C for 16S rRNA, and 36 sec at 72°C with a single fluorescent measurement at the end of each elongation step) and dissociation protocol (60°C to 95°C by 1°C increments followed by a 30-sec hold and fluorescent measurement). No-template control tubes (NTC), containing water instead of template mRNA, were run under the same conditions for each gene.

3.3.6 Data analysis

Real-time PCR efficiencies were calculated from the slopes of the standard curves according to the equation $E = 10^{(-1/slope)}$ (Rasmussen, 2001). A slope of -3.32 is the theoretical maximal slope obtained by regression analysis of a PCR reaction of optimal

efficiency. The relative standard curve method was used to calculate the fold change different between test samples (day 1-day 7 tissues) and a calibrator sample (day 0 tissue). The standard curves were plotted between C_T value and log copy number. A measurement of the mRNA copy numbers in an experimental sample for each gene of interest was determined by interpolating the cDNA copy number from the plasmid cDNA standard curve. The determination of RNA copy numbers assumed that reverse transcription of RNA to cDNA was 100% efficient. The relative quantification calculations are as followed:

Copy number = $(C_T\text{-}C)/M$

Where C_T is the C_T at linearity of the unknown gene, M is the slope, and C is the y-intercept.

$$\text{Normalized target (test sample, calibrator sample)} = \frac{\text{Copy number of Target}}{\text{Copy number of endogenous control}}$$

$$\text{Fold difference} = \frac{\text{Normalized target (test sample)}}{\text{Normalized target (calibrator sample)}}$$

For the relative quantification of each transcript, average comparative gene expression was determined from duplicate qPCRs. Standard deviations were reported with the fold-difference result.

3.3.7 Statistical analysis

Differences of mRNA expression levels of Dippu-AstR in *D. punctata* between day 0 and those at the different day after adult emergence were statistically analyzed by one-way ANOVA and independent-sample *t*-test respectively, with a significance level at the $p < 0.05$ level.

3.4. Results

3.4.1 Standard curves

Standard curves were generated from serial dilutions of plasmid cDNA standards for Dippu-AstR and 16S rRNA (Fig. A.3). Standard curves for each plasmid cDNA standard demonstrated linearity over the complete range of dilution series with correlation coefficients of 0.99. No amplification was observed in the no-template control (NTC) reactions up to a maximum of 45 cycles. The most concentrated and the most diluted cDNA sample of each mRNA provided C_T values, spanning the range of C_T values expected for unknown samples. Primer dimers were never detected for any gene assayed using either melt-curve analysis or agarose gel analysis (Fig. A.4).

3.4.2 Relative expression of Dippu-AstR

3.4.2.1 Brain

Expression of Dippu-AstR mRNA in brains of mated females remained relatively constant until day 6 when mRNA levels significantly increased almost 5-fold (Fig. 3.1). This pattern of expression was different from that of Dippu-AST mRNA expression which was steadily declined to significantly lower on day 5. After day 5 Dippu-AST

mRNA level increased to a maximum on day 6 and declined thereafter as did Dippu-AstR (Fig. 3.1). In comparison, Dippu-AstR mRNA levels in virgin females remained relatively low and never exhibited the peak of enhanced expression on day 6 after adult emergence (Fig. 3.2). Expression of Dippu-AST fell to significantly low levels by day 5 (Fig. 3.2).

3.4.2.2 Corpora allata (CA)

Expression levels of Dippu-AstR mRNA in CA of mated females significantly decreased after emergence then remained relatively constant. Expression levels of mRNA significantly increased about 4-fold on day 6 compared to those measured on day 0 (Fig. 3.3). In virgin female CA, Dippu-AstR mRNA levels were relatively low after adult emergence, and then continued to rise to significantly higher levels on day 7 at which Dippu-AstR mRNA levels were elevated about 40-fold compared to those measured on day 0 (Fig. 3.4).

3.4.2.3 Midgut

Midgut Dippu-AstR mRNA expression of mated females significantly increased about 8-fold on day 2 followed by a decline by day 4. The mRNA levels remained relatively constant for the rest of the gonadotropic cycle (Fig. 3.5). Expression of Dippu-AST mRNA revealed an opposite relationship. On day 2, when Dippu-AstR showed the highest level of expression, Dippu-AST was expressed at the lowest level. Similarly, the maximal increase in Dippu-AST mRNA expression was found on day 6 when Dippu-AstR returned to a low level (Fig. 3.5). Virgin midgut Dippu-AstR mRNA expression levels gradually increased after emergence and rose to a maximum on day 6 (90-fold

compared to those measured on day 0) (Fig. 3.6).This expression pattern differed from Dippu-AST expression which exhibited a peak of expression on day 3 then returned to low levels (Fig. 3.6).

3.4.2.4 Abdominal ganglion

Low levels of Dippu-AstR mRNA were detected on all days tested in abdominal ganglion of both virgin and mated females (Fig. 3.7). No significant changes were noted.

3.4.2.5 Ovaries

In ovarian tissue of mated females, minor variations in levels of Dippu-AstR mRNA expression were found. No significant changes were noted. The levels of Dippu-AST fell rapidly, reaching a minimum by day 2 but were elevated thereafter to levels comparable to day 0 (Fig. 3.8).

3.4.2.6 Testes

Expression of Dippu-AstR in testes appeared to significantly increased by approximately 3-fold on day 4, then declined before increasing again on day 7 reaching a 4-fold level of elevation of expression relative to day 0 (Fig. 3.9).

3.5. Discussion

In the present study, we have chosen tissues for RT-PCR analyses that are either known targets of Dippu-AST action or sites of its localization: i.e. brain, corpora allata, gut, ovary, abdominal ganglia and testes. Our study shows that Dippu-AstR is expressed in these tissues during the first seven days of adult life. JH biosynthesis is maximal on day 5 in mated females and expression of Dippu-AstR mRNA reaches its highest levels one day later in brain and corpora allata. Expression of Dippu-AstR is maximal in midgut of day 2 mated females and these high levels may function in the inhibition of feeding at this stage. In virgin females, expression of Dippu-AstR mRNA in midgut and CA is dramatically elevated on days 6 and 7, respectively. No significant fluctuations in Dippu-AstR expression was noted in the abdominal ganglia of either mated or virgin females. Similarly, no significant differences were found in expression of ovarian Dippu-AstR in mated females throughout the gonadotrophic cycle. Testes also express Dippu-AstR, with maximal levels on days 4 and 7.

4.1 JH biosynthesis in Diploptera

The distribution of Dippu-AST expression in brain is extensive and thus, Dippu-ASTs likely regulate more functions than have currently been assayed. In one circuit, Dippu-ASTs are translated and processed in medial neurosecretory cells that arborize with lateral Dippu-AST-expressing neurosecretory cells of the protocerebrum (Ding et al., 1995; Stay et al., 1992). Axons arising from these lateral cells innervate the CA. Accordingly, Dippu-AstR could be critical for communication between Dippu-AST-expressing brain neurons and in the regulation of JH biosynthesis.

Previous studies (Johnson et al., 1993; Tobe, 1980) showed a precise relationship between oocyte length and rate of JH production during the first gonadotrophic cycle. Following adult emergence, basal oocytes are previtellogenic and JH biosynthesis is correspondingly low. By days 2-3, the oocytes become vitellogenic and rates of JH biosynthesis begin to rise, reaching a maximum on day 4-5 as vitellogenesis continues (Stay et al., 1977; Tobe et al., 1977). On days 5 and 6, rates of JH biosynthesis decline rapidly, while vitellogenesis and chorionation of oocytes is completed. Oviposition occurs on day 7, by which time JH biosynthesis is low. Mating and the ovary are required for the cycle of JH biosynthesis (Stay et al., 1978; Stay et al., 1983) and accordingly, virgin *D. punctata* do not show a cycle of JH biosynthesis associated with oocyte maturation (Stay et al., 1977; Stay et al., 1978).

4.2 Dippu-AstR expression in brain and CA

The pattern of Dippu-AstR mRNA expression is similar in brains and CA of mated females. Dippu-AstR mRNA expression in brain and CA is relatively constant for the first five days, dramatically increases on day 6 and then declines. This decrease in receptor level parallels the loss in Dippu-AST sensitivity that occurs following ovulation (Stay et al., 1991). The relatively constant level of Dippu-AstR mRNA expression in mated female brain and CA between days 1 to 5 suggests that this receptor may not be required to suppress JH biosynthesis during this period but may be necessary to lower rates of JH biosynthesis following the peak that typically occurs on day 4-5.

The CA of virgin females produce JH at low rates that are similar to those of pre- and post-vitellogenic females (Stay et al., 1977). Although the brains of virgin females express a low level of Dippu-AstR, virgin CA show a 40-fold increase in Dippu-AstR expression on day 7 and a 90-fold increase in expression on day 6. It is rare for virgins to mate after the first two days of adult life (Stay et al., 1977) and accordingly, reduced rates of JH production might be expected. Because Dippu-AstR expression is relatively low on the first six days post-eclosion, this may, in part, account for the low rates of JH biosynthesis in virgins. However, JH biosynthesis in virgins continues at a basal level and these animals gradually produce mature but non-viable eggs.

4.3 Dippu-AstR expression in Gut

Expression of Dippu-AstR mRNA in midgut of mated female peaks on day 2 and then declines rapidly prior to the elevation in JH production. This observed rise is consistent with an increase in food intake by mated females in preparation for oocyte maturation.

Food consumption might also be expected to decrease in virgins as a result of the much reduced rate of oocyte maturation. The peak in expression of Dippu-AST mRNA on day 3 may also result in production of ASTs that reduce feeding with increasing Dippu-AstR expression. The expression of Dippu-AstR in midgut may be complex as these receptors may serve both to inhibit midgut contraction, yet may stimulate secretion of digestive enzymes (Fuse et al., 1999) to enhance the efficiency of food absorption. The role played by midgut Dippu-AstR may be further complicated by interactions with other receptors

such as that of leucomyosuppressin that functions as an inhibitor of feeding in *Blattella germanica* (Aguilar et al., 2004).

4.4 Dippu-AstR expression in the Reproductive system

In day 1-4 mated females, Dippu-AST expression is found in 45 pairs of ventrally and dorsally located cells in the terminal abdominal ganglion (TAG). Four large ventral and two dorsal unpaired medial neurons as well as ventral nerve 7 that innervate the ovary also express Dippu-AST (Garside et al., 2002). Dippu-AST expression was observed in 40 or more TAG cells and in the posterior and anterior branches of the proctodeal nerve exiting the TAG and innervating the muscles of the rectum and anterior hindgut of virgin females (Lange et al., 1993). Given the apparent central function of Dippu-AST innervation of both ovaries and hindgut and associated musculature arising from the TAG, it may be that Dippu-AstR is present at a fairly constant level to shut down particular neuronal pathways. The *Drosophila* AstR has recently been shown to effect selective and quickly reversible neuronal inhibition in mammalian systems (Lechner et al., 2002; Tan et al., 2006).

Ovaries and oviducts of *D. punctata* express Dippu-ASTs. However, Dippu-ASTs are found in nerves branching on the surface of the oviduct, but not in the lateral or common oviducts or ovary (Garside et al., 2002). Dippu-ASTs also occur in the oocyte cytoplasm at the micropyle and on the surface of the chorion, in the lumen of the follicle cell sheath and the lateral oviducts, in epithelial cells of the oviduct, in nerves leading to the muscles of the oviduct and in yolk cells. Localization suggests that Dippu-ASTs may function in

ovulation, and in yolk utilization in the fertilized egg (Woodhead et al., 2003). No role has been ascribed to AST in testes of *D. punctata*. However, the fluctuating levels of Dippu-AstR in adult males suggest that ASTs may also have a function in these animals. This role may be in the regulation of development, since the silencing of AST expression in crickets through RNA inhibition resulted in reduced development of testes (Meyering-Vos et al., 2006).

The ability to detect the Dippu-AstR in all tissues tested at times and levels that correlate with the relative levels of Dippu-AST expression may suggest that that only one receptor is necessary to coordinate the functions of the ASTs. However, certain inconsistencies such as midgut expression in mated females in which the levels of neuropeptide mRNA and receptor mRNA do not entirely coincide may suggest that a second receptor is operative.

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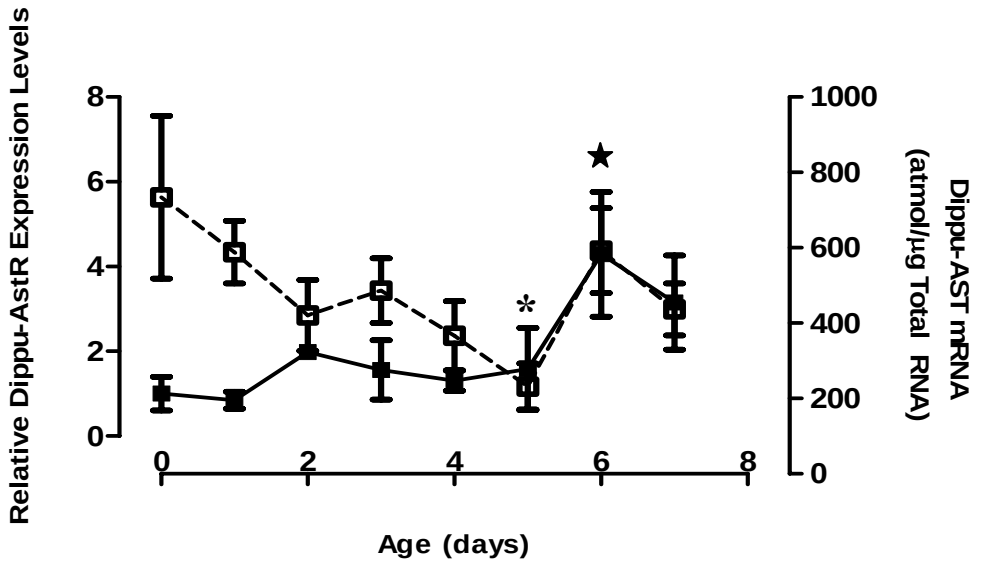

Fig. 3.1 Relative expression levels of Dippu-AstR mRNA (filled squares) in brains of mated female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 30 – 50 brains. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Statistical analysis was performed using ANOVA and student's t-test, respectively. $P<0.05$ was considered as significant. Star indicates values significantly different from day 0 values for Dippu-AstR mRNA. QC-RT-PCR quantification of Dippu-AST expression (open squares) is plotted for comparison. Asterisk indicates values significantly different from day 0 values for Dippu-AST mRNA (data from Garside et al. 2003).

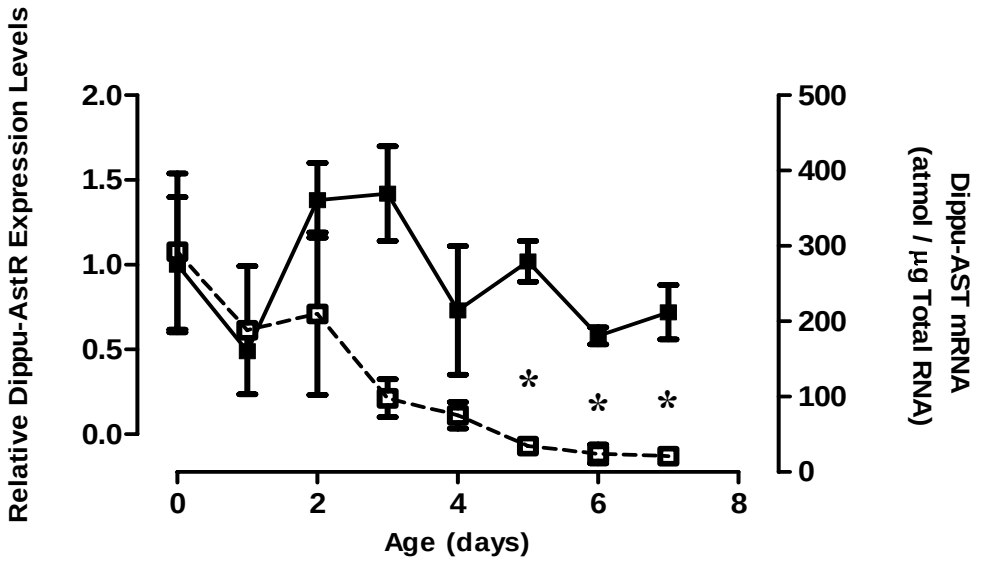

Fig. 3.2 Relative expression levels of Dippu-AstR mRNA (filled squares) in brains of virgin female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 30 – 50 brains. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Statistical analysis was performed using ANOVA and student's t-test, respectively. $P<0.05$ was considered as significant. QC-RT-PCR quantification of Dippu-AST expression (open squares) is plotted for comparison. Asterisk indicates values significantly different from day 0 values for Dippu-AST mRNA (data from Garside et al. 2003).

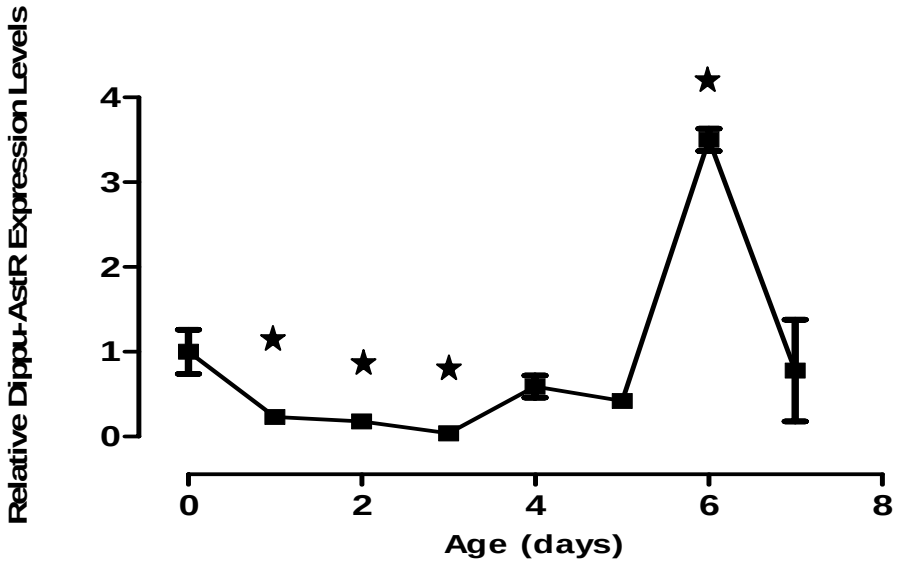

Fig. 3.3 Relative expression levels of Dippu-AstR mRNA in CA of mated female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 70 - 80 CA. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Star indicates values significantly different from day 0 values using ANOVA and student's t-test, respectively ($P < 0.05$).

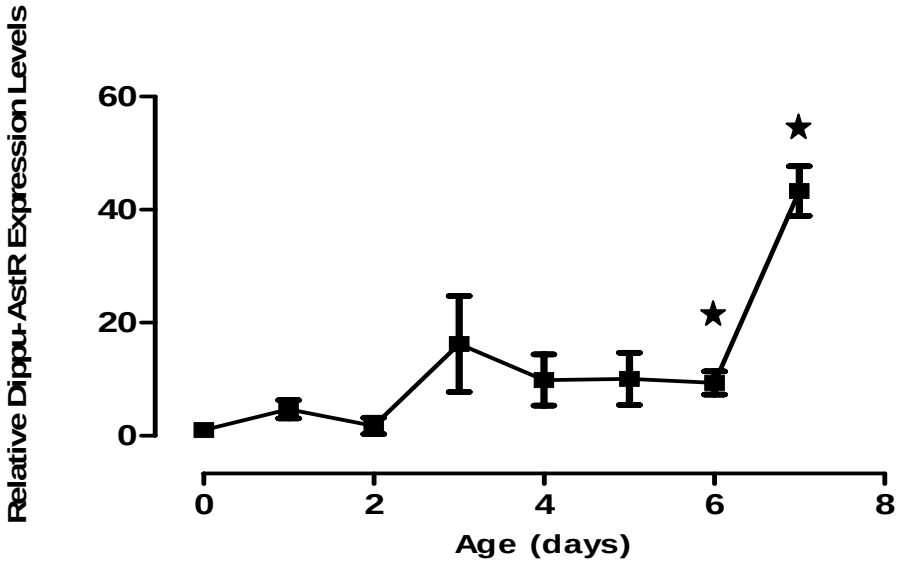

Fig. 3.4 Relative expression levels of Dippu-AstR mRNA in CA of virgin female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 70 - 80 CA. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Star indicates values significantly different from day 0 values using ANOVA and student's t-test, respectively ($P < 0.05$).

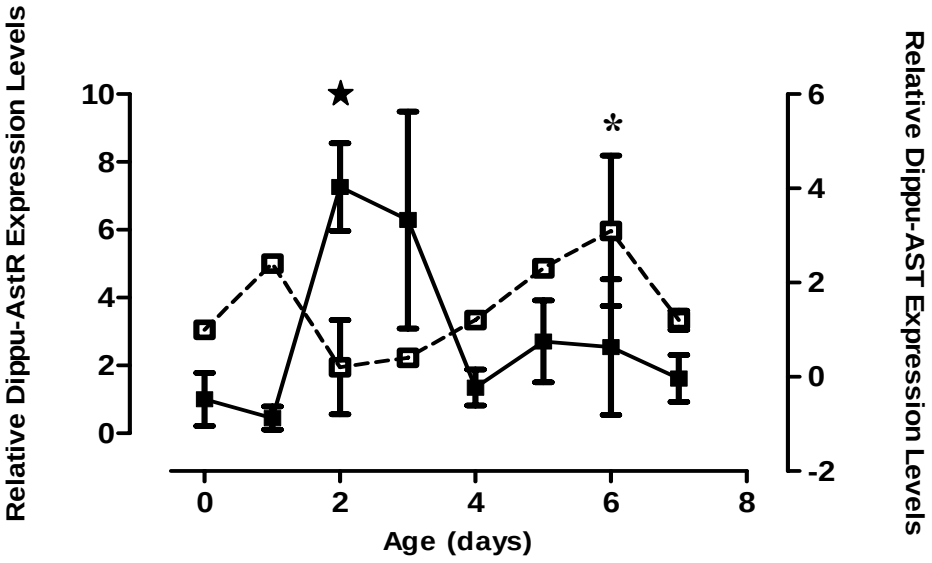

Fig. 3.5 Relative expression levels of Dippu-AstR mRNA (filled squares) in midgut of mated female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 10 midguts. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Statistical analysis was performed using ANOVA and student's t-test, respectively. $P<0.05$ was considered as significant. Star indicates values significantly different from day 0 values for Dippu-AstR mRNA. qPCR quantification of Dippu-AST expression (open squares) is plotted for comparison. Asterisk indicates values significantly different from day 0 values for Dippu-AST mRNA.

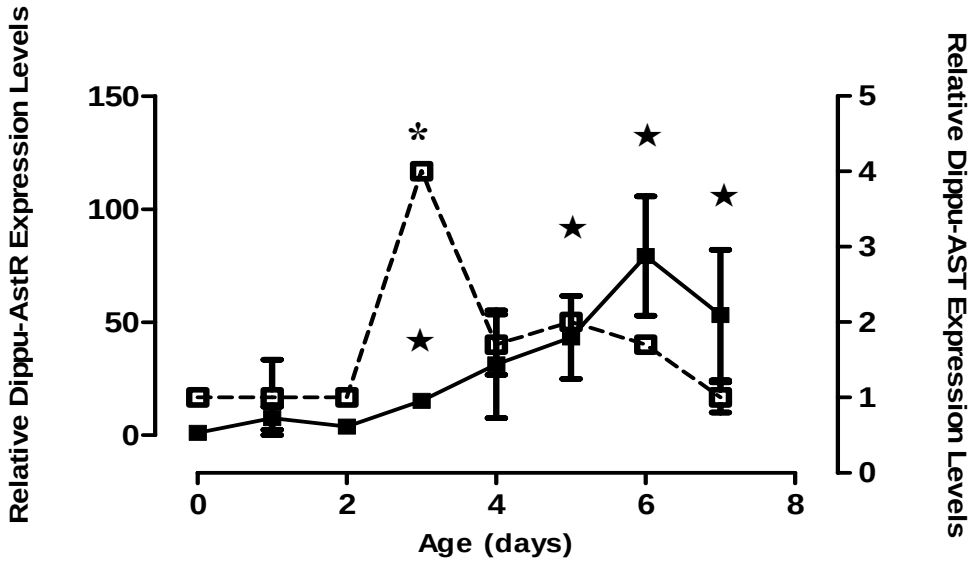

Fig. 3.6 Relative expression levels of Dippu-AstR mRNA (filled squares) in midgut of virgin female *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 10 midguts. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Statistical analysis was performed using ANOVA and student's t-test, respectively. $P<0.05$ was considered as significant. Star indicates values significantly different from day 0 values for Dippu-AstR mRNA. qPCR quantification of Dippu-AST expression (open squares) is plotted for comparison. Asterisk indicates values significantly different from day 0 values for Dippu-AST mRNA.

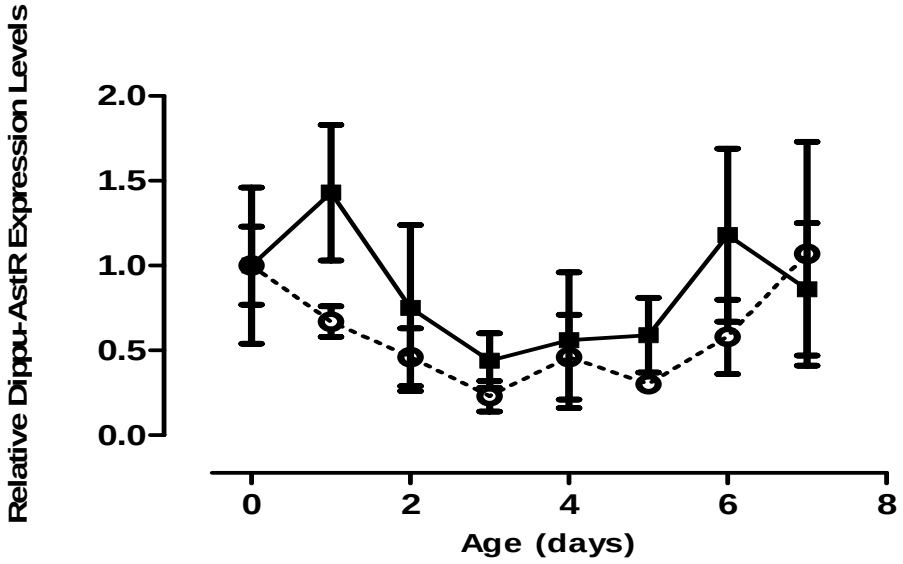

Fig. 3.7 Relative expression levels of Dippu-AstR mRNA in abdominal ganglion day 0 – day 7 post-emergence of mated (filled squares) and virgin (open circles) female *D. punctata*. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 50 - 70 abdominal ganglion. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Data shown are not significantly different ($P > 0.05$). Statistical analysis was performed using ANOVA and student's t-test, respectively.

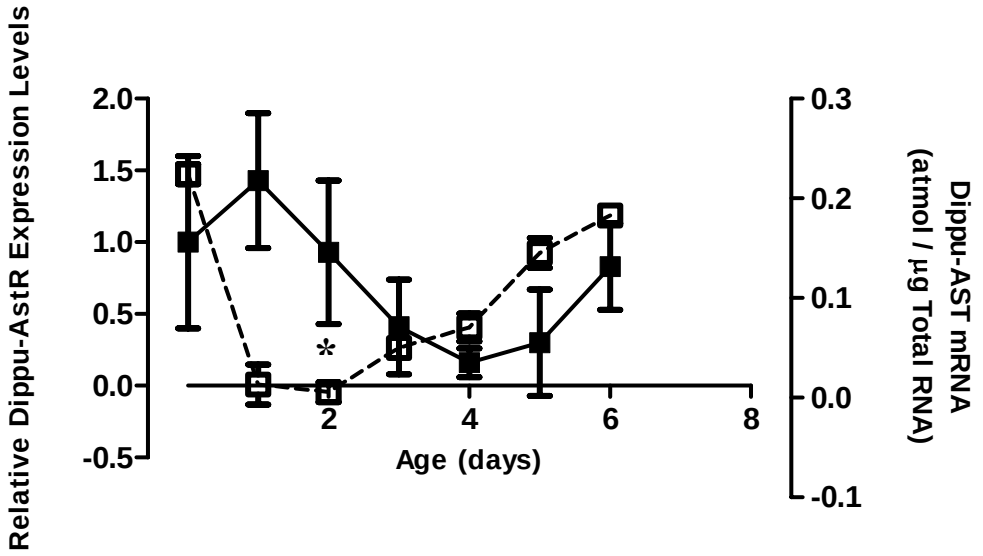

Fig. 3.8 Relative expression levels of Dippu-AstR mRNA (filled squares) in ovaries of mated *D. punctata* day 0 – day 6 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 30 – 40 ovaries. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Statistical analysis was performed using ANOVA and student's t-test, respectively. $P<0.05$ was considered as significant. QC-RT-PCR quantification of Dippu-AST expression (open squares) is plotted for comparison. Asterisk indicates values significantly different from day 0 values for Dippu-AST mRNA (data from Garside et al. 2002).

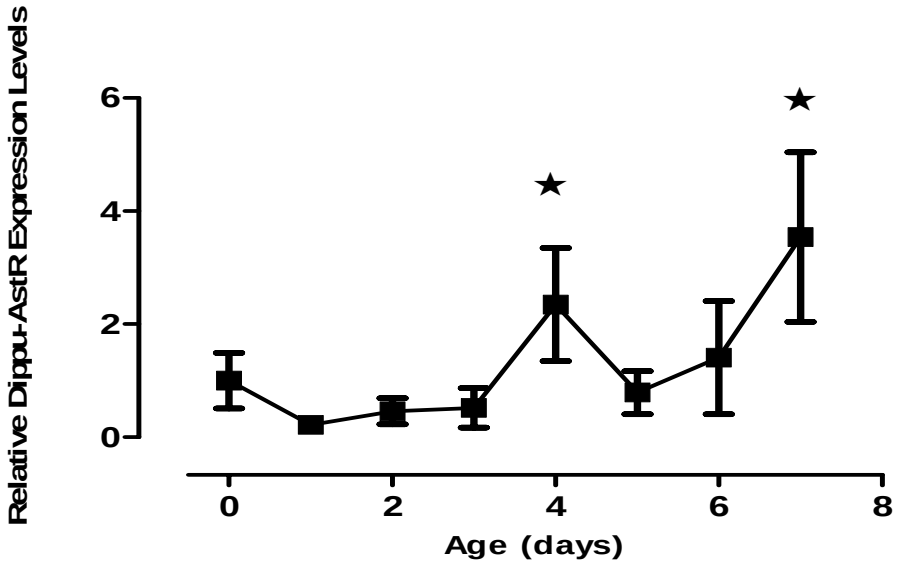

Fig. 3.9 Relative expression levels of Dippu-AstR mRNA in testes of *D. punctata* day 0 – day 7 post-emergence. Each point represents the mean ± SEM of two independent RNA extractions that were each assayed by qPCR three times. For each time point, RNA was isolated from 40 - 50 testes. Expression levels were normalized using 16S rRNA as a standard. Relative expression was calculated by comparison to RNA expression values of day 0 animals. Star indicates values significantly different from day 0 values using ANOVA and student's t-test, respectively ($P < 0.05$).

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CHAPTER 4:

General discussion

4.1 Structure of the cockroach *D. punctata* allatostatin receptor (Dippu-AstR)

The goal of this thesis was to isolate an allatostatin receptor from the cockroach *D. punctata*. *D. punctata* AST receptor is a GPCR of 425 amino acid that is similar to the cockroach *P. americana* AST receptor. Seven of the N-linked glycosylation sites are predicted, three in the extracellular NH_2-terminus, one between TM VI and VII, and three in the intracellular carboxyl-terminus which are conserved between Dippu-AstR and *P. americana* AST receptor. Intracellular sites are unlikely to be glycosylated. The presence of N-glycosylation sites in the N-terminal domain is a common characteristic of GPCRs. For example, studies with N-glycosylation of the rat angiotensin II receptor (Deslauriers *et al*. 1999) showed that receptors with all three defective N-glycosylation sites are not expressed on the plasma membrane, but instead are accumulated in the endoplasmic reticulum. Another study (Davis *et al*. 1995) also reported that removal of six N-glycosylation sites on rat lutropin receptor had no effect on binding to human chorionic gonadotropin, although glycosylation may be involved in proper receptor folding (Davis *et al*. 1997).

In the present study, we observe nine PKC phosphorylation sites and five PKA phosphorylation sites in the intracellular loop of the Dippu-AstR. GPCRs play an important role in many physiological processes. The receptor is regulated after the initial

response to prevent further G-protein-mediated signaling in a process called desensitization (Lefkowitz 1988). Desensitization starts by phosphorylation of the intracellular carboxy terminus and the third loop of the receptor either by G-protein receptor kinases (GRKs) or cellular kinases, including protein kinase A (PKA) and protein kinase C (PKC). PKC requires Ca^{2+}, diacylglycerol (DAG), and a phospholipid such as phosphatidylcholine for activation. PKA and PKC are directly activated by many cellular GPCRs, and the activation of these kinases can lead to the desensitization of any GPCR containing phosphorylation sites for these particular kinases.

As means of testing the function of the AST-like receptor, the technique of double-stranded RNA interference (RNAi) was tested. RNAi is a powerful genetic tool to silence gene expression. We injected RNAi, made to the AstR, into adult cockroaches and measured JH biosynthesis and oocyte length. Measurable differences were noted but the degree of inhibition of AstR mRNA was not measured in CA. Thus, although our data is suggestive of this receptor being reduced with a subsequent increase in JH biosynthesis further work is necessary to prove this reduction is due to the RNAi inhibition. I have shown that RNAi is capable of reducing midgut AstR levels in a dose dependent manner.

4.2 Expression patterns of Dippu-AstR mRNA

4.2.1 Comparison of conventional RT-PCR and quantitative real-time pcr (qPCR)

Real-time RT-PCR was used to quantitate Dippu-AstR mRNA levels in various tissues of adult *D. punctata*. Unlike conventional PCR that detects the PCR product amplification at the end-point analysis. Quantitative real-time PCR (qPCR) is a preferable alternative to

other forms of quantitative PCR. qPCR system is based on the detection and quantitation of a fluorescent reporter so the signal increases in direct proportion to the amount of PCR product in the reaction. The sensitivity of real-time RT-PCR overcomes the drawback of other less sensitive methods, particularly with the analysis of low abundance mRNAs, such as northern blot analysis. In this study, duplicates of total RNA extraction, reverse transcription, and RT-PCR reaction show evidence of a high degree of reproducibility. Although no measurement was made to determine the efficiency of first-strand cDNA synthesis from total RNA, we assume that the efficiency of the reverse-transcription reaction is constant for all samples. Even so, small differences in cDNA levels can result in large changes in the calculation of fold. This must be taken into account when interpreting data obtained by real-time RT-PCR analysis. The use of an endogenous reference to control for analytical variation is important for normalization from different tissues. The quality of RNA and the efficiency of reverse transcription are known to account for most of the variability in qRT-PCR. To minimize these errors and to correct for sample-to-sample variation, mRNA amounts were normalized against an endogenous reference.

The results of Dippu-AstR mRNA expression in various tissues of adult *D. punctata* were confirmed by the conventional RT-PCR and the qPCR. The results suggest that the Dippu-AstR gene is expressed in brain, gut, testes, and ovaries. Expression levels of Dippu-AstR mRNA during the first gonadotrophic cycle in mated female of cockroach *D. punctata* were investigated using qPCR. Virgin females were assayed at the same age for comparison. The receptor was expressed in all the tissues studied and the timing of

expression of the *D. punctata* AST receptor is as would be expected for a receptor that would act to inhibit the peak of JH production.

4.2.2 qPCR analysis of Dippu-AstR mRNA in various tissues

It was demonstrated that levels of Dippu-AST mRNA in the brain of mated and virgin females of *D. punctata* change significantly during early adult life (Garside *et al*. 2003). The cellular pattern of brain Dippu-AST expression is extensive and thus exerts more regulatory functions than have currently been assayed. In one circuit, Dippu-ASTs are translated and processed in medial neurosecretory cells that arborize with lateral Dippu-AST expressing neurosecretory cells of the protocerebrum (Ding et al., 1995; Stay et al., 1992). These lateral cells send projections that innervate the CA. Dippu-AstR should thus be critical for communication between Dippu-AST expressing brain neurons as well as integrating Dippu-AST release from several tissue sources.

The inverse relationship between levels of Dippu-AST mRNA and the titer of JH biosynthesis was observed in mated female. We investigated the pattern of expression levels of Dippu-AstR mRNA using qPCR with the results showing close correlation with that observed for Dippu-AST, and titer of JH biosynthesis. In mated females, a low JH titer is found in the first part of the first gonadotrophic cycle, when Dippu-AST and Dippu-AstR mRNA levels are relatively high. JH titer then reaches the maximum level on days 4-5, when Dippu-AST mRNA level is at the lowest, and Dippu-AstR mRNA is relatively low. JH titer is declining rapidly to low titer after day 6, whereas Dippu-AST and Dippu-AstR reaches the maximum level of mRNA expression. These data suggest

that Dippu-AST and its receptor do regulate the rates of JH biosynthesis during the gonadotrophic cycle of mated females.

Previous study in virgin females showed the general pattern of Dippu-AST observed in mated female is preserved but at lower levels (Garside *et al*. 2003). We observed the same trend of Dippu-AstR mRNA expression in this present study. qPCR of Dippu-AstR mRNA expression in virgin females *D. punctata* shows the closely correlation to those observed in levels of expression in Dippu-AST mRNA. After adult emergence Dippu-AST mRNA levels are relatively high as in Dippu-AstR. Levels of Dippu-AST begin to decline on day 1 and continue to fall to the lower levels on day 5-7 (Garside *et al*. 2003). The same expression pattern can be observed in Dippu-AstR. The levels of Dippu-AstR in mated females are higher than those observed in virgin females. These data support the suggestion from Garside *et al*. (2003) that mated females respond very quickly to the stimuli of mating and that the high titer of JH in day 4-5 mated females leads to a stimulation of Dippu-AST expression.

It has been suggested that the changes in sensitivity of CA to ASTs in *D. punctata* are the result of variations in the density of AST receptors within the CA (Stay *et al*. 1991). Previous study showed that the sensitivity of isolated CA to AST-1 during the first gonadotrophic cycle of mated females changes dramatically (Pratt *et al*. 1990; Stay *et al*. 1991). After the emergence, sensitivity declines, reaching a minimum on day 4, at the peak of JH biosynthesis. We observe the decline of Dippu-AstR after adult emergence, reaching a minimum on day 3. Dippu-AST then increases, reaching a peak on days 6-7,

as JH biosynthesis declines. The Dippu-AstR mRNA expression levels also increase, reaching a peak on day 6. In contrast, virgin females produce JH throughout the first gonadotrophic cycle at very low levels (Stay *et al*. 1991). CA of virgin females show greater sensitivity to AST treatment compared to mated female throughout this period. The expression levels of Dippu-AstR correlate well with the levels of sensitivity of CA to AST treatment, which higher levels of the receptor observed throughout the first period of gonadotrophic cycle. These data suggest that the regulation of AST receptor accounts for the change in sensitivity; for example CA showing low sensitivity to AST would have lower receptor sites than sensitive CA.

In virgin cockroach *D. punctata*, the Dippu-AST-like-immunoreactivity (DAIR) has been shown in the terminal abdominal ganglion (TAG) (Lange *et al*. 1993). The presence of DAIR was also observed in the TAG in day 1-4 of mated female *D. punctata,* as well as in the ventral nerve 7 (VN7) (Garside *et al*. 2002). The study showed that VN7, the second lateral nerve from the TAG, innervates the reproductive structures in *D. punctata* using methylene blue staining. In this study, we observe the existence of Dippu-AstR in very low levels throughout the first 7 days of the gonadotrophic cycle in both mated and virgin female *D. punctata.* The functions of ASTs throughout the ganglion are yet to be determined.

In addition to the ability of ASTs to inhibit JH biosynthesis, ASTs are able to inhibit muscle contraction of the hindgut and the antennal pulsatile organ (Lange *et al*. 1995; Lange *et al*. 1993). It has been reported that the FGL-AST AST 7 was found to stimulate

activity of both invertase and α-amylase in a dose-dependent manner in the lumen of midguts of *D. punctata in vitro* (Fuse *et al*. 1999).

qPCR analysis show that Dippu-AST mRNA expression in the mated female midgut changed significantly during the first gonadotrophic cycle. Dippu-AST mRNA levels show a decrease around the middle of the gonadotrophic cycle. The same result was observed in the cockroach *B. germanica*, which the mRNA levels of Dippu-AST were high after adult evergence, declined around the middle of the gonadotrophic cycle, and recovered thereafter (Aguilar *et al*. 2003). However, the mRNA pattern of Dippu-AST is inverse to that of mRNA levels in the virgin midgut *D. punctata*, when the levels of Dippu-AST mRNA reach the highest on day 3. Further results show the expression levels of Dippu-AstR during the gonadotrophic cycle in both virgin and mated midgut female. However, the pattern of Dippu-AstR mRNA shows no correlation to Dippu-AST.

Taken together, these data suggest that in the midgut of *D. punctata* another AST receptor may exist or ASTs may be involved in other functions rather than JH biosynthesis, such as muscle contraction or regulation of feeding and nutrition.

In addition to the brain, the ovary seems to be an important organ which regulates CA activity in both a stimulatory and an inhibitory manner (Bylemans *et al*. 1998; Stay *et al*. 1994; Stay *et al*. 1996; Unnithan *et al*. 1998; Lanzrein *et al*.1981). A relationship exists during the first gonadotrophic cycle between oocyte length and the rate of JH production (Tobe, 1980; Johnson *et al*. 1985). Basal oocytes are previtellogenic and JH biosynthesis

is low after the adult emergence. On days 2-3 the oocytes become vitellogenic and rates of JH biosynthesis begin to rise. JH biosynthesis reaches the maximum on day 4-5 when oocytes are continuing to grow. On days 5 and 6, rates of JH biosynthesis decline rapidly, while the oocytes continue to grow and become chorionated. Oviposition occurs and the oocytes are retracted into the brood sac on day 7, at which JH biosynthesis is low. Mating and the ovary are required for the cycle of JH biosynthesis (Stay and Tobe 1978; Stay and Tobe 1981). Oocytes of the virgin *D. punctata* do not mature at a rapid rate and do not show the cycle in JH biosynthesis as described in mated females (Stay and Tobe 1977).

ASTs expression occurs in the ovary. It was shown by RT-PCR that the FGL-AST gene was expressed not only in ovaries but also in oviducts of *D. punctata*. However, immunoreactivity was only detected in nerves branching on the surface of the oviduct not in the lateral or common oviducts or ovary (Garside *et al*. 2002). The immunoreactivity of the FGL-AST was also reported in the oocyte cytoplasm of *D. punctata* at the micropyle and on the surface of the chorion, in the lumen of the follicle cell sheath, in the lumen of the lateral oviduct below the ovary, in epithelial cells of the oviduct, in nerves leading to the muscles of the oviduct, and in yolk cells, suggesting that FGL-ASTs in the oocyte/oviduct may function in the process of ovulation, and after ovulation, ASTs in the fertilized egg may function in yolk utilization (Woodhead *et al*. 2003).

The pattern of Dippu-AstR expression in our study is found closely related to that observed in the *Spodoptera frugiperda* AST (Spofr-AS), in which the expression levels observed in the ovary were high during the first 3 days after emergence and dropped

thereafter (Abdel-Latief *et al*. 2004). Moreover, Dippu-AstR mRNA expression pattern is also correlated well with the levels of Dippu-AST mRNA, and titer of JH biosynthesis in *D. punctata* (Garside *et al*. 2002; Tobe and Stay 1977). In the ovary of mated female during the first gonadotrophic cycle, Dippu-AST mRNA levels are relatively low. qPCR of Dippu-AstR mRNA showed that the receptor expression are also very low during the first 6 days of the gonadotrophic cycle. After adult emergence, JH is low, Dippu-AST levels are high, and Dippu-AstR levels are relatively high. Levels of Dippu-AST drop and remaining low until day 4, 5 when JH biosynthesis reach a maximum on day 4,5. We observed the lowest level of Dippu-AstR at the same time, day 4, 5. The Dippu-AST mRNA levels are rising by day 5, when the Dippu-AstR mRNA levels are increasing, and JH biosynthesis is decreasing (Garside *et al*. 2002; Tobe and Stay 1977). Though the very low levels of Dippu-AST and Dippu-AstR mRNA expression were observed during the first gonadotrophic cycle, the closely correlation of Dippu-AST, Dippu-AstR, and the titer of JH biosynthesis suggest that Dippu-ASTs and the receptor in the ovary partly controls JH biosynthesis.

The expression of Spofr-AS has been shown in the reproductive tissues in both males and female moths (Abdel-Latief *et al*. 2004). Spofr-AS expression rates in the testes could be detected during the first 24 h after emergence. To date, no evidence is available for the expression pattern of AST in testes of *D. punctata*. In this study, we demonstrate that Dippu-AstR mRNA is expressed in testes of *D. punctata* in the first 6 days of adult life. The ASTs function in testes is yet known. In *P. americana*, a sex peptide is transferred in the ejaculate by males, which increase the JH titer in females haemolymph (McNeil and

Tobe 2001). Further investigation is needed to help understand the mode of action of AST and its receptor in testes.

CHAPTER 5:

Summary and Future aspects

5.1 Summary

1. By PCR screening of CA cDNA library and by Rapid Amplification of cDNA Ends (RACE), a 1722 bp cDNA sequence of the *D. punctata* allatostatin receptor was cloned. This cDNA fragment encodes a putative receptor of 425 amino acids with N-linked glycosylation, potential PKA and PKC sites, which are GPCRs typical signatures.

2. *Diploptera punctata* allatostatin receptor (Dippu-AstR), a G-protein coupled receptor, shares the most similarity to *Periplaneta americana* allatostatin receptor. To understand the mechanisms and function of the pleiotropic allatostatins, it is necessary to characterize their receptor gene and study the spatial and temporal expression patterns of the gene.

3. Dippu-AstR RNAi was tested on ability to effect JH biosynthesis and oocyte growth. Small but significant effects were noted. These results are, however, preliminary as quantitative reduction of Dippu-AstR mRNA by RNAi was not measured in CA.

4. By RT-PCR and real-time PCR technique, it could be demonstrated that the Dippu-AstR mRNA is expressed in various tissues of mated and virgin female adult cockroaches. The results on Dippu-AstR mRNA expression as obtained by RT-PCR were

confirmed by real-time PCR. The expression of Dippu-AstR mRNA in various tissues of adult female cockroaches varied in age-dependent manner.

5. Our study shows that Dippu-AstR is expressed in these tissues during the first seven days of adult life. JH biosynthesis is maximal on day 5 in mated females and expression of Dippu-AstR mRNA reaches its highest levels one day later in brain and corpora allata. Expression of Dippu-AstR is maximal in midgut of day 2 mated females and these high levels may function in the inhibition of feeding at this stage. In virgin females, expression of Dippu-AstR mRNA in midgut and CA is dramatically elevated on days 6 and 7, respectively. No significant fluctuations in Dippu-AstR expression was noted in the abdominal ganglia of either mated or virgin females. Similarly, no significant differences were found in expression of ovarian Dippu-AstR in mated females throughout the gonadotrophic cycle. Testes express Dippu-AstR, with maximal levels on days 4 and 7.

5.2 Future aspects

Isolation of an AST-receptor in *D. punctata* in this report may lead to an understanding of how multiple FGL-ASTs sequence bind and activate inhibitory fuctions. *In vivo* expression of the AST receptors in either mammalian cells or Xenopus oocytes may allow us to analyze the binding requirements for AST receptor to its cognate ligands. Functional expression of the cockroach *P. americana* AST receptor in Xenopus oocytes showed dose-response relationship for the activation of *Periplaneta* ASTR by several *D. punctata* AST peptides. All ASTs tested activated the receptor with high affinity (Auerswald *et al*. 2001). This is in contrast to the varying activity ASTs were found to

have in stimulating JH biosynthesis (Tobe *et al*. 2000). Using the peptide lacking the C-terminal amide results in no activation of inward currents, suggesting that the C-terminal pentapeptide is sufficient for agonistic activity and the amidation of this sequence is a necessary requirement for receptor activation (Auerswald *et al*. 2001).

Knowledge of the receptor sequence and an expression system may allow testing of residues important for binding of peptide ligands. Identifying the "binding pockets" may lead to design of non-peptide analogs that may function as insecticides. Analogs have already been designed that allow ASTs to not degrade in the hemolymph due to proteases (Nachman and Tobe 1999).

Is there a second or multiple AST receptors? Evidence supports the existence of multiple receptors. Possibly further screening of tissues specific libraries may lead to an alternate form. Possibly the second type of receptor does not resemble a known *Drosophila* receptor. This will require reverse physiology assays (Birgul *et al*. 1999) that was succesful in uncovering the first AST receptor.

RNAi with further controls may be a promising approach. Further work to optimize the reduction may allow identification of the male of this AST receptor in a variety of tissues.

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Appendix

LB Broth (1 liter)

10 g NaCl

10 g Bacto-tryptone

5 g Yeast extract

Adjust volume to 1 liter with dH_2O

Cockroach Saline (1 liter)

8.77 g NaCl

0.89 g KCl

1.47 g $CaCl_2.2H_2O$

0.61 g $MgCl_2.6H_2O$

2.38 g HEPES

7.21 g glucose

Adjust pH to 7.2-7.4

Injection Buffer (for RNAi) (100 ml)

0.24 g. Tris

0.88 g. NaCl

Adjust pH to 7.8 with HCl

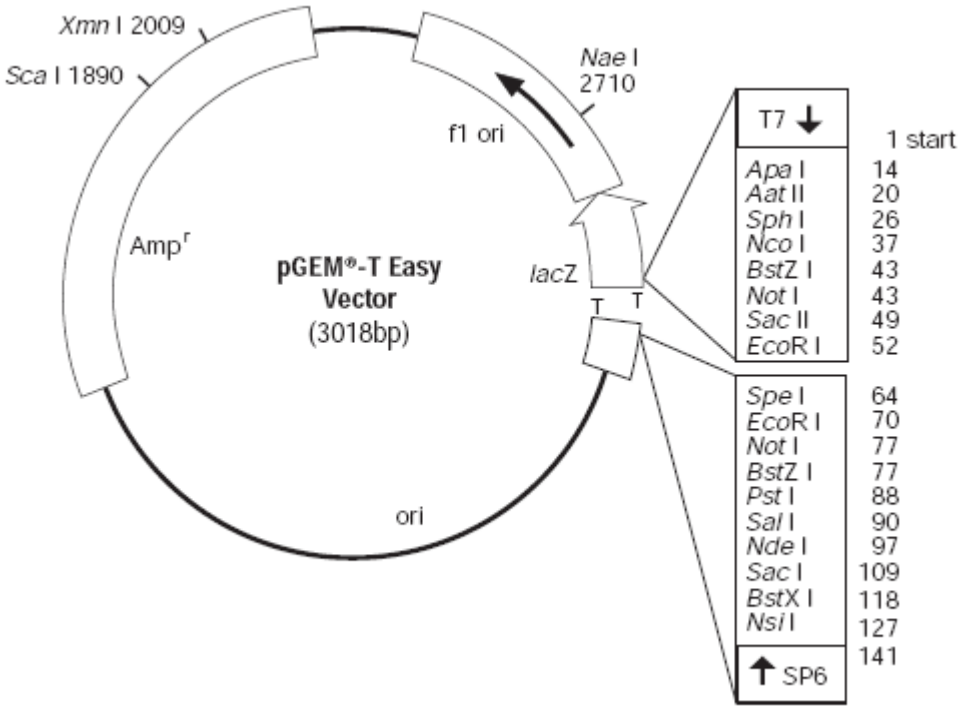
5 X Annealing Buffer (for RNAi) (100 ml)

0.60 g. Tris

0.58 g. NaCl

0.19 g. EDTA

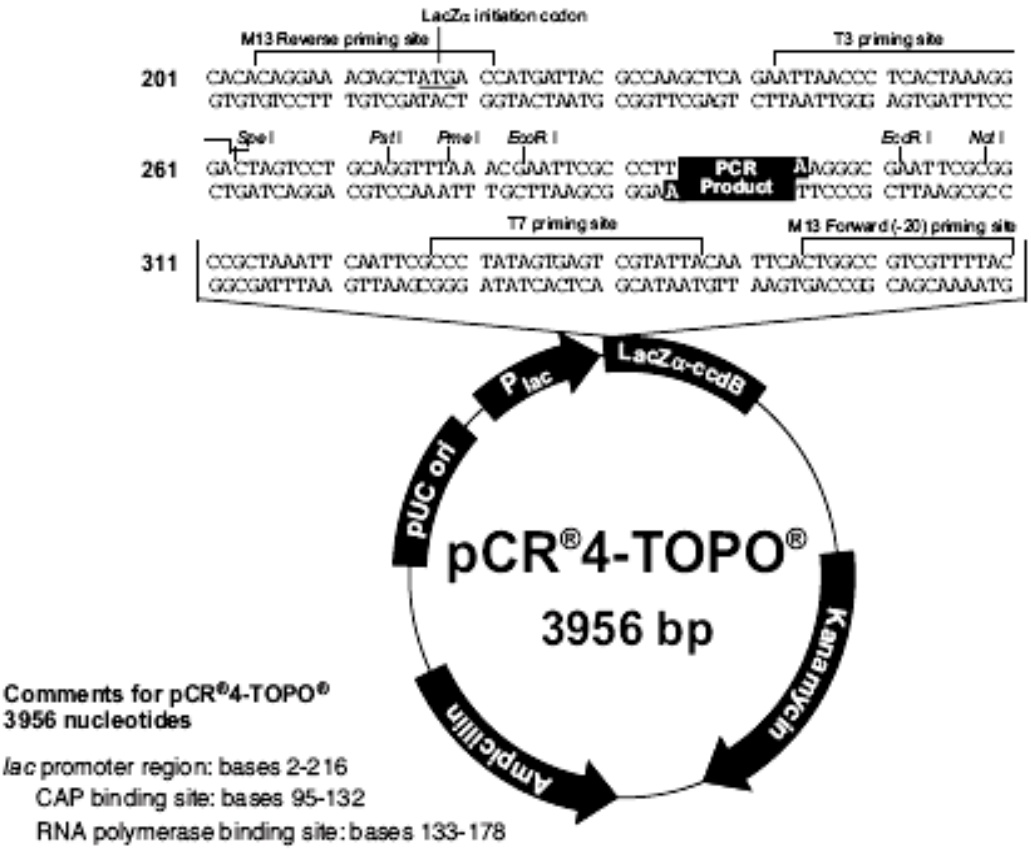
Adjust pH to 8.0 with HCl



pGEM®-T Easy Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter	3002-6
SP6 RNA Polymerase promoter	136-158
multiple cloning site	10-128
*lac*Z start codon	180
lac operon sequences	2839-2999, 166-395
lac operator	100-216
β-lactamase coding region	1337-2197
phage f1 region	2383-2838
binding site of pUC/M13 Forward Sequencing Primer	2959-2975
binding site of pUC/M13 Reverse Sequencing Primer	176-192

Fig. A.1 pGEM-T Easy plasmid map



Comments for pCR®4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
CAP binding site: bases 95-132
RNA polymerase binding site: bases 133-178
Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
LacZα portion of fusion: bases 217-497
*ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

Fig. A.2 pCR 4 – TOPO plasmid map

Oligonucleotide primers for Dippu-AstR cloning

Degenerate primers

ASTR-F: 5’**-** AGGGAATTCGCNGTNGTNCAYCC -3’

ASTR-R: 5’**–** GCCGGATCCAANGCRTANARDATNGGRTT – 3’

3’END

F: 5’ - CAA CAG ACC TGA TGG CTA CAA C – 3’

R: T7 primer

5’END

F: 5’ – GGTCTGCCACAGCGAGGTTGATTA – 3’

F: 5’ – CACCTTCTGGATGAGTTGGAG – 3’

Oligonucleotide primers for RT-PCR

16S rRNA

F: 5’ - TTA CGG TGT TAT CCC TAA GGT AA – 3’

R: 5’ - CGC CTG TTT AAC AAA AAC AT – 3’

R1a-R1s

F: 5’ - CAA CAG ACC TGA TGG CTA CAA C – 3’

R: 5’ - ATG ACG CTG GTG TTA GTG ATG T – 3’

Oligonucleotide primers for quantitative real-time pcr

16S rRNA for real-time PCR

16S rRNA-F: 5’ - TTA CGG TGT TAT CCC TAA GGT AA – 3’

RT-16S rRNA-R: 5’ - GTT GGA CTA CTG TCT TAA TT – 3’

Dippu-AstR for real-time pCR

F 745: 5’ - ATC CTG GTG CTG AAG AGT GCG GAC C – 3’

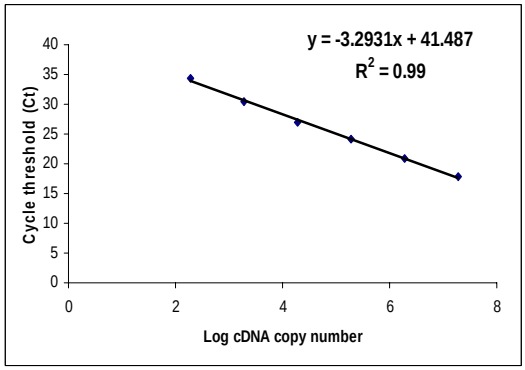
R 894: 5’ - GCG GAA TGC TTT GCG GAA GTG G – 3’

Dippu-AST for real-time pCR

F 609: 5’ - TTG GGC AAA CGA GCA CCA TC – 3’

R768: 5’ - CAG AGG ACC TTC CAG AGT TGA CTG – 3’

A.



B.

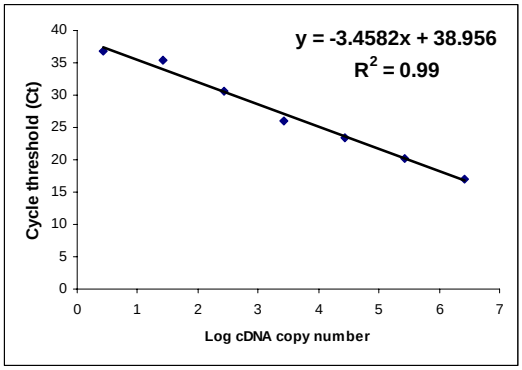


Fig. A.3 Standard curves of (A) 16srRNA, (B) Dippu-AstR

cDNA was serially diluted before amplification with the appropriate primers. Each sample was assayed in duplicate and re-assayed one week later. The standard curve is a product of both assays. The equation for the line-of-best-fit, y = slope (±S.D.)x + intercept (±S.D.) and the coefficient of determination (R^2) are shown for each standard curve.

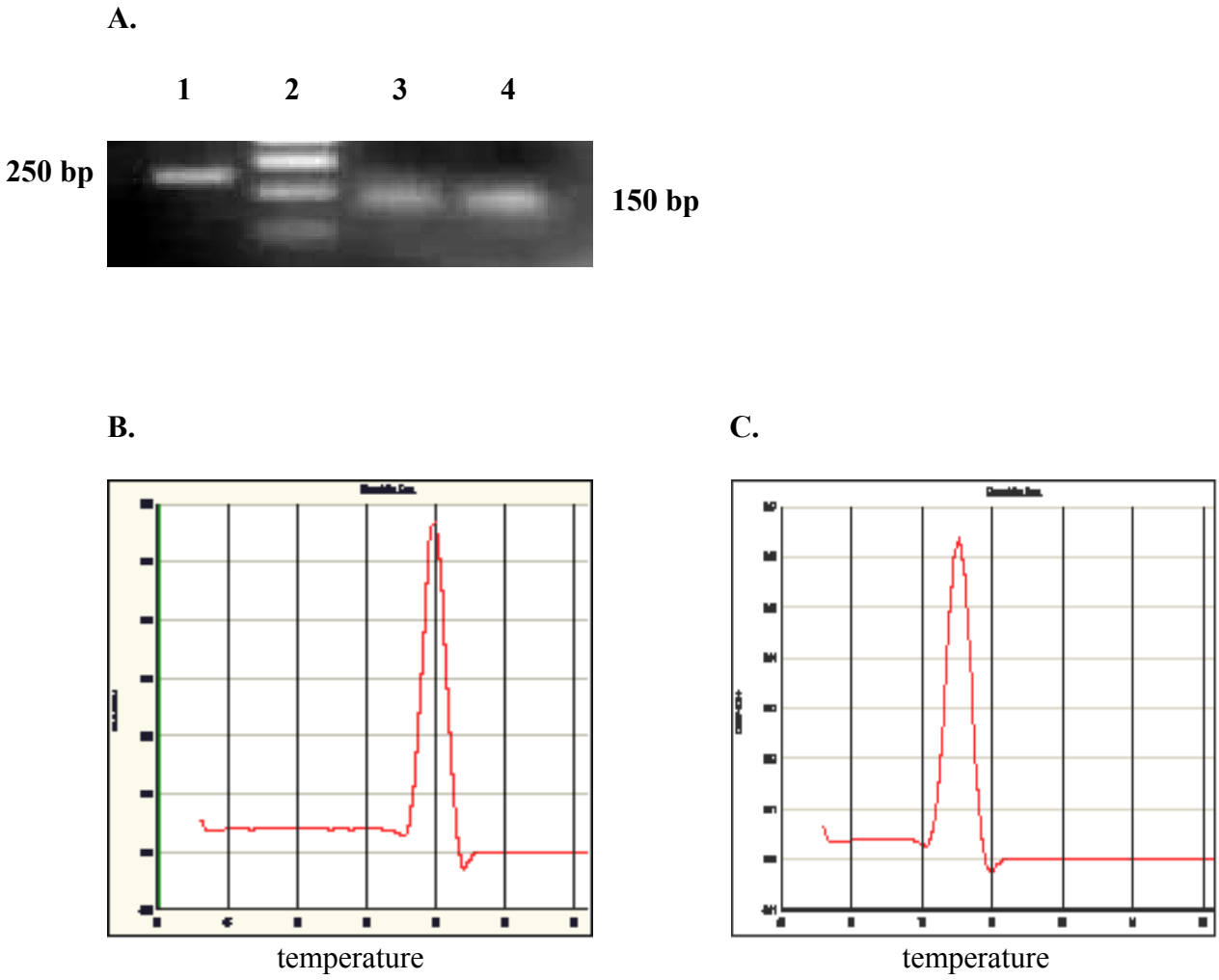


Fig. A.4 A) A 1% agarose gel electrophoresis carried out at end point following qPCR. Lane1 = 1000 bp marker, lane2 = 100 bp marker, lane3 = Dippu-AstR cDNA (150 bp), lane4 = 16S rRNA cDNA (150 bp). B) Melt curve analysis, conducted at end point of Dippu-AstR cDNA amplification. C) Melt curve analysis, conducted at end point of 16S rRNA cDNA amplification.

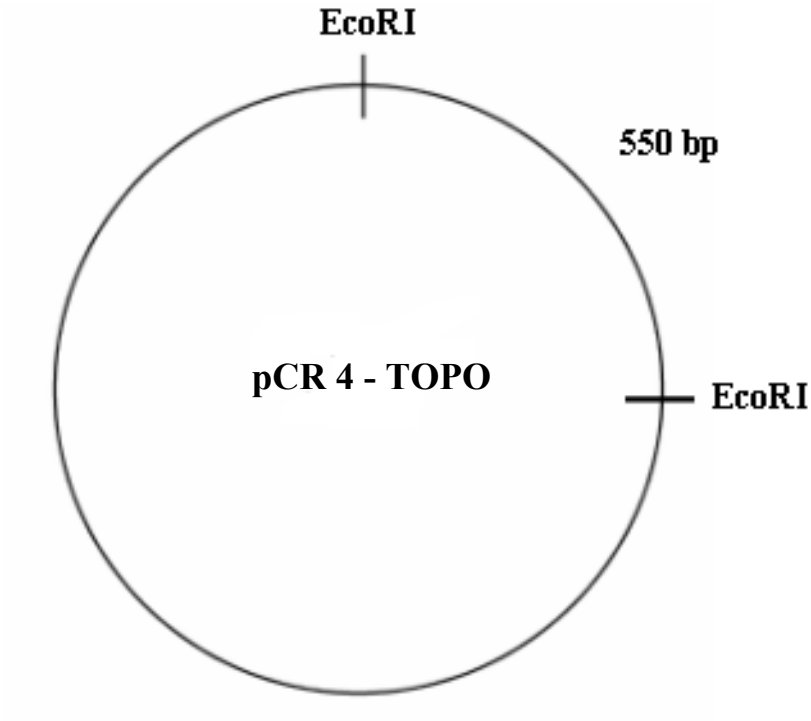


Fig. A.5 RNAi construct (pASTR, pCA2) (Amp^R)

To make RNA sense strand: use T7 RNA Polymerase, digest plasmid with SpeI

To make RNA antisense strand: use T3 RNA Polymerase, digest plasmid with NotI

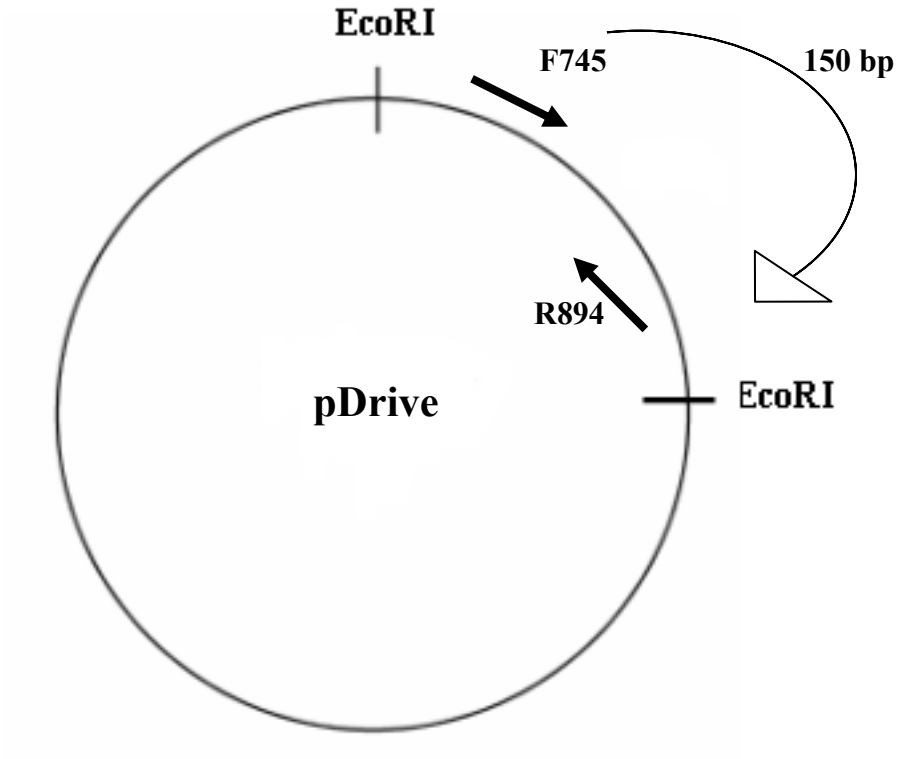


Fig. A.6 Real-time PCR construct for Dippu-AstR: p3'ASTR) (Amp^R)

Forward primer (F745), Reverse primer (R894): product size = 150 bp

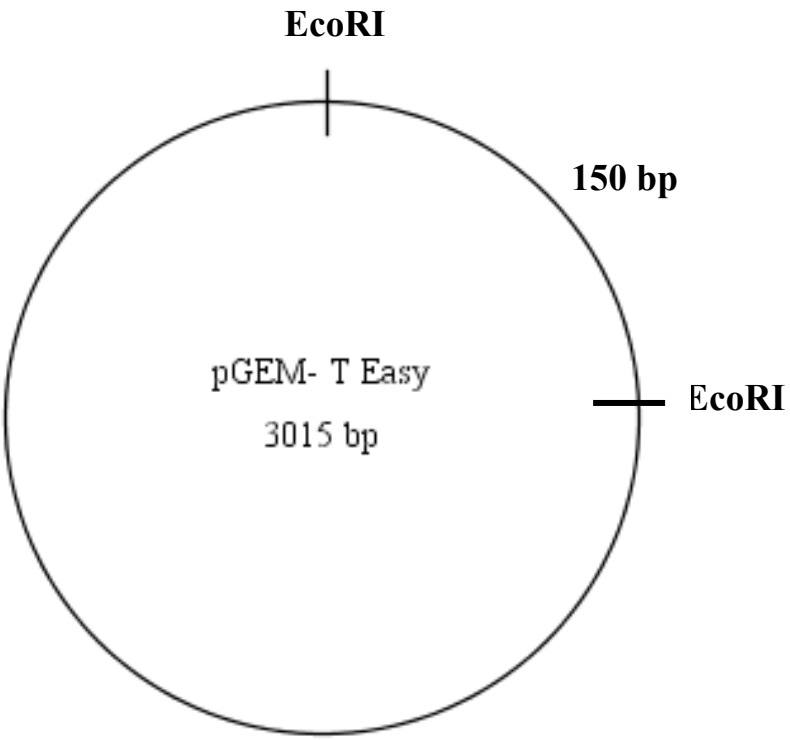


Fig. A.7 Real-time PCR construct for Dippu-AST: pDippu-AST) (Amp^R)

Forward primer (F609)

Reverse primer (R768)

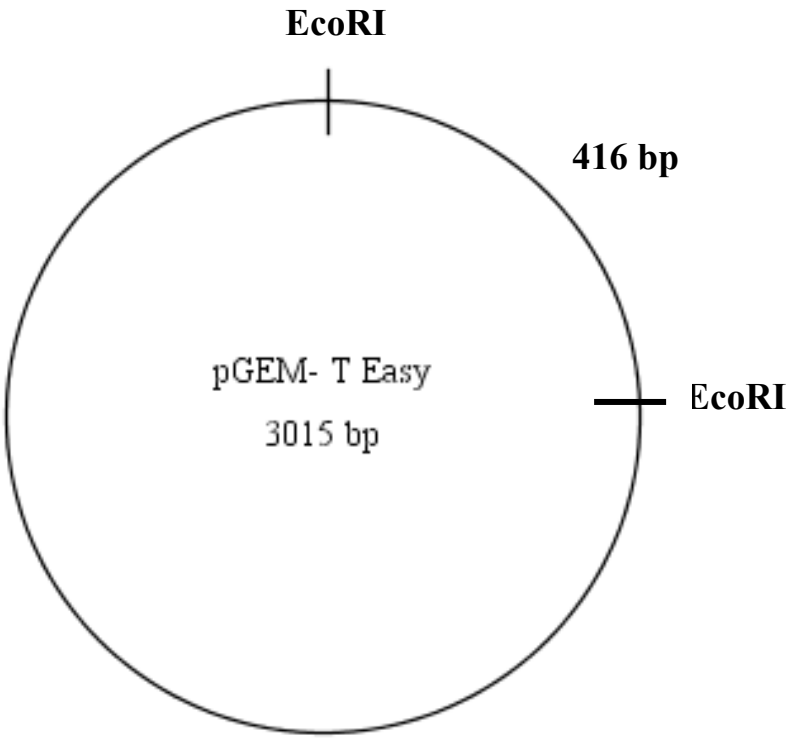


Fig. A.8 Real-time PCR construct for 16S rRNA: pDippu-16SrRNA) (Amp^R)

Forward primer (16S rRNA-F)

Reverse primer (RT-16s rRNA-R)