The Allatoregulatory Neuropeptides and their Genes in the Fall Armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Contents

Introduction

Humans have always been intrigued by insects such as butterflies and moths, not only because of their general beauty and striking colours, but also because of the spectacular changes that occur during their life cycles. Insects represent the largest group of animals (more than 70% of all animal species are supposed to be insects) and are ecologically and economically extremely important, e.g., because most flowering plants depend on insects for their pollination (honeybees alone pollinate 20 billion dollars worth of crop yearly in the United States). Insects, however, can also be severe pests in agriculture, destroying 30% of our potential annual harvest, and can be vectors for major diseases such as malaria, sleeping disease, and yellow fever. During the last century a huge amount of classical chemical insecticides from different chemical groups were used to control the populations of insect pests. With the time these insecticides caused severe problems because of their side effects on non-target animals as well as on the general ecological environment. Progress has been made in the last 30 years with the development of natural and synthetic compounds which are capable of interfering with the processes of growth, development and metamorphosis of the target insects. These chemicals have been called insect growth regulators (IGR), some of which theoretically depend on introducing the insects own hormones or like-hormones to control pest populations, thus applying insect hormones in pest control. Insect growth regulators disrupt the normal development of insects by mimicking juvenile hormone (JH) and/or moulting hormone (20-hydroxyecdysone) effects, or by interfering with chitin synthesis.

Despite the economic importance of insects, their molecular endocrinology is not well understood. With this in mind, research has been forced during the last decade into structure elucidation, genetics, biochemistry and physiology of biologically active peptides that are produced in the neurosecretory cells of insects. These neuropeptides control most of the critical metabolic, homoeostatic, developmental, reproductive and behavioural events during insect life. To interfere with these physiological events, and use the neuropeptides in a more safe and rational manner of insect pest control, it is essential to characterize these various neuropeptides and understand their functions. Pest management technologies based on neuropeptides potentially offer a degree of biological activity, target specificity and environmental compatibility that are lacking in conventional neurotoxic insecticides (Menn et al., 1989). However, insect neuropeptides are unlikely to be rapidly absorbed through the insect cuticle, and are likely to be prone to proteolysis and rapid degradation both in

environment and within the insect. Nevertheless, we expect that more stable synthetic peptide analogs may help to overcome these problems.

Juvenile hormone (JH)

Juvenile hormones (JH) are unique humoral agents, sesquiterpenoids, which play a role in almost every aspect of insect development and reproduction, including embryogenesis, larval moulting, metamorphosis, vitellogenin synthesis and ovarian development in maturing females and spermatogenesis in males, caste determination in social insects, phase determination in locusts and aphids, larval and adult diapause regulation, colour polymorphism and various aspects of metabolism associated with these functions (Nijhout, 1994; Riddiford, 1994; Gäde et al., 1997). The morphogenetic role of JH (Neotenin, status quo hormone) was first detected by observing that insect larval development was accelerated when the corpora allata (CA) were removed. The CA are endocrine glands in the posterior region of the head which are closely associated with the stomatogastric nervous system. They are of ectodermal origin, arising in the embryonic development (Tobe and Stay, 1985).

Juvenile hormones derived their name from the fact that they block metamorphosis of nymphs into imagoes or the development of pupae into an adult insect. As a developmental hormone, JH controls switches between alternative pathways at several points in the life cycle. In ascribing a role to JH in the control of such developmental switches, it summed to be that different concentrations of JH are responsible for specifying the different pathways (Nijhout, 1994; Gäde et al., 1997).

During the last decade, interest has been focused on factors (neuropeptides) that regulate JH biosynthesis by the CA (Stay et al., 1994). JH biosynthesis can be regulated by both peptidergic and aminergic inputs (Rachinsky and Tobe, 1996). Depending upon the species and developmental stages their signals may be either stimulatory or inhibitory (allatotropin and allatostatin) and the signals may reach the glands via the haemolymph or via nervous connections.

Allatostatins (AST or AS) are insect neuropeptides that inhibit JH biosynthesis *in vitro* by the CA. The known allatostatic neuropeptides can be subdivided into three types (for review see Bendena et al., 1997; Gäde et al., 1997; Weaver et al., 1998; Hoffmann et al., 1999; Stay, 2000; Gäde, 2002; Gäde and Goldsworthy, 2003).

Allatostatin type-A (cockroach allatostatin)

Type-A allatostatins, at first were isolated from the brains of virgin females of the cockroach *Diploptera punctata* (Woodhead et al., 1989; Pratt et al., 1991a) and have

subsequently been shown to occur in multiple forms in a large number of insect orders (Nässel, 2002; Gäde, 2002). They are characterized by the common C-terminal pentapeptide sequence Y/FXFGL-amide. This C-terminal pentapeptide sequence has been demonstrated to be the minimum sequence capable of inhibiting JH biosynthesis (Stay et al., 1991; Pratt et al., 1991b). By using molecular biological techniques, precursor genes for the type-A allatostatins were isolated from various species: in *D. punctata* the gene encodes for 13 AST A-type peptides (Dippu-AST 1-13) (Donly et al., 1993), and in other cockroaches even 14 peptides (Ding et al., 1995; Bellés et al., 1999). The Mediterranean field cricket *Gryllus bimaculatus* also possesses a preprohormone including 14 putative allatostatins (Meyering-Vos et al., 2001). In the preprohormone of the mosquito *Aedes aegypti* five FGL-amides are present (Veenstra et al., 1997), and in *Drosophila melanogaster* also five putative allatostatins were found (Lenz et al., 2000c). In the locust *Schistocerca gregaria* the gene encodes for ten allatostatin-like peptides (Vanden Broeck et al., 1996).

More than 40 peptides belonging to the Y/FXFGL-amide allatostatin superfamily have been isolated and identified from the central nervous system (CNS) of some crustacean species such as the tiger prawn, *Penaeus monodon* (Duve et al., 2002).

In 1997a, Duve et al. isolated and purified members of the allatostatin peptide superfamily from some lepidopteran species, the codling moth, *Cydia pomonella*, and the bollworm, *Helicoverpa armigera*. The peptides, designated cydiastatins and helicostatins, respectively, were monitored during purification with a radioimmunoassay based on the callatostatin structure for the blowfly *Calliphora vomitoria*. Eight peptides were identified from each of the two species, with four identical in both species and three differing by a single amino acid. The gene which encodes the helicostatin peptide family was later isolated from *H. armigera* genomic DNA library (Davey et al., 1999). The gene consists of three exons and encodes a precursor of 225 amino acids that contains three blocks of tandemly arranged helicostatin peptides. A comparison of the helicostatin precursor with that of the cockroaches, locusts and flies revealed a variation in size, sequence and organization of the allatostatin precursors across different insect orders. Within the lepidopterans, another gene precursor of the FGL-amide allatostatin family was cloned for the silkworm *Bombyx mori* (Secher et al., 2001). This allatostatin precursor contains eight A-type allatostatins.

The allatostatic function of the type-A allatostatins seems to be restricted to cockroaches and crickets (Hoffmann et al., 1999). In all other species where A-type allatostatins were found, including blowflies, bees, moths and stick insects, they do not have any effect on the biosynthesis of JH. However, these peptides may affect visceral muscles by inhibiting their spontaneous contraction, inhibit the heartbeat of certain cockroaches, and

inhibit vitellogenin synthesis and the release of vitellogenin from the fat body of cockroaches. At least in some insects, these peptides may stimulate the activity of midgut carbohydrate-digesting enzymes (for review see Gäde, 2002).

Immunocytochemical studies showed that type-A allatostatins are widely distributed, not only in the central nervous system but also in peripheral nerves projecting to visceral muscles, in midgut endocrine cells, and in haemocytes (Stay, 2000). Recently, type-A AST immunoreactivity was also shown in the cortical cytoplasm of *G. bimaculatus* oocytes (Witek and Hoffmann, 2001). Using semi-quantitative RT-PCR it was demonstrated that the type-A allatostatin gene is strongly expressed in the brain and the caecum of *G. bimaculatus*, and to lower extent also in other parts of the digestive tract (ileum, midgut, colon) and in various other tissues such as fat body, ovaries and female accessory reproductive glands (Meyering-Vos and Hoffmann, 2003). Using quantitative competitive reverse-transcriptase polymerase chain reaction technique (QC-RT-PCR) a strong expression of the allatostatin type-A preprohormone in lateral and common oviducts as well as in the ovary of *D. punctata* was shown. The pattern of expression as observed in the oviduct and the ovary well correlates with physiological changes occurring during the reproductive cycle. Specifically expression of the allatostatins was drastically reduced during the time of maximal vitellogenin uptake by the oocytes, with higher levels measured prior to and following vitellogenesis (Garside et al., 2002). In the embryos of *D. punctata*, Dippu-AST 7 inhibited sesquiterpenoid production by the CA of mid to late embryos, whereas it exerted a dose-dependent stimulatory effect in the early embryo (Stay et al., 2002).

Already a decade ago, receptor proteins for binding allatostatins were thought to be identified with the use of photoaffinity label, but only recently two receptors have been identified in *Drosophila* (DAR-1 and -2) (Birgül et al., 1999; Lenz et al., 2000a; Lenz et al., 2000b). Receptors similar to those from *Drosophila* have been cloned from the cockroach *Periplaneta americana* and the moth *B. mori* (Auerswald et al., 2001; Secher et al., 2001). Structurally, the transmembrane regions of the AST type-A receptors are most closely related to the mammalian galanin receptors.

In conclusion, members of the type-A allatostatins are present in each insect species tested so far. Their allatoregulating function, however, seems to be restricted to cockroaches and crickets, but other functions such as myoinhibition on visceral muscles may exist in a number of insects.

Allatostatin type-B (cricket allatostatin)

The second group of allatostatins are called type-B allatostatins. These allatostatins were at first isolated by four HPLC steps from methanolic extracts of brains of the cricket *G. bimaculatus* (Lorenz et al., 1995). Their structure was characterized as $W(X)_6$ W-amides. The peptides show high sequence similarity to the *Locusta* and *Manduca* myoinhibiting peptides (Locmi-MIP, Manse-MIP), respectively (Blackburn et al., 1995; 2001), but are structurally different from other allatostatins. Recently, an AST B-type peptide was isolated from the cockroach *P. americana* (Predel et al., 2001). The peptide inhibited spontaneous muscle activity in the foregut, but also in other visceral muscles. A prothoracicostatic peptide which shows the conserved $W(X)_6$ W-amides structure was isolated from brains of *B. mori* (Bommo-PTSP) (Hua et al., 1999). Neuropeptides with a similar structure were also isolated from the stick insect, *Caraosius morosus*, but their function is still questionable (Lorenz et al., 2000).

To date only one hormone precursor gene has been published, which encodes the B-type allatostatins from *D. melanogaster* genome DNA. The precursor is 211 amino acid residues long and contains one copy of each of the following putative allatostatins: AWQSLQSSW-amide, AWKSMNVAW-amide, pEAQGWNKFRGAW-amide, EPTWNNLKGMW-amide, and DQWQKLHGGW-amide (Williamson et al., 2001a). From larval brains of *B. mori* the cDNA which encodes the prothoracicostatic peptide (Bommo-PTSP) was cloned (Hua et al., unpublished data; Gene bank accession no. AB073563). The predicted open reading frame encoded 288 amino acids including five repeats of the Bommo-PTSP and five other structurally related peptides.

In conclusion, B-type allatostatins are also widespread in insects and pleiotropic in function. Their allatoinhibiting function seems to be restricted to crickets. Receptors for the B-type allatostatins are not yet known.

Allatostatin type-C (*Manduca sexta* allatostatin)

The third group of allatostatins is represented by the C-type or *Manduca sexta* allatostatin (Manse-AST). This peptide was first purified from head extracts of pharate adults of *M. sexta* and strongly inhibited JH biosynthesis *in vitro* by the CA from fifth instar larvae and adult females (Kramer et al., 1991). The peptide has the primary structure pEVRFRQCYFNPISCF-OH (where pE is pyroglutamate). The sequence shows no similarity to other allatostatins. The synthetic free acid and the amidated form of the peptide did not differ in their activity *in vitro*. The ED_{50} of synthetic *M. sexta* allatostatin on CA from early fifth instar larvae was about 2 nM and the inhibiting effect was fully reversible. *M. sexta* allatostatin also inhibited the activity of the CA from adult *Heliothis virescens* and *Pseudaletia unipuncta*, but had no effect on the activity of the CA from the beetle, *Tenebrio*

molitor, the grasshopper, *Melanoplus sanguinipes*, or the cockroach, *P. americana.* This suggests that the action of this peptide may be lepidopteran-specific (Kramer et al., 1991).

From a *P. unipuncta* brain cDNA library, a cDNA was isolated that encodes a 125 amino acid polypeptide containing the Manse-AST (Pseun-AST) sequence. The *Pseun-AST* gene is present as a single copy per haploid genome. Expression of this gene was low in *P. unipuncta* sixth instar larvae, prepupae and early pupae, but was relatively high in late pupae and in 1 to 3 d-old adults both sexes (Jansons et al., 1996). This pattern expression does not well correlate with the profile of JH biosynthesis in the CA of *Pseudaletia*. Audsley et al. (1998) reported on the presence of Manse-AST immunoreactivity in the nervous system and the haemolymph of the tomato moth *Lacanobia oleracea.* Injection of Manse-AST into fifth or sixth instar larvae of *L. oleracea* resulted in a retardation of larval growth, reduction in feeding and increased their mortality, but had no effect on non-feeding (day 7) sixth instar larvae (Audsley et al., 2001). These results suggest that Manse-AST is not only acting on the CA by inhibiting JH biosynthesis, but most likely by myoinhibiting actions on the foregut. Inhibition of foregut peristalsis by Manse-AST *in vivo* appears to suppress feeding, resulting in increased mortality. Foregut peristalsis may be inhibited by intact peptides as well as by peptide fragments produced through cleavage of Manse-AST by haemolymph enzymes (Audsley et al., 2001).

Incubation of the retrocereberal complex from adult females of the moth *H. virescens* during the first 24 hours after eclosion with the synthetic Manse-AST did not reduce the production of JH. However, incubation of the same tissues from 3 d-old females with Manse-AST significantly reduced the production of JH (Teal, 2002). Incubation of the retrocerebral complex from *H. virescens* females with Manse-AST plus farnesol or JH III acid resulted in a significant increase in the production of JH III, but neither JH I nor JH II were detected. These findings indicate that Manse-AST acts prior to the formation of the sesquiterpene alcohol precursors of JH. From *D. melanogaster* a cDNA that encodes a 121 amino acid residues was cloned, which contains one copy of the sequence pEVRYRQCYFNPISCF-OH (drostatin-C). This peptide differs in only one amino acid residue (F→Y in position 4) from the Manse-AST. The gene has three introns and four exons and is located at chromosome position 32D2-3. Northern blots showed that the gene is strongly expressed in larvae and adult flies but less in pupae and embryos. *In situ* hybridization studies with larvae showed that the gene is expressed in various neurons of the brain and abdominal ganglion, but also in endocrine cells of the midgut (Williamson et al., 2001b). *In vitro* studies revealed that the peptide dramatically decreases spontaneous visceral muscle contraction and it was therefore named the peptide flatline (FLT) (Price et al., 2002). The activity studies indicated that the FLT

peptide is a potent myotropin but does not act as an allatostatin in *Drosophila*. Despite its profound myotropic effect, pupae of *D. melanogaster* injected with FLT eclosed. Immunocytochemical studies revealed that the peptide is localized in the median neurosecretory cell group of the pars intercerebralis of dipteran brains (Shiga, 2003). Recently, from the genome project of *Anopheles gambiae* a peptide of the allatostatin C-type has been also identified (Riehle et al. 2002). It shows the sequence pEIRYRQCYFNPISCF-OH, which differs in one position (V→I in position 2) from the *Drosophila* peptide.

In conclusion, also the C-type allatostatins seem to represent brain-gut peptides whose allatostatic activity is restricted to lepidopterans. Kreienkamp and co-workers (2002) have recently identified two novel G-protein-coupled receptors from *D. melanogaster*, which are structurally related to the mammalian opioid/somatostatin receptor family and are both activated by the drostatin-C peptide.

Allatotropin (AT)

To date only one peptide with allatotropic activity has been isolated and identified, the *M. sexta* allatotropin (Manse-AT; GFKNVEMMTARGF-NH_2). This peptide is an amidated tridecapeptide that was at first purified from extracts of heads of the pharate adult of *M. sexta*. It stimulates the secretion of JH from the CA of adult, but not of larval tobacco hornworms (Kataoka et al., 1989). Manse-AT also stimulated JH biosynthesis in the CA of *H. virescens,* but not in *T. molitor*, *Schistocerca nitens* or *P. americana*, indicating a lepidopteran-specifity. Studies with NH_2-terminal truncated sequences of the synthetic peptide suggested that the amino acid residues 6-13 represent the biologically active core. Manse-AT was also isolated from head extracts of *Spodoptera frugiperda* where it activated JH biosynthesis up to sevenfold (Oeh et al., 2000). In *L. oleracea,* synthetic Manse-AT (10 µM) caused a 37% stimulation of the CA activity (Audsley et al., 1999). Manse-AT stimulated JH production by the CA of 0 to 6-d old adult females of *P. unipuncta* (Koladich et al., 2002). Finally, Manse-AT showed a stimulatory effect on CA of some larval stages of the honeybee *Apis mellifera* (Rachinsky and Feldlaufer, 2000; Rachinsky et al., 2000). In addition to its effect in stimulating JH production, Manse-AT is a potent cardioaccelerating peptide in pharate adults of *M. sexta* (Veenstra et al., 1994).

Manse-AT was shown to be present not only in the brain and retrocerebral complex but also in the ventral nerve cord of *M. sexta* (Veenstra et al., 1994). The peptide also occurs in one or two pairs of ipsilateral lateral cells (La_l) of the larval brain, two pairs of contralateral cells (III) and in numerous axons in the corpora cardiaca (CC) and the CA (Zitnán et al., 1995). Immunoreactivity and *in situ* hybridization studies indicated that the allatotropin may

also occur in other nerves of the central nervous system and in frontal ganglion cells projecting to the gut (Bhatt and Horodyski, 1999). Other functional roles of Manse-AT are the inhibition of ion transport in the midgut of *M. sexta* (Lee et al., 1998) and the stimulation of foregut contraction in the moths *H. armigera* and *L. oleracea* (Duve et al., 1999, 2000). Finally, Manse-AT was shown to play a role in circuits relaying photic information from circadian photoreceptors to the central pacemaker in the cockroach *Leucophaea maderae* (Petri et al., 2002).

Several other allatotropic neuropeptides seem to exist in various insect species but have not yet been identified (Hoffmann et al., 1999; Elekonich and Horodyski, 2003). A Manse-AT-like peptide was isolated from the mosquito *Aedes aegypti* and its structure determined to be APFRNSEMMRARGF-NH_2 (Aedae-AT) (Veenstra and Costes, 1999). The same peptide sequence was identified in two genes from the genome project of *A. gambiae* (Riehle et al., 2002). Treating the CC/CA complexes from 1 d-old females of *A. aegypti* with the synthetic Aedae-AT resulted in a strong and dose-dependent stimulation of JH synthesis suggesting that the peptide is a true allatotropin (Li et al., 2003).

The gene encoding Manse-AT was at first isolated from the genomic DNA of *M. sexta* (Taylor et al., 1996). The *Manse-AT* gene is expressed as three mRNAs which differ from each other by alternative splicing (Taylor et al., 1996; Horodyski et al., 2001). These mRNAs are predicted to encode three distinct prohormones, each containing one copy of Manse-AT. The specific mRNA isoforms differ in a tissue- and developmental-specific manner (Lee et al., 2002).

Additional Manse-AT-like peptides (ATL) are predicated from other regions of the precursor whose functions are yet unknown (Taylor et al., 1996). The Manse-AT-like sequences may be derived from duplication of an ancestral Manse-AT-like sequence followed by divergence (Horodyski et al., 2001). *In situ* hybridization and immunocytochemistry studies showed that *Manse-AT* gene is expressed in both the central and enteric nervous systems. In larvae of *M. sexta*, Manse-AT mRNA was most abundant in two cells of the frontal ganglion, which project their axons down the recurrent nerve toward the gut, and in cells of the terminal abdominal ganglion (Bhatt and Horodyski, 1999). Lower levels of Manse-AT mRNA were detected in the brain and suboesophageal ganglion. In pupae and pharate adults, Manse-AT mRNA was also detected in cells of the abdominal ganglia and in additional cells of the terminal abdominal ganglion. This pattern of expression suggests that Manse-AT may mediate multiple physiological functions during the entire life cycle of the insect, including the larval stage in which no function has yet been described for the peptide. The gene encoding Manse-AT has been cloned from *B. mori, P. unipuncta*, and *Agrius*

convolvuli for (review see Elekonich and Horodyski, 2003). No allatotropin precursor sequence has been found in the *D. melanogaster* genome (Hewes and Taghert, 2001; Vanden Broeck, 2001).

In conclusion, Manse-AT seems to be ubiquitous in the Lepidoptera, but it is not yet known whether additional allatostimulating peptides exist in this order. Further allatotropic neuropeptides will certainly be detected in other insect orders. The fact, that Manse-AT has fundamental actions unrelated to JH synthesis mirrors the trend observed for the three types of allatostatins to act as a pleiotropic hormone and the question arises, whether allatoregulating effects are the primary functions of these molecules. No allatotropin receptor is known so far.

Research gaps and the aims of this thesis

The fall armyworm, *S. frugiperda* (Lepidoptera: Noctuidae) represents a major pest of corn, sorgum and bermudagrass in southeastern United States. It is also an occasional pest on cotton, millet, alfalfa, rye, rice and soybeans. In Arkansas, for example, the fall armyworm is the third most important noctuid pest on cotton requiring 150,000 acres of cotton to be treated with insecticides and causing a loss of approximately 3,800 bales per year. Little is known about the allatoregulating neuropeptides or the control of JH biosynthesis in general in this insect. Therefore, it was of interest to know which allatoregulatory neuropeptides (allatostatin and allatotropin) may exist in this insect species and what are their biological functions. Uwe Oeh from our group at first isolated a peptide from methanolic brain extracts of adult *S. frugiperda* by high performance liquid chromatography (HPLC) that strongly stimulated the JH biosynthesis *in vitro* by CA from adult females. This peptide turned out to be identical to the *M. sexta* allatotropin (Manse-AT) and was code-named Spofr-AT (Oeh et al., 2000). Its stimulatory effect on the JH biosynthesis by the CA was dose-dependent and reversible. The stimulatory effect could be inhibited in a dose-dependent manner by the synthetic Manse-AST, but we were not able to detect Manse-AST or a Manse-AST-like peptide in the *Spodoptera* brain extract. Injections of Manse-AT (Spofr-AT) into penultimate and last instar larvae of *S. frugiperda* reduced their weight gain and increased mortality (Oeh et al., 2001). Injections of the peptide into adult females reduced the oviposition rate but had no effect on JH biosynthesis *in vitro* of the CA. Injections of Manse-AST into penultimate and last instar larvae of *S. frugiperda* hardly affected growth and development of the animals, whereas combined injections of Manse-AST and Manse-AT resulted in effects similar to those using Manse-AT alone (Oeh et al., 2001).

By using a combination of reversed phase (RP)- and normal phase (NP)-HPLC, Range et al. (2002) could show that the CA of *S. frugiperda* synthesized and released two major

products under *in vitro* conditions. One of the compounds co-migrated with JH II diol, the second compound with JH III diol. Gland extracts also contained both the major products. Addition of Spofr-AT to the incubation medium increased the synthesis and release of both compounds.

Since we hope that novel pest control strategies, based on disruption of the insect endocrine system, will aid in finding new, promising substances that can be used in agricultural applications (Hoffmann and Lorenz, 1998; Cusson, 2000; Gäde and Goldsworthy 2003), it is essential to obtain more detailed information about neuropeptides that regulate JH biosynthesis in potential pest species. Novel molecular biological techniques offer the possibility to clone preprohormone precursors and this means to identify the full set of neuropeptides of a hormone family within an insect species, to unravel the gene structure of prohormones and hormone receptors, or to deduce the physiological (pleiotroic) role(s) of neuropeptides from hybridization and gene expression studies. With these techniques at hand, it was the objective of my thesis to elucidate the complete equipment of *S. frugiperda* with "allatoregulating" neuropeptides and to study their putative physiological functions. To reach these aims, the following experiments were performed:

1. Molecular cloning of the allatotropin gene in *S. frugiperda* (*Spofr-AT*), in order to confirm the structure of the recently identified Manse-AT which had previously been isolated from brain extracts with conventional chromatographic techniques.
2. Molecular cloning of the C-type allatostatin gene (*Spofr-AST*) in *S. frugiperda*. The existence of a Manse-AST peptide was deduced from biological activity studies, but such a hormone could not be isolated from brain extracts with conventional chromatographic techniques.
3. Molecular cloning of the FGL-amide (type-A allatostatin) peptide precursor gene (*Spofr-AST A*) in *S. frugiperda*. Members of this peptide family were found in various lepidopteran species but their physiological role remained unclear.
4. Whole-mount *in situ* hybridization studies in order to demonstrate the tissue-specific localization of the genes in *S. frugiperda*.
5. Detailed studies on the tissue- and time-specific expression of the genes by using one step RT-PCR. Such studies may shed light on the multifunctional role of these neuropeptides in *S. frugiperda.*
6. Molecular cloning of novel allatoregulating neuropeptides in *S. frugiperda* and verification of their biological activity by gene expression, *in situ* hybridization, and bioassay studies.

Synopsis

Aim of my work was to elucidate the complete equipment of "allatoregulating" neuropeptides in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae) and to reveal their putative physiological functions in this insect species by the use of molecular biological methods.

A neuropeptide that strongly stimulates JH biosynthesis *in vitro* by the corpora allata (CA) from adult female *S. frugiperda* had recently been isolated in our group from methanolic brain extracts by conventional chromatographic techniques and its primary structure was elucidated as *M. sexta* allatotropin (Manse-AT; GFKNVEMMTARGFa) by Edman degradation and mass spectrometry (Oeh et al., 2000). However, using the same techniques we were not able to isolate and sequence any neuropeptide with allatostatic activity from brain extracts of *S. frugiperda*.

In the first paper (**paper 1)** I confirmed the primary structure of the previously identified Manse-AT (GFKNVEMMTARGFa) in *S. frugiperda*, and elucidated the existence of a C-type allatostatin in this species by cloning the cDNAs, which encode the precursors of Spofr-AT and Spofr-AST, respectively. The basic organization of the Spofr-AT precursor is similar to that of *A. convolvuli*, *M. sexta*, *P. unipuncta*, and *B. mori* with 83 to 93 % amino acid sequence identity. The *Spofr-AT* gene is expressed in three mRNA isoforms with 134, 171 and 200 amino acids, differing from each other by alternative splicing. The Spofr-AT mature peptide is located between amino acid residues Arg^{38} and Lys^{53} on each of the three precursors, flanked by potential Arg and Lys-Arg endoproteolytic cleavage sites and ends with a glycine residue, the signal for carboxy-terminal amidation by a peptidyl-α-amidating monooxygenase (Eipper et al., 1992). The Spofr-AT peptide sequence is present as a single copy within the precursor. Recognition of all possible proteolytic cleavage sites would result in the production of three additional peptides of 15, 58 and 20 amino acids on the first AT mRNA precursor (134 amino acids), five additional peptides of 15, 18, 14, 59 and 20 amino acids on the second AT mRNA precursor (171 amino acids), and four additional peptides of 15, 68, 54 and 20 amino acids on the third AT mRNA precursor (200 amino acids). The cleavage site in the second mRNA isoform at amino acid position 75 can also be RR instead of RK, which might be supported by the His residue two positions upstream of the cleavage site (HARR). This would imply a Lys residue as the first amino acid of the following peptide (15-residue peptide). The 14- or 15-residue peptide of the second isoform is of special interest as that peptide ends with a glycine, the signal for carboxy-terminal amidation, which is

characteristic for many bioactive peptides. The peptide shares 8 of 14 (15) amino acids with the Manse-AT-like III peptide which was predicted for *M. sexta* (Horodyski et al., 2001).

A cDNA that encodes 125 amino acid residues including one copy of the Manse-AST peptide has been cloned from *S. frugiperda* (Spofr-AST; QVRFRQCYFNPISCF-OH). The basic organization of the Spofr-AST precursor is similar to that of *P. unipuncta* with 85% amino acid sequence identity. The complete *Spofr-AST* cDNA sequence consists of 699 nucleotides. It contains a 5' untranslated region of 215 nucleotides upstream of an open reading frame of 125 amino acids. The assigned initiator codon is located at positions 216-218. It is possible that the translation initiation occurs at methionines further downstream (Met^{16}, Met^{29}). Initiation at these sites would, however, yield a precursor without an appropriate signal peptide. The open reading frame is followed by a 106-nucleotide 3' untranslated region including the poly (A) tail. A consensus polyadenylation signal (AATAAA) is found after position 648, which is 19 nucleotides upstream from the poly (A) tail. The Spofr-AST peptide sequence is located at the carboxy-terminus between Arg^{108} and Arg^{124} and is flanked by potential Lys-Arg and Arg-Lys endoproteolytic cleavage sites. The *Spofr-AST* gene is present as a single copy.

Semi-quantitative one-step RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the three mRNAs of the *Spofr-AT* gene (**paper 2)**. The results demonstrate that the *Spofr-AT* gene is expressed in brains of all developmental stages of *S. frugiperda* studied, but with variable intensity. The shortest (1^{st} Spofr-AT) mRNA isoform was highly expressed in 5^{th} instar larvae, 10 d-old pupae, and 1 to 5 d-old adult females and males, respectively, whereas it was expressed at a lower level in the prepupal stage and in young pupae. The 2^{nd} Spofr-AT mRNA isoform was highly expressed in the 5^{th} larval stage, whereas no expression was detected in 10 d-old pupae. Significant expression of the 3^{rd} mRNA isoform was found only in 5^{th} instar larvae, young pupae, and young adult females and males. In adult females and males, generally all three mRNA isoforms of the *Spofr-AT* gene were expressed during the first 5 days after eclosion, and always in the order of 1^{st} mRNA > 2^{nd} mRNA > 3^{rd} mRNA. No significant quantitative changes in total amounts of AT mRNA were observed.

Spofr-AST expression studies using one-step RT-PCR for semi-quantification of mRNA products demonstrated high expression rates in larvae (L4 to L6), but low expression during the pupal phase. A clear sex-specific expression of the *Spofr-AST* gene can be seen in older imagoes, with high expression rates in males (d4/d5), but negligible values in females (d5).

In the midgut of *S. frugiperda*, AT expression could be detected in all developmental stages, but with considerable qualitative and quantitative variability. In general, the 2nd mRNA isoform was present in all stages and significant amounts of the 3rd mRNA isoform were found in prepupae and in older pupae. Spofr-AST midgut expression was highest in L4 larvae, but low during the pupal stage. No clear time-dependent expression profile could be found for the imagoes.

Spofr-AT expression was high in ovaries and testes of young adults (day 1 to day 3), and dropped to negligible values thereafter. Within the testes, the shortest (1st Spofr-AT) mRNA isoform was most expressed, whereas isoform 3 could not be detected. In the ovary, the 2nd mRNA isoform represents the dominating one. Spofr-AST expression rates in the ovary were high during the first 3 days after ecdysis and dropped thereafter, whereas for the testes a high precursor expression could be detected only during the first 24 h after ecdysis.

In situ hybridization (ISH) using digoxigenin-labelled cRNA revealed that *Spofr-AT* mRNA is distributed in several groups of cells in the brain of 2 d-old adult female. In *S. frugiperda,* the suboesophageal ganglion (SOG) lies directly below the cerebral ganglion and is fused to the ventral sides of the brain. There were two relatively large clusters of cells in the anterior-lateral region of the SOG, which displayed strong expression of *Spofr-AT* mRNA. Spofr-AST was expressed in a cluster of cells at the coxa region of the antenna in the deuterocerebrum. Another cluster of positive cells was found in the posterior-ventral region in each of the optical lobes adjacent to the protocerebrum. *In situ* hybridization of *Spofr-AT* and *Spofr-AST* mRNA in the digestive tract showed that both genes are expressed in various parts of the intestine, especially in the posterior part of the crop and the anterior part of the midgut. In *S. frugiperda,* ovarioles are of the polytrophic type. *Spofr-AT* gene expression was clearly localized in the follicle cells between the oocytes. The *Spofr-AST* gene was localized in the outer region of the oocytes (follicle cells), and some slight hybridization was seen between the oocytes.

Spofr-AT and Spofr-AST expression rates in the brain of *S. frugiperda* are supposed to be correlated with the allatoregulating activity of the corresponding neuropeptides during larval and adult development. Both hormone precursors were slightly or not expressed during the pupal phase, whereas in the late larval stages (L5 and L6), high expression of the allatostatin in combination with low expression of the allatotropin may lead to the inevitable reduction in CA activity at that time. In young adult females and males, increasing haemolymph JH titers will be necessary to induce vitellogenesis and spermatogenesis, respectively. This increase in JH biosynthesis is accomplished by relatively high rates of expression for both hormone precursors in females and males during the first 24 h after

eclosion. The results confirm the earlier speculation (Oeh et al., 2000, 2001) that Spofr-AT and Spofr-AST strongly interfere while regulating JH biosynthesis. Low expression rates of the Spofr-AST in the brain of older females (day 5) together with high expression for Spofr-AT, well agree with earlier measurements of low JH biosynthesis by the CA at that time (Range et al., 2002), because the brain AST expression should be low when JH biosynthesis (and vitellogenesis) is high and vice versa. The role of ovarian and testes Spofr-AT and Spofr-AST in the regulation of the reproductive cycle is not clear. It is unknown whether these peptides will be released into the haemolymph, and thus may affect CA activity through an endocrine mechanism, or if they may act in a more paracrine way, and thus affect oviduct and semiduct contraction and/or egg laying, growth of the male accessory reproductive glands, and deposition of the spermatophores (Paemen et al., 1990, 1992). In locusts, an inhibitory effect of Y/FXFGL-amide allatostatins on lateral oviduct contraction is known (Schoofs et al., 1997). Garside et al. (2002) and Meyering-Vos and Hoffmann (2003) recently demonstrated the expression of type-A allatostatins in the common and lateral oviducts as well as in the ovary of mated females of *D. punctata* and *G. bimaculatus,* respectively.

Distinct changes in the rate of expression of Spofr-AT and Spofr-AST in the midgut of larvae and adults confirm the suggested roles of lepidopteran allatotropin and allatostatin, respectively, as brain/gut neuropeptides (for reviews see Hoffmann et al., 1999). Manse-AT was shown to inhibit active ion transport across the larval midgut epithelium of *M. sexta* (Lee et al., 1998; Chamberlin and King, 1998) and it stimulated foregut contractions in *H. armigera* (Duve et al*.,* 1999). Manse-AST was shown to inhibit spontaneous foregut contractions in *L. oleracea* (Duve et al*.,* 2000). Both peptides may play a role related to feeding and nutrition (Oeh et al., 2001).

The results from the whole mount *in situ* hybridization studies are in agreement with the semi-quantitative RT-PCR data. Both genes (*Spofr-AT* and *Spofr-AST*) are localized in distinct cells or cell groups of the brain, midgut, and ovary of larval and/or adult *S. frugiperda*. In the brain of 2 d-old females, Spofr-AT hybridizing cells were found in the lateral protocerebrum as well as within the medial neurosecretory region, consistent with areas associated with innervation of the CA and modulation of JH biosynthesis (Carrow et al., 1984; Orchard and Loughton, 1985; Homberg et al., 1991). Expression was also detected in several cells of the SOG that are suggested to innervate the CA through the NCA II nerve (Truesdell et al., 2000). In contrast to *Spofr-AT*, the *Spofr-AST* gene was highly expressed in a cluster of cells of the coxa region of the antenna and in the posterior region of the SOG. Possibly, the allatostatin is involved in regulating the movement of the antenna and/or sensory perception.

Strong hybridization of both *Spofr-AT* and *Spofr-AST* mRNAs in epithelial cells of the crop and midgut (*Spofr-AST* only) as well as in longitudinal and circular muscles of the midgut support the evidence that both peptides may control the peristaltic movements of the digestive tract as well as the ion transport through the epithelial cells. As in most other tissues studied, Spofr-AT and Spofr-AST did not show co-localization in the ovary of a 2 d-old unmated female. Whereas *Spofr-AT* mRNA was restricted to the follicle cells between the oocytes, *Spofr-AST* mRNA was mainly found at the outer membrane of the oocytes. In another polyandrous lepidopteran species with JH as the main gonadotropic hormone, *P. unipuncta*, females seem to be able to detect the quality of the males through the time of mating and the size of the spermatophores. Males may transfer a sex peptide in the ejaculate through copulation which increases the haemolymph JH titer in the females, as observed shortly after mating (McNeil et al., 2000). This peptide could be an allatotropin or an allatotropin-related peptide. Truesdell et al. (2000) observed a marked sexual dimorphism in the levels of the allatotropin mRNA in the thoracic ganglia of *P. unipuncta* adults.

In conclusion, the present data strengthen the hypothesis that both Spofr-AT and Spofr-AST exhibit multiple functions, which may be tissue-specific as well as specific to a particular life stage. Although allatotropin and allatostatin in *S. frugiperda* are identical in its primary structures to those in *M. sexta* and *P. unipuncta,* differences in activity between species may exist which would reflect differences in the life strategy of the species.

Several allatostatins, structurally unrelated to Manse-AST, were identified in various insect orders including the lepidopterans (e.g. Duve et al., 1997a). The allatostatin A-type peptide family currently comprises more than 60 members isolated from insects and some crustacean species (Duve et al., 1997b, 2002). The peptides share the C-terminally amidated pentapeptide sequence Y/FXFGL-amide (A-type allatostatins; allatostatin superfamily). Their allatostatic function seems to be restricted to cockroaches and crickets (Hoffmann et al., 1999). The gene encoding the *S. frugiperda* allatostatin type-A peptide family (Y/FXFGL-amides) was isolated from *S. frugiperda* brain cDNA in the third paper (**paper 3**). The gene encodes a precursor of 231 amino acids containing nine (or ten) Y/FXFGL-amide peptides which are tandemly arranged in three blocks. All except one of the *S. frugiperda* peptides had previously been deduced from the cDNA sequences of two other lepidopteran species (Davey et al., 1999; Secher et al., 2001). Spofr-AST A 9 (ERDMHGFSFGLa) differs in its primary structure from the homologous peptides found in two other lepidopteran species, *H. armigera* and *B. mori*, by one and two amino acids, respectively. The cDNA contains 132 nucleotides of an untranslated sequence upstream of a single open reading frame beginning at position 133 and ending at position 826 with the translation stop codon TAA. The *Spofr-AST A* precursor

begins with a hydrophobic domain that represents a probable signal peptide with the most likely site of cleavage between residues 18 and 19 (Von Heijne, 1986). The precursor contains the unprocessed sequences of nine Y/FXFGL-amide peptides, which are located at residues 286-312, 319-375, 382-408, 526-552, 592-618, 625-651, 658-684, 691-720, and 727-762, respectively. All of the putative peptides have a signal glycine residue at the C-terminal end that provides a substrate for peptidyl-glycine-α-amidating monooxygenase (Eipper et al., 1992). Cleavage of Spofr-AST A 2 at amino acid positions 71/72 would lead to a tenth peptide.

The *Spofr-AST A* gene is strongly expressed as one transcript in the brains of 4th to 6th instar larvae as well as in the early and late pupal stage, whereas the expression signal was significantly lower in the brains of the prepupal stage. In brains from adult moths, a sex-specific expression of the *Spofr-AST A* gene can be observed. Expression was high in the brain of adult females shortly after eclosion (day 1) and decreased thereafter, whereas in males the gene expression was low after eclosion but increased up to day 4 after moulting. In the midgut of *S. frugiperda*, allatostatin type-A expression could be detected in all developmental stages (from 4th instar larvae to 5 d-old adults of both sexes), but with considerable variability. High expression rates were observed in last larval instars (L6) and in prepupae, but also in the midgut of adult moths (day 3 to day 5). The *Spofr-AST A* gene was also expressed in the reproductive tissues of both sexes. Expression rates in the ovary were high at day 2 and day 3 after ecdysis and dropped to day 4, whereas for the testes high expression was detected at the first day after eclosion, but decreased thereafter.

In situ hybridization (ISH) using digoxigenin-labelled cRNA was carried out for the digestive tract, oviduct and the ovary from 2 d-old adult females and showed the cellular distribution of two populations of hybridizing cells in the midgut, one located at the anterior end of the midgut, and the second at the extreme posterior end. The *Spofr-AST A* mRNA was also localized in the wall of the crop. In the ovaries, the *Spofr-AST A* gene was localized within the oocytes with stronger labelling in older follicles. A distinct expression of the *Spofr-AST A* mRNA was also localized in the lateral oviduct.

The general organization of the allatostatin A-type precursor structure (total number of amino acids, number and arrangement of encoded peptides, number and localization of acidic spacers) seems to be conserved within the order Lepidoptera, although some specific family differences may exist. This results in a 84% amino acid residue identity between the precursor proteins of *S. frugiperda* and *H. armigera* (both Noctuidae), but only a 57 % identity between *S. frugiperda* and the silkworm *B. mori* (Bombycidae). Such family-specific differences have already been shown for other major neuropeptide families such as the

adipokinetic hormones (see Gäde et al., 1997) and, more recently, for diapause hormones and pheromone biosynthesis-activating neuropeptides (PBAN) (Iglesias et al., 2002).

One-step RT-PCR analyses demonstrated that the *Spofr-AST A* gene is expressed in different tissues of the moth in a developmental- and sex-specific manner. The distinct changes in the rates of expression of the gene in brain and midgut confirm the allatostatin A-type peptides as insect brain-gut myomodulatory hormones. The marked sex-specific brain expression in adult animals during the first four days after eclosion is similar to that found for Spofr-AST (C-type allatostatin). However, the physiological meaning of the sex specific brain expression of Spofr-AST A peptide is not yet clear. The *Spofr-AST A* gene localization within the oocytes might suggest a role of the peptides during embryonic development. Gene localization in the midgut of adult *S. frugiperda* was similar to that described for the helicostatin gene expression in *H. armigera* larvae (Davey et al., 1999). Knowledge of the allatostatin type-A peptide precursor sequence has opened the way for synthesis of all members of the peptide family in *S. frugiperda* for detailed physiological and functional studies.

Until this PhD thesis became in progress only one peptide with allatotropic activity had been isolated and identified, the *M. sexta* allatotropin peptide (Manse-AT; GFKNVEMMTARGF-NH_2). This peptide was purified from extracts of heads of the pharate adult of *M. sexta* and it stimulated the secretion of JH from the CA of various lepidopteran species. Data presented in the fourth paper (**paper 4**) demonstrate the isolation of a cDNA that encodes a novel *S. frugiperda* prepro-allatotropin. The cDNA encodes 53 amino acids, including one copy of the RVRGNPISCF-OH peptide. This peptide strongly stimulated the synthesis and release of JH *in vitro* by the CA of *S. frugiperda* and was code-named Spofr-AT 2. The cDNA consists of 307 nucleotides and contains a 5' untranslated region of 15 nucleotides upstream of an open reading frame. The assigned initiation codon is located at nucleotide positions 16 to 18. Within the amino-terminus of the precursor a single potential signal peptide cleavage site downstream Ala^{32} was found (Von Heijne, 1986). The open reading frame is followed by a 130-nucleotide 3' untranslated region including the poly (A) tail. A consensus polyadenylation signal (AATAAA) was found after position 260, which is 12 nucleotides upstream from the poly (A) tail. The novel allatotropin is located between amino acid residues Arg^{36} and Arg^{47} and is flanked by potential Lys-Arg and Arg-Lys endoproteolytic cleavage sites. The amino acid sequence of the peptide is identical at its C-terminus (-NPISCF) with the *M. sexta* allatostatin (Manse-AST), but the synthetic peptide showed strong allatotropic activity in the radiochemical bioassay for measuring JH biosynthesis by the CA *in vitro*.

Northern blot analysis demonstrated that the gene is expressed in only one transcript of ca. 0.366 kb in the brains of 6th instar larvae and 2 d-old adult females, but also in the midgut and ovary of 2 d-old females. RT-PCR analyses confirmed the expression of the gene as demonstrated by Northern blotting. A clear expression signal was found in the brains of 4th (L4), 5th (L5), and 6th instar larvae (L6), but also in prepupae, young pupae, 10 d-old pupae, and 0 to 3 d-old adult females. In contrast, no expression signal could be seen for the brains of 0 to 5 d-old adult males. A distinct gene expression was found in the midgut and the ovary of the females during the first 5 days of adult lifespan, but no signal could be detected in the midgut of the preimaginal stages and of adult males. Whole-mount *in situ* hybridization studies demonstrated that the *Spofr-AT 2* mRNA is localized in two large clusters of cells in the anterior-lateral region of the suboesophageal ganglion (SOG). In the ovary of a 2 d-old adult female the Spofr-AT 2 gene transcript was localized within the oocytes.

According to the C-terminal hexapeptide sequence –NPISCF, which the Spofr-AT 2 peptide shares with Manse-AST, an allatostatic activity was expected when incubating the CA from adult females in the presence of the synthetic peptide. Unexpectedly, however, when the CA from 2 and 3 d-old adult females, after a 2 h preincubation in medium without peptide, were transferred into medium containing the peptide at concentrations ranging from 10^{-8} to 10^{-4} M, a strong and dose-dependent allatotropic effect was observed. The peptide caused an approximately tenfold rise in JH release at a concentration of 10^{-4} M, from about 4 pmol · h^{-1} · CA^{-1} to ca. 40 pmol · h^{-1} · CA^{-1}. The stimulation was dose-dependent with an apparent EC_{50} of ca. 10^{-7} M. CA that were activated with Spofr-AT 2 could be inhibited by the addition of synthetic Manse-AST. Glands that had been activated with 10^{-6} M Spofr-AT 2 in the first incubation and then transferred to a peptide-free medium for a second 2 h incubation still produced and released significantly more JH ($p<0.001$) than untreated control glands. Simultaneous addition of Spofr-AT 2 (10^{-6} to 10^{-4} M) and Manse-AT (10^{-6} M) resulted in a cumulative effect; glands released significantly more JH ($p<0.01$ and $p<0.001$, respectively) than when incubated in the presence of only one of the peptides.

For the present studies on JH biosynthesis and release by the CA *in vitro* we have used ^{14}C-labelled acetate as a tracer. The rates of JH release by the CA from 2 d-old females of *S. frugiperda* as well as the effects of Manse-AT, Manse-AST, and Manse-AT plus Manse-AST did not differ from the results from previous experiments (Oeh et al., 2000; Range et al., 2002) where L-[methyl-^{3}H]-methionine was used as the marker.

In conclusion the novel peptide seems to represent a brain/gut neuropeptide which is also expressed in the female reproductive organs. The tissue distribution as well as its clear expression in the anterior-lateral region of the SOG and in the oocytes corresponds to that

recently demonstrated for Manse-AT and Manse-AST in *S. frugiperda* (see paper 2). This is the first report on the presence and function of two different allatotropins in an insect species.

The similarity in the primary structures of Manse-AST (with strong allatostatic activity in the radiochemical assay *in vitro*) and Spofr-AT 2 (with its strong allatotropic activity) is striking. In Manse-AST the Cys^{7} to Phe^{15} C-terminal sequence seems to define the active core of the peptide (disulfide bridge between Cys^{7} and Cys^{14}). In Manse-AT, the C-terminal octapeptide EMMTARGF-amide represents the biologically active core, which is completely different from the C-terminus in Spofr-AT 2. Further experiments have to be carried out using deletion analogs of Spofr-AT 2 to provide insight into the active core for this peptide. More experiments will also be necessary to explain the physiological role of Spofr-AT 2 as well as its possible interactions with Manse-AT and Manse-AST in the regulation of JH biosynthesis in *S. frugiperda*. Finally, it should be tried to isolate the Spofr-AT 2 peptide from *S. frugiperda* brain extracts and to look for the occurrence and probably pleiotropic functions of this peptide in other lepidopteran and non-lepidopteran species.

References

Audsley, N.; Weaver, R.J.; Edwards, J.P. (1998): Enzyme linked immunosorbent assay for *Manduca sexta* allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in *Lacanobia oleracea*. Insect Biochem. Molec. Biol. **28**, 775-784.

Audsley, N.; Weaver, R.J.; Edwards, J.P. (1999): Juvenile hormone synthesis by corpora allata of the tomato moth, *Lacanobia oleracea* (Lepidoptera: Noctuidae), and effects of allatostatins and allatotropin *in vitro*. Eur. J. Entomol. **96**, 287-293.

Audsley, N.; Weaver, R.J.; Edwards, J.P. (2001): *In vivo* effects of *Manduca sexta* allatostatin and allatotropin on larvae of the tomato moth, *Lacanobia oleracea*. Physiol. Entomol. **26**, 181–188.

Auerswald, L.; Birgül, N.; Gäde, G.; Kreienkamp, H-J.; Richter, D. (2001): Structural, functional, and evolutionary characterization of novel members of the allatostatin receptor family from insects. Biochem. Biophys. Res. Commun. **282**, 904-909.

Bellés, X.; Graham, L.; Bendena, W.G.; Ding, Q.; Edwards, J.P.; Weaver, R.J.; Tobe, S.S. (1999): The molecular evolution of the allatostatin precursor in cockroaches. Peptides **20**, 11-22.

Bendena, W.G.; Garside, C.S.; Yu, C.G.; Tobe, S.S. (1997): Allatostatins: diversity in structure and function of an insect neuropeptide family. Ann. N. Y. Acad. Sci. **814**, 53-66.

Bhatt, T.R.; Horodyski, F.M. (1999): Expression of the *Manduca sexta* allatotropin gene in cells of the central and enteric nervous systems. J. Comp. Neurol. **403**, 407-420.

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

Blackburn, M.B.; Wagner, R.M.; Kochansky, J.P.; Harrison, D.J.; Thomas-Laemont, P.; Raina, A.K. (1995): The identification of two myoinhibitory peptides, with sequence similarities to the galanins, isolated from ventral nerve cord of *Manduca sexta*. Reg. Pept. **57**, 213-219.

Blackburn, M.B.; Jaffe, H.; Kochansky, J.P.; Raina, A.K. (2001): Identification of four additional myoinhibitory peptides (MIPs) from the ventral nerve cord of *Manduca sexta*. Arch. Insect Biochem. Physiol. **48**, 121-128.

Carrow, G.M.; Calabrese, R.L.; Williams, C.M. (1984): Architecture and physiology of insect cerebral neurosecretory cells. J. Neurosci. **4**, 1034-1044.

Chamberlin, M.E.; King, M.E. (1998): Changes in midgut active ion transport and metabolism during the fifth instar of the tobacco hornworm (*Manduca sexta*). J. Exp. Zool. **280**, 135-141.

Cusson, M. (2000): Can juvenile hormone research help rejuvenate integrated pest management? Can. Entomol. **132**, 263-280.

Davey, M.; Duve, H.; Thorpe, A.; East, P. (1999): Characterization of the helicostatin peptide precursor gene from *Helicoverpa armigera* (Lepidoptera: Noctuidae). Insect Biochem. Molec. Biol. **29**, 1119-1127.

Ding, Q.; Donly, B.C.; Tobe, S.S.; Bendena, W.G. (1995): Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach, *Periplaneta americana*. Eur. J. Biochem. **234**, 737-746.

Donly, B.C.; Ding Q.; Tobe, S.S.; Bendena, W.G. (1993): Molecular cloning of the gene for allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. Proc. Nat. Acad. Sci. USA **90**, 8807-8811.

Duve, H.; Johnsen, A.H.; Maestro, J-H.; Scott, A.G.; Crook, N.; Winstanley, D. (1997 a): Identification, tissue localization and physiological effect *in vitro* of a neuroendocrine peptide

identical to a dipteran Leu-callatostatin in the codling moth *Cydia pomonella* (Tortricidae: Lepidoptera). Cell Tissue Res. **289**, 73-83.

Duve, H.; Johnsen, A.H.; Maestro, J.L.; Scott, A.G.; Jaros, P.P.; Thorpe, A. (1997 b): Isolation and identification of multiple neuropeptides of the allatostatin superfamily in the shore crab *Carcinus maenas*. Eur. J. Biochem. **250**, 727-734.

Duve, H.; East, P.D.; Thorpe, A. (1999): Regulation of lepidopteran foregut movement by allatostatins and allatotropin from the frontal ganglion. J. Comp. Neurol. **413**, 405-416.

Duve, H.; Audsley, N.; Weaver, R.J.; Thorpe, A. (2000): Triple co-localisation of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran *Lacanobia oleracea* (Noctuidae): innervation and action on the foregut. Cell Tissues Res. *300*, 153–163.

Duve, H.; Johnsen, A.H.; Scott, A.G.; Thorpe, A. (2002): Allatostatins of the tiger prawn, *Penaeus monodon* (Crustacea: Penaeidea). Peptides **23**, 1039-1051.

Eipper, B.A.; Stoffers, D.A.; Mains, R.E. (1992): The biosynthesis of neuropeptides: peptide-amidation. Annu. Rev. Neurosci. **15**, 57-85.

Elekonich, M.M.; Horodyski, F.M. (2003): Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. Peptides **24**, 1623-1632.

Gäde, G.; Hoffmann, K.H.; Spring, J. (1997): Hormonal regulation in insects: facts, gaps and future directions. Physiol. Rev. **77**, 963-1032.

Gäde, G. (2002): Allatoregulatory peptides-molecules with multiple functions. Invertebr. Reprod. Dev. **41**, 127-135.

Gäde, G.; Goldworthy, G.J. (2003): Insect peptide hormones: a selective review of their physiology and potential application for pest control. Pest Managem. Sci. **59**, 1063-1075.

Garside, C.S.; Koladish, P.M.; Bendena, W.G.; Tobe, S.S. (2002): Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. Insect Biochem. Molec. Biol. **32**, 1089-1099.

Hewes, R.S.; Taghert, P.H. (2001): Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res. **11**, 1126-1142.

Hoffmann, K.H.; Lorenz, M.W. (1998): Recent advances on hormones in insect pest control. Phytoparasitica **26**, 323-330.

Hoffmann, K.H.; Meyering-Vos, M.; Lorenz, M.W. (1999): Allatostatins and allatotropins: is regulation of corpora allata activity their primary function? Eur. J. Entomol. **96**, 255-266.

Homberg, U.; Davies, N.T.; Hildebrand, J.G. (1991): Peptide-immunocytochemistry of neurosecretory cells in the brain and retrocerebral complex of the sphinx moth *Manduca sexta*. J. Comp. Neurol. **303**, 35-52.

Horodyski, F.M.; Bhatt, S.R.; Lee, K.Y. (2001): Alternative splicing of transcripts by *Manduca sexta* allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. Peptides **22**, 263-269.

Hua, Y-J.; Tanaka, Y.; Nakamura, K.; Sakakibara, M.; Nagata, S.; Kataoke, H. (1999): Identification of prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. J. Biol. Chem. **274**, 31169-31173.

Iglesias, F.; Marco, P.; François, M-C.; Camps, F.; Fabrias, G.; Jaquin-Joly, G. (2002): A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. Insect Biochem. Molec. Biol. **32**, 901-908.

Jansons, I.S.; Cusson, M.; McNeil, J.W.; Tobe, S.S.; Bendena, W.G. (1996): Molecular characterisation of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). Insect Biochem. Molec. Biol. **26**, 767-773.

Kataoka, H.; Toschi, A.; Li, J.P.; Carney, R.L.; Schooley, D.A.; Kramer, S.J. (1989): Identification of an allatotropin from adult *Manduca sexta*. Science 243, 1481-1483.

Koladich, P.M.; Cusson, M.; Bendena, W.G.; Tobe, S.S.; McNeil, J.N. (2002): Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth, *Pseudaletia unipuncta*. Peptides **23**, 645-651.

Kramer, S.J.; Toschi, A.; Miller, C.A.; Kataoka, H.; Quistad, G.B.; Li, J.P.; Carney, R.L.; Schooley, D.A. (1991): Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. Proc. Nat. Acad. Sci. USA **88**, 9458-9462.

Kreienkamp, H-J.; Larusson, H.J.; Witte, I.; Roeder, T.; Birgül, N.; Hönck, H-H.; Harder, S.; Ellinghausen, G.; Buck, F.; Richter, D. (2002): Functional annotation of two orphan G-protein-coupled receptors, Drostar 1 and -2, from *Drosophila melanogaster* and their ligands by reverse pharmacology. J. Biol. Chem. **277**, 39937-39943.

Lee, K-Y.; Horodyski, F.M.; Chamberlin, M.E. (1998): Inhibition of midgut ion transport by allatotropin (Mas-AT) and Manduca FLRF-amides in the tobacco hornworm *Manduca sexta*. J. Exp. Biol. **201**, 3067-3074.

Lee, K-Y.; Chamberlin, M.E.; Horodyski, F.M. (2002): Biological activity of *Manduca sexta* allatotropin-like peptides, predicted products of tissue-specific and developmentally regulated alternatively spliced mRNAs. Peptides **23**, 1933-1941.

Lenz, C.; Søndergaard, L.; Grimmelikhuijzen, C.J.P. (2000a): Molecular cloning and genomic organization of a novel receptor from *Drosophila melanogaster* structurally related to mammalian galanin receptors. Biochem. Biophys. Res. Commun. **269**, 91-96.

Lenz, C.; Williamson, M.; Grimmelikhuijzen, C.J.P. (2000b): Molecular cloning and genomic organization of a second probable allatostatin receptor from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. **273**, 571-577.

Lenz, C.; Williamson, M.; Grimmelikhuijzen, C.J.P. (2000c): Molecular cloning and genomic organization of an allatostatin preprohormone from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. **273**, 1126-1131.

Li, Y.; Unnithan, G.C.; Veenstra, J.A.; Feyereisen, R.; Noeriega, F.G. (2003): Stimulation of JH biosynthesis by the corpora allata of adult female *Aedes aegypti in vitro*: effect of farnesoic acid and *Aedes* allatotropin. J. Exp. Biol. **206**, 1825-1832.

Lorenz, M.W.; Kellner, R.; Hoffmann, K.H. (1995): A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, *Gryllus bimaculatus*. J. Biol. Chem. **270**, 21103-21108.

Lorenz, M.W.; Kellner, R.; Hoffmann, K.H.; Gäde, G. (2000): Identification of multiple peptides homologous to cockroach and cricket allatostatins in the stick insect *Carausius morosus*. Insect Biochem. Molec. Biol. **30**, 711-718.

McNeil, J.N.; Miller, D.; Laforge, M.; Cusson, M. (2000): Dynamics of juvenile hormone biosynthesis, degradation and titers in females of the true armyworm: a comparison of migratory and non-migratory populations. Physiol. Entomol. **25**, 103-111.

Menn, J.J.; Raina, A.K.; Edwards, J.P. (1989): Juvenoids and neuropeptides as insect control agents: retrospect and prospects. In Progress and Prospects in Insect Control, ed. N.R. McFarlane, BCPC Monograph No. **43** pp. 89-106. Farnham: British Crop Protection Council.

Meyering-Vos, M.; Wu, X.; Huang, J.; Jindra, M.; Hoffmann, K.H.; Sehnal, F. (2001): The allatostatin gene of the cricket *Gryllus bimaculatus* (Ensifera: Gryllidae). Molec. Cel. Endocrinol. **184**, 103-114.

Meyering-Vos, M.; Hoffmann, K.H. (2003): Expression of allatostatins in the Mediterranean field cricket, *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae). Comp. Biochem. Physiol. B **136**, 207-215.

Nässel, R. (2002): Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Progr. Neurobiol. **68**, 1-84.

Nijhout, H.F. (1994): Insect Hormones. Princeton University. Press, Princeton, N.J.

Oeh, U.; Lorenz, M.W.; Dyker, H.; Lösel, P.; Hoffmann, K.H. (2000): Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. Insect Biochem. Molec. Biol. **30**, 719-727.

Oeh, U.; Dyker, H.; Lösel, P.; Hoffmann, K.H. (2001): *In vivo* effects of *Manduca sexta* allatotropin and allatostatin on the development and reproduction in the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Invertebr. Reprod. Dev. **39**, 239-247.

Orchard, I.; Loughton, B. (1985): Neurosecretion. In: Kerkut, G.A. and Gilbert, L.I., Editors, Comp. Insect Physiol. Biochem. Pharmacol. **7**, 61–108.

Paemen, L.; Schoofs, L.; De Loof, A. (1990): Presence of myotropic peptides in the male accessory reproductive glands of *Locusta migratoria*. J. Insect Physiol. **36**, 861-867.

Paemen, L.; Schoofs, L.; De Loof, A. (1992): Localisation of the Lom-AG-myotropin 1-like substances in the male reproductive and nervous tissues of the locust, *Locust migratoria*. Cell Tissue Res. **268**, 91-97.

Petri, B.; Homberg, U.; Loseel, R.; Stengl, M. (2002): Evidence for a role of GABA and Mas-allatotropin in photic entrainment of the circadian clock of the cockroach *Leucophaea maderae*. J. Exp. Biol. **205**, 1459-1469.

Pratt, G.E.; Farnsworth, D.E.; Fok, K.F.; Siegel, N.R.; McCormack, A.L.; Shabanowitz, J.; Hunt, D.S.; Feyereisen, R. (1991a): Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. Proc. Nat. Acad. Sci. USA **88**, 2412-2416.

Pratt, G.E.; Farnsworth, D.E.; Siegel, N.R.; Fok, K.F.; Feyereisen, R. (1991b): Two types of allatostatic peptides from brains of cockroach *Diploptera punctata*. In Menn J.J., Kelly T.J. and Mader E. (eds): Insect Neuropeptides. ACS Symposium Series **453**. American Chemical Society, Washington, pp. 177-192.

Predel, R.; Rapus, J.; Eckert, M. (2001): Myoinhibitory neuropeptides in the American cockroach. Peptides **22**, 199-208.

Price, M.D.; Merte, J.; Nichols, R.; Koladich, P.M.; Tobe, S.S.; Bendena, W.G. (2002): *Drosophila melanogaster* flatline encodes a myotropin orthologue to *Manduca sexta* allatostatin. Peptides **23**, 787-794.

Rachinsky, A.; Feldlaufer, M.F. (2000): Responsiveness of honey bee (*Apis mellifera* L.) corpora allata to allatoregulatory peptides from four insect species. J. Insect Physiol. **46**, 41-46.

Rachinsky, A.; Tobe, S.S.; Feldlaufer, M.F. (2000): Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera* L.): developmental changes in sensitivity to JH precursor and allatotropin. Insect Biochem. Molec. Biol. **30**, 729-737.

Rachinsky, A.; Tobe, S.S. (1996): Role of second messengers in the regulation of juvenile hormone production in insects, with particular emphasis on calcium and phosphoinositide signaling. Arch. Insect Biochem. Physiol. **33**, 259-282.

Range, S.; Oeh, U.; Lorenz, M.W.; Etzel, W.; Nauen, R.; Hoffmann, K.H. (2002): Juvenile hormone biosynthesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). Comp. Biochem. Physiol. B **132**, 191-202.

Riddiford, L.M. (1994): Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. **24**, 213-274.

Riehle, M.A.; Garczynski, S.F.; Crim, J.W.; Hill, C.A.; Brown, M.R. (2002): Neuropeptides and peptides hormones in *Anopheles gambiae*. Science **289**, 172-175.

Schoofs, L.; Veelaert, D.; Vanden Broeck, J.; De Loof, A. (1997): Peptides in the locusts, *Locusta migratoria* and *Schistocerca gregaria*. Peptides **18**, 145–156.

Secher, T.; Lenz, C.; Cazzamali, G.; Sorensen, G.; Williamson, M.; Hansen, G.N.; Svane, P.; Grimmelikhuijzen, C.J.P. (2001): Molecular cloning of the functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. J. Biol. Chem. **276**, 47052-47060.

Shiga, S. (2003): Anatomy and functions of brain neurosecretory cells in Diptera. Microsc. Res. Tech. **62**, 114-131.

Stay, B.; Joshi, S.; Woodhead, A.P. (1991): Senstivity to allatostatins of corpora allata from larval and adult female *Diploptera punctata*. J. Insect Physiol. **37**, 36-70.

Stay, B.; Tobe, S.S.; Bendena, W.G. (1994): Allatostatins: identification, primary structures, functions and distribution. Adv. Insect Physiol. **25**, 267-337.

Stay, B. (2000): A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem. Molec. Biol.**30**, 653-662.

Stay, B.; Zhang, J.R.; Tobe, S.S. (2002): Methyl farnesoate and juvenile hormone production in embryos of *Diploptera punctata* in relation to innervation of corpora allata and their sensitivity to allatostatin. Peptides **23**, 1981-1990.

Taylor, P.A.; Bhatt, T.R.; Horodyski, F.M. (1996): Molecular characterization and expression analysis of *Manduca sexta* allatotropin. Eur. J. Biochem. **239**, 588-596.

Teal, P.E.A. (2002): Effects of allatotropin and allatostatin on *in vitro* production of juvenile hormones by the corpora allata of virgin females of the moths of *Heliothis virescens* and *Manduca sexta*. Peptides **23**, 663-669.

Tobe, S.S.; Stay, B. (1985): Structure and regulation of the corpora allatum. Adv. Insect Physiol. **18**, 305-432.

Truesdell, P.F.; Koladich, P.M.; Kataoka, H.; Kojima, K.; Suzuki, A.; McNeil, J.N.; Mizoguchi, A.; Tobe, S.S.; Bendena, W.G. (2000): Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta* encoding *Manduca sexta* allatotropin peptide. Insect Biochem. Molec. Biol. **30**, 691-702.

Vanden Broeck, J. (2001): Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. Peptides **22**, 241-254.

Vanden Broeck, J.; Veelaert, D.; Bendena, W.G.; Tobe, S.S.; De Loof, A. (1996): Molecular cloning of the precursor cDNA for schistostatins, locust allatostatin-like peptides with myoinhibiting properties. Molec. Cell. Endocrinol. **122**, 191-198.

Veenstra, J.A.; Lehman, H.K.; Davis, N.T. (1994): Allatotropin is a cardioacceleratory peptide in *Manduca sexta*. J. Exp. Biol. **188**, 347-354.

Veenstra, J.A.; Noriega, F.G.; Graf, R.; Feyereisen, R. (1997): Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*. Peptides **18**, 937-942.

Veenstra, J.A.; Costes, L. (1999): Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. Peptides **20**, 1145-1151.

Von Heijne, G. (1986): A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. **14**, 4683-4690.

Weaver, R.J.; Edwards, J.P.; Bendena, W.G.; Tobe, S.S. (1998): Structures, functions and occurrence of insect allatostatic peptides. In: Coast, G.M., Webster, S.G. (eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge. Pp, 3-32.

Williamson, M.; Lenz, C.; Winther, M.E.; Nässel, D.R.; Grimmelikhuijzen, C.J.P. (2001a): Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. **281**, 544-550.

Williamson, M.; Lenz, C.; Winther, M.E.; Nässel, D.R.; Grimmelikhuijzen, C.J.P. (2001b): Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. **282**, 124-130.

Witek, G.; Hoffmann, K.H. (2001): Immunological evidence for FGL-amide and W^2W^9-allatostatins in the ovary of *Gryllus bimaculatus* (Ensifera, Gryllidae). Physiol. Entomol. **26**, 49-57.

Woodhead, A.P.; Stay, B.; Seidel, S.L.; Khan, M.A.; Tobe, S.S. (1989): Primary structure of four allatostatins: neuropeptide inhibitoris of juvenile hormone synthesis. Proc. Nat. Acad. Sci. USA **86**, 5997-6001.

Žitňan, D.; Kingan, T.G.; Kramer, S.J.; Beckage, N.E. (1995): Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. J. Comp. Neurol. **365**, 83-100.

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Molecular characterisation of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides☆

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Abstract

Allatotropin (AT) is a 13-residue amidated neuropeptide, first isolated from pharate adult heads of the tobacco hornworm, *Manduca sexta* (Manse-AT), which strongly stimulates the biosynthesis of juvenile hormones (JH) in the corpora allata (CA) of adult moths. In *Spodoptera frugiperda*, a cDNA that encodes 134 amino acids, including an AT peptide, has been cloned. The *S. frugiperda* allatotropin mature peptide (Spofr-AT) [GFKNVEMMTARGFa] is identical to that isolated from *M. sexta*. The basic organization of the Spofr-AT precursor is similar to that of *Agrius convolvuli*, *M. sexta*, *Pseudaletia unipuncta*, and *Bombyx mori* with 83–93% amino acid sequence identity. The Spofr-AT gene is expressed in at least three mRNA isoforms with 134, 171 and 200 amino acids, differing from each other by alternative splicing.

All allatostatins (AS) have an inhibitory action on the JH biosynthesis in the CA. A cDNA that encodes 125 amino acid residues including one copy of the Manse-AS peptide has been cloned from *S. frugiperda* (Spofr-AS; QVRFRQCYFNPISCF). The basic organization of the Spofr-AS precursor is similar to that of *P. unipuncta* with 85% amino acid sequence identity.

Using one step RT-PCR for semi-quantification of the gene expression, we showed that the three mRNAs of the Spofr-AT gene and the Spofr-AS gene are expressed in brains of last instar larvae, prepupae, pupae, and adults of both sexes of *S. frugiperda* with variable intensity.

Keywords: Lepidoptera; Allatostatin; Allatotropin; Hormone processing; Prohormone; Neuropeptide

1. Introduction

The development and reproduction of insects are regulated, to a large extent, by juvenile hormones (JH). During the larval stages these hormones control moulting and metamorphosis, whereas in adult insects they are involved in the regulation of vitellogenesis in females and spermatogenesis and growth of the accessory reproductive glands in males (Koeppe et al., 1985; Nijhout, 1994; Riddiford, 1994; Gäde et al., 1997). Juvenile hormones are synthesized and released from the corpora allata (CA), which are present in all insect stages. During the last decade, interest has focused on factors that regulate JH biosynthesis by CA (Stay et al., 1994; Hoffmann et al., 1999; Bendena et al., 1999; Stay, 2000). Depending upon species and development stages, the signals may be either stimulatory (allatotropin, AT) or inhibitory (allatostatin, AS), and they may reach the glands via haemolymph or via nervous connections.

Since 1989, more than 60 neuropeptides that inhibit JH production by the CA in homologous or heterologous bioassays in vitro have been isolated from brains of several insect taxa (moths, cockroaches, locusts, crickets, flies and bees), and also from crustaceans (for review see Bendena et al., 1997; Gäde et al., 1997; Weaver et al., 1998; Hoffmann et al., 1999). The allatostatins form three different peptide families. Members of the allatostatin A family are characterised by a common C-terminal

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Y/FXFGL/I-a pentapeptide sequence. Some members of the allatostatin A family were deduced from prohormone gene sequences (Donly et al., 1993; Ding et al., 1995; East et al., 1996; Vanden Broeck et al., 1996; Veenstra et al., 1997; Bellés et al., 1999; Davey et al., 1999; Lenz et al., 2000; Meyering-Vos et al., 2001). In some species these peptides have no effect on the CA of the source insect, but instead exhibit myo-inhibiting properties (Duve and Thorpe, 1994). The second family consists of peptides, which were at first isolated from *Gryllus bimaculatus* and all have a common amino acid (Trp) at positions two and nine (allatostatin B family; W^2W^9-amide peptide family; $W(X)_6W$-amides) (Lorenz et al., 1995). A B-type allatostatin preprohormone from *Drosophila melanogaster* (DAP-B) has recently been cloned, which encodes 211 amino acid residues and contains one copy each of five putative allatostatin-B peptides (Williamson et al., 2001a).

Within the Lepidoptera, a non-amidated allatostatin was first characterised from the tobacco hornworm, *Manduca sexta*. This peptide, *M. sexta* allatostatin (C-type allatostatin; Manse-AS; pEVRFRQCYFNPISCF-OH) inhibits JH biosynthesis in vitro by the CA of larvae and adult females of *M. sexta*, and adult females of *Heliothis virescens*, *H. zea* and *Lacanobia oleracea* (Kramer et al., 1991; Audsley et al., 1999, 2000; Edwards et al., 2001; Teal, 2002). Manse-AS did not affect the CA of two orthopteroid species, *Periplaneta americana* and *Melanoplus sanguinipes*, or the CA of the beetle *Tenebrio molitor* (Kramer et al., 1991). The peptide shows no sequence similarity to members of any other allatostatin family. In *Pseudaletia unipuncta*, a cDNA has been characterised, which encodes 125 amino acid residues including the 15-residue peptide of the allatostatin C-type (Jansons et al., 1996). The Pseun-AS did not inhibit JH biosynthesis in 6th instar larvae or newly emerged (0 day) adults, but inhibited the CA activity of 5 day-old adult females of *P. unipuncta* by 60%. Expression of the gene was low in 6th instar larvae, prepupae and early pupae, but relatively high in late pupae and in 1 day- and 3 day-old adults of both sexes (Jansons et al., 1996). In *Spodoptera littoralis* (Audsley et al., 1999) and in *L. oleracea* (Audsley et al., 1998), a peptide that seems to be identical with Manse-AS was detected in larval brain extracts. Very recently, the allatostatin C-type preprohormone has been characterised from *D. melanogaster* (drostatin-C). The drostatin-C peptide differs in only one amino acid residue (F to Y in position 4) from the Manse-AS. The gene was strongly expressed in larvae and adult flies, but less in pupae and embryos (Williamson et al., 2001b).

In the fall armyworm *S. frugiperda*, synthetic Manse-AS did not affect the in vitro rate of JH secretion from the CA of adult moths. However, when the CA had previously been activated by Manse-AT, addition of Manse-AS reduced JH biosynthesis by about 70%. This allatostatic effect of Manse-AS on allatotropin-activated glands was dose-dependent and reversible (Oeh et al., 2000). However, until now, Manse-AS has not been detected in *S. frugiperda*.

To date, only one allatotropin is known (GFKNVEMMTARGFa), which has been demonstrated to stimulate the CA. It was first isolated from the head of pharate adults of *M. sexta* (Kataoka et al., 1989), for which the gene encoding the peptide has also been cloned (Taylor et al., 1996). This gene is expressed in at least three mRNA isoforms that differ from each other by alternative splicing regulated in a tissue-specific manner (Horodyski et al., 2001). Manse-AT has also been isolated from larval and adult brains of *L. oleracea* (Audsley et al., 2000). From the mosquito *Aedes aegypti* an allatotropin immunoreactive peptide has been isolated and its structure determined as APFRNSEMMTARGFa. Furthermore, the cDNA encoding this peptide has been identified (Veenstra and Costes, 1999). A cDNA isolated from the true armyworm, *P. unipuncta*, encodes a 135 amino acids sequence containing the Pseun-AT which is identical to that isolated from *M. sexta*. The AT mRNA was highly expressed in older pupae and 3- and 5-day-old adults of *P. unipuncta* (Truesdell et al., 2000). A precursor, which encodes a 130 amino acid polypeptide, has been cloned from a midgut cDNA library of *B. mori*, and this precursor also contained the Manse-AT peptide sequence (Park et al., 2002).

A peptide that strongly stimulates JH biosynthesis in vitro by the adult female CA was recently isolated from methanolic brain extracts of adult *S. frugiperda* and was identified as *M. sexta* allatotropin (Manse-AT) (Oeh et al., 2000). Injections of Manse-AT twice daily into penultimate and last instar larvae of *S. frugiperda* drastically reduced their weight gain and increased mortality, shortened the life span of the adult females and decreased the total number of deposited eggs (Oeh et al., 2001). Functional assays and distributional studies support additional roles for Manse-AT to that of JH regulation. Manse-AT seems to function as a cardioacceleratory peptide in adult *M. sexta* (Veenstra et al., 1994) and *P. unipuncta* (Koladich et al., 2002) and it inhibits midgut ion transport in day 2 fifth instar larvae of *M. sexta* (Lee et al., 1998). Multiple functional activities are suggested by the distribution of Manse-AT immunoreactive cells in neural tissues of various insect species (Veenstra and Hagedorn, 1993; Veenstra et al., 1994; Žitnan et al., 1993, 1995; Persson and Nässel, 1999; Rudwall et al., 2000; Tu et al., 2001).

In the present study we confirm the structure of the recently identified Manse-AT in *S. frugiperda*, and elucidate the existence of a C-type allatostatin in this species by cloning the cDNAs, which encode the precursors of Spofr-AT and Spofr-AS, respectively. Both the genes were variably expressed in the brains of larvae, pupae and adult moths.

2. Materials and methods

2.1. Animals

Pupae and eggs of *S. frugiperda* were provided by Bayer AG (Leverkusen) and reared at 27 °C and 70% relative humidity under a L 16: D 8 photoperiod as previously described (Oeh et al., 2000). Larvae and adults were reared as described by Oeh et al. (2000). Under these conditions, the 6th (last) larval stage lasts for about 4 days. Each pupa was individually kept in a separate compartment of assortment boxes (9 × 32 × 36 - mm per compartment; Licefa, Bad Salzuflen, Germany) until emergence. Freshly emerged females were kept in 20 × 20 × 10 - cm plastic boxes and fed with 10% sugar solution until dissection.

2.2. Preparation of the cDNA by RACE

Brains (cerebral and subesophageal ganglia) of 530 female adult fall armyworms, 2–3 days old, were dissected in modified cricket saline (Lorenz et al., 1997), frozen immediately in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab Biotechnologie GmbH). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen GmbH). The Smart™ RACE cDNA amplification kit (Clontech) was used to amplify the cDNA. 1 μg mRNA was added as a template for each RACE reaction. A 3′-RACE was carried out using degenerate oligonucleotide primers ATf4 (5′-GGN TTY AAR AAY GTN GAR ATG ATG A-3′) corresponding to nucleotide positions 397–421 of Fig. 1 and ASf5 (5′-GTN MGN TTY MGN CAR TGY TAY TTY AA-3′) corresponding to nucleotide positions 543-569 of Fig. 4, respectively, in combination with the universal primer (5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3′) that recognises the primer binding sequence, introduced by the Smart RACE kit components. The cDNA was further amplified by using gene specific primers ATf7 corresponding to nucleotide positions 397–421 and ATr6 corresponding to nucleotide positions 418–444 of Fig. 1 to get the 5′ end and the 3′ end of the AT preprohormone and ASf6 corresponding to nucleotide positions 543–569 and ASr10 corresponding to nucleotide positions 569–593 of Fig. 4 in order to get the 5′ end and the 3′ end of the AS precursor, respectively. The PCR program was 95 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 68 °C for 45 s decreased with 1 °C per cycle, followed by 35 cycles of 94 °C for 30 s, 58 °C for AS or 60 °C for AT for 45 s, 68 °C for 1.5 min, and a final extension step of 68 °C for 10 min.

2.3. Cloning and sequencing

The PCR products were eluted from low melting point agarose gel (Biozym) with GFX™ purification kit (Pharmacia), and ligated into plasmids with the pGEM-T easy system kit (Promega). The vector with the inserts was grown in *E. coli* JM109 (Promega) or XL1-blue (Stratagene), respectively. Plasmid DNA was purified using QIAprep® Spin Miniprep kit (Qiagen). The templates were sequenced by MWG Biotech (Ebersberg, Germany) or GATC (Konstanz, Germany). Sequences were analysed with the GCG software (Wisconsin Package).

2.4. RT-PCR analysis

For RT-PCR 20 ng mRNA from brains of 6th instar larvae (<12 h), pre-pupae (PP), young pupae (P0), 10-day-old pupae (P10), 2-day-old adult males (M2) and 2-day-old adult females (F2) were used with specific primers ATf7-ATr9 corresponding to nucleotide positions 397–421 and 598–621 of Fig. 1A and primers ASf6-ASr9 corresponding to nucleotide positions 543–569 and 644–670 of Fig. 4, respectively, in 10 μl of the RT-PCR mixture (TITANIUM® One Step RT-PCR Kit, CLONTECH Laboratories, Inc.). The PCR program for amplification of the fragment was 50 °C for 1 h followed by 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 60 °C for AT or 58 °C for AS for 45 s, 68 °C for 1 min, and a final extension step of 68 °C for 2 min. The DNA products were analysed using 1.5% agarose gel at 8 V/cm for 1.5 h, transferred to Hybond-N^+ membrane (Amersham) and hybridised to the probe (ATf7-ATr9) 225 bp corresponding to positions 397–621 of Fig. 1 and probe (ASf6-ASr9) 127 bp corresponding to positions 543–670 of Fig. 4, respectively, labelled by the PCR method with Dig dUTP (Roche). As a control the same quantities (20 ng) of mRNA from different developmental stages were used as a template in combination with two specific primers ACTf1 corresponding to nucleotide positions 1–25 and ACTr2 corresponding to nucleotide positions 469–493 from the nucleotide sequence of *S. littoralis* mRNA for beta-actin (partial; EMBL Nucleotide Sequence Data Base, accession number Z46873), which yields a 494 bp amplified fragment. Expressions of AT and AS were normalized relative to that of beta-actin. A negative control for genomic DNA has been done using RT-PCR program strategy 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 45 s, 68 ° for 1 min, and a final extension step of 68 °C for 2 min. Densitometry was preformed using an ImageMaster® VDS (Pharmacia Biotech) and the ImageMaster 1D Database software. A series of 5 to 20 ng mRNA (Spofr-AT and Spofr-AS from 6th instar larvae and 2-day-old adult females, respectively) was used to produce a standard curve by RT-PCR.

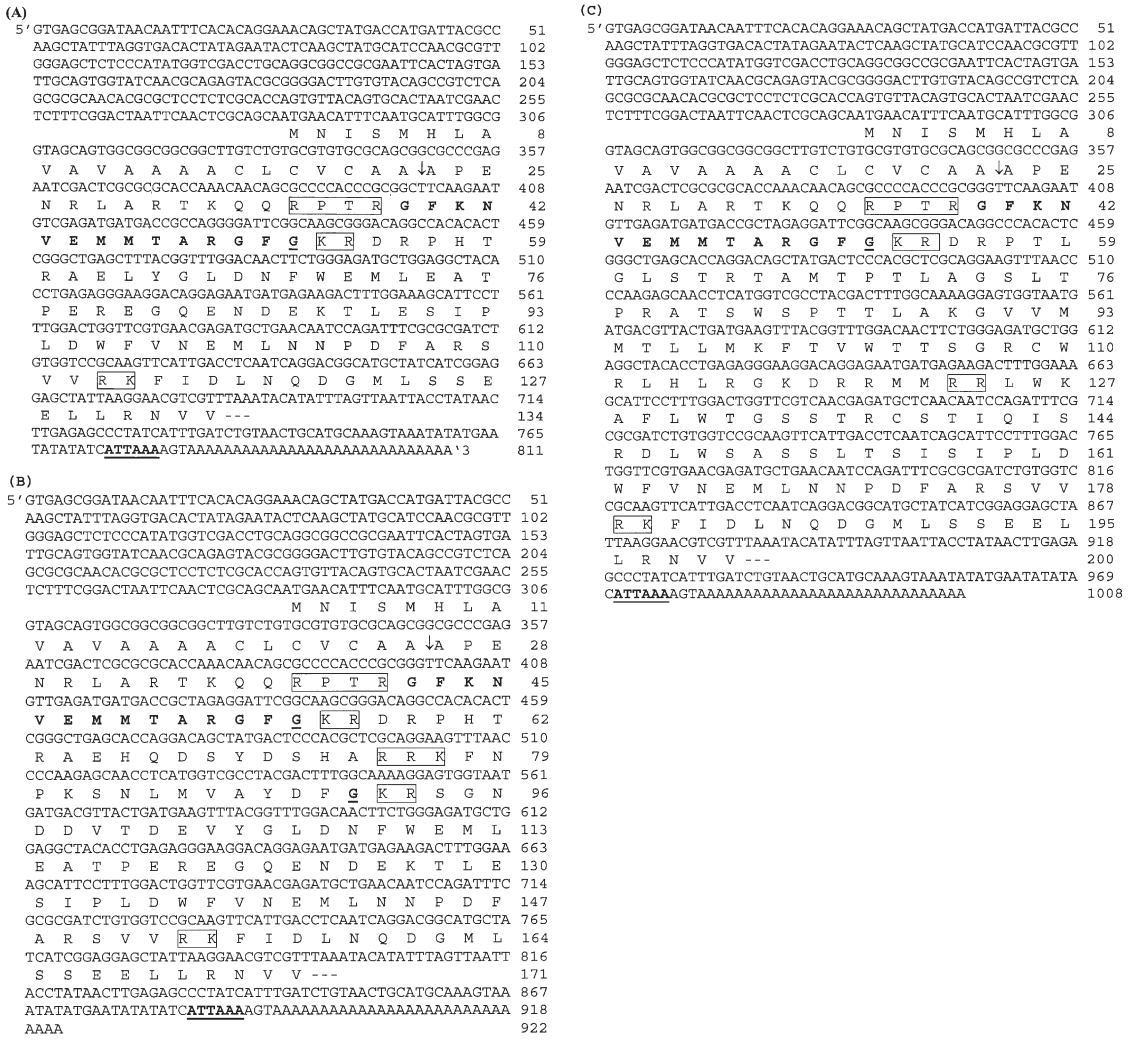

Fig. 1. Spofr-AT sequence data. (A) Nucleotide and the deduced amino acid sequences of the *S. frugiperda* AT cDNA. The sequences are numbered at the right. The Spofr-AT amino acid sequence is shown in bold type. Possible proteolytic cleavage sites are in boxes and the glycine residue required for amidation is underlined. The potential polyadenylation signal is shown in bold type and underlined; --- represents the stop codon. A signal peptide cleavage site is indicated by a downward arrow. (B) Nucleotide and deduced amino acid sequences of the second Spofr-AT precursor mRNA. (C) Nucleotide and deduced amino acid sequences of the third Spofr-AT precursor mRNA.

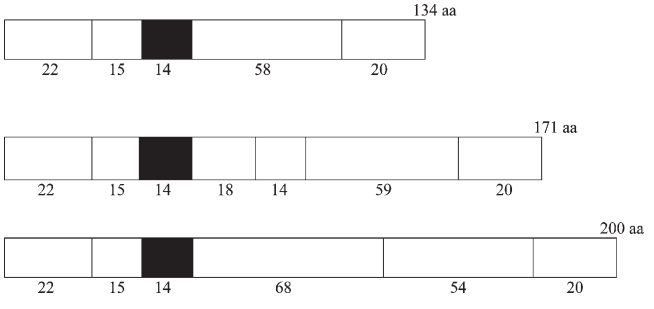

Fig. 2. The organisation of the predicted peptides encoded by the three Spofr-AT mRNAs. All three predicted forms contain a 22-residue signal peptide, a 15-residue peptide, the 14-residue Manse-AT peptide, including the glycine to be amidated, and a 80, 117 or 146-residue C-teminal peptide, which may be further processed.

3. Results

3.1. S. frugiperda *allatotropin (AT) cDNA*

The *S. frugiperda* AT gene is expressed in at least three mRNA isoforms. The cDNA AT sequences with 811, 922 and 1008 nucleotides, respectively, are shown in Fig. 1A–C. The cDNAs contain a 5′ untranslated region of 282 nucleotides and an open reading frame of 134, 171 and 200 amino acids, respectively, corresponding to nucleotides 283–684, 283–795 and 283–882. The assigned initiator codon is located at position 283–285. The stop codon is followed by a 124-nucleotide 3′ untranslated region including the 29-nucleotide poly (A) tail. A consensus polyadenylation signal (ATTAAA) is found after position 773, 884 and 970, respectively,

```
Spofr-AT MNISMHLAVAVAAAACLCVCAA↓APENRLARTKQQRP  36
Bommo-AT **LT*Q*E*I**V--**VLAEG**DV**V*******   34
Pseun-AT **F******V*********V*****G**T*******  36
Manse-AT **LT*Q***I**V--***LAEG**DV**T*******   34
Agrco-AT **LT*Q**MI**V--****AEG**DV**********   34

Spofr-AT TRGFKNVEMMTARGFGKRDRPHTRAELYGLDNFWEM   72
Bommo-AT ***********************P**********V*  70
Pseun-AT ***********************************   72
Manse-AT **********************P*************   70
Agrco-AT **********************P*************   70

Spofr-AT LEATPEREGQE-NDEKTLESIPLDWFVNEMLNNPDF  107
Bommo-AT **PSP***V**-V****F******************  105
Pseun-AT **SA*******T************************  108
Manse-AT **TS****V**V-************************  105
Agrco-AT **TS****V**VV************************  106

Spofr-AT ARSVVRKFIDLNQDGMLSSEELLRNVV           134
Bommo-AT **F**E*****************-***-           130
Pseun-AT *****H*********************           135
Manse-AT *************************F           131
Agrco-AT *************************             132
```

Fig. 3. Alignment of the AT precursor peptides of *S. frugiperda* (Spofr-AT), *B. mori* (Bommo-AT) (Park et al., 2002), *P. unipuncta* (Pseun-AT) (Truesdell et al., 2000), *M. sexta* (Manse-AT) (Taylor et al., 1996), and *A. convolvuli* (Agrco-AT) (H. Kataoka, unpublished). Asterisks represent amino acids identical to each of the precursors. The mature AT peptide is shown in bold type. A signal peptide cleavage site is indicated by a downward arrow.

```
5′CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGTACGCGG   51
  GGTCATTTGCTTTCAAAAACCTTCGAGGGAGACCACGGAAGCACCAACTAC  102
  AACCTTCCGTAGTTACTAGTAAAAGTGATCCGTACTTTATTTCACGTAAAC  153
  GAAACTCAGTTAGCTGCTGAATTTATTTCGACGTGCAGATTTATAAAGTTC  204
  GAAGGATCACAATGAAAACGAGCGCGTACAACGTGTACCTGGGAGTCGTGG  256
              M  K  T  S  A  Y  N  V  Y  L  G  V  V     13
  CCGCCATGTTGGCTCTACTGTTCGTCACAATTAATGCTGCGCCAATGGAGG  307
  A  A  M  L  A  L  L  F  V  T  I  N  A↓A  P  M  E     30
  CGGACGATGAGACGGCTGAGAACACCCTCGTGGCGCATCCCGATGGTGACA  358
  A  D  D  E  T  A  E  N  T  L  V  A  H  P  D  G  D     47
  TGGAGCTCTCAGGCCCCTGGGATGCTATCAACACTGCCGCTCTACGCAAAC  409
  M  E  L  S  G  P  W  D  A  I  N  T  A  A  L  R  K     64
  TGCTGCTGCAACTTGATGCAGAGGACAGGATGGGCGGGGTGACCCGCTCGT  460
  L  L  L  Q  L  D  A  E  D  R  M  G  G  V  T  R  S     81
  GGCCCCAAGCTGAGCCCCGCGGTTGGGGTCTGCGGGCGTTGGACAGCCGTC  511
  W  P  Q  A  E  P  R  G  W  G  L  R  A  L  D  S  R     98
  TGGCGCGGCAGTGGAGGGCAGACAAGCGGCAGGTGCGATTCCGCCAGTGCT  562
  L  A  R  Q  W  R  A  D  K  R  Q  V  R  F  R  Q  C    115
  ACTTCAACCCAATTTCCTGCTTCCGCAAGTGAAAACAGCACCAACTCAACG  613
  Y  F  N  P  I  S  C  F  R  K ---                     125
  ACGCATCGACCCTTTGACCTAGGGTAGCAAGAACGAATAAAACGTCGCCAT  664
  AATCTCCCGAAAAAAAAAAAAAAAAAAAAAAAAAAAA′3              699
```

Fig. 4. Nucleotide and the deduced amino acid sequences of the *S. frugiperda* AS cDNA. The sequences are numbered at the right. The amino acid sequence of the Spofr-AS is shown in bold type. Possible dibasic proteolytic cleavage sites are in boxes. The possible site for cleavage of the signal sequence is marked with a downward arrow. The potential polyadenylation signal is shown in bold type and underlined; --- represents the stop codon.

which is three nucleotides upstream from the poly (A) tail.

The *S. frugiperda* neuropeptide precursor contains a peptide sequence identical to Manse-AT. The Spofr-AT mature peptide is located between amino acid residues Arg^{38} and Lys^{53} on each of the three precursors (Fig. 1A–C and 3), flanked by potential Arg and Lys-Arg endoproteolytic cleavage sites and ends with a glycine residue, the signal for carboxy-terminal amidation by peptidyl-α-amidating monooxygenase (Eipper et al., 1992). The Spofr-AT sequence is present as a single copy. Recognition of all possible proteolytic cleavage sites would result in the production of three additional peptides of 15, 58 and 20 amino acids on the first precursor (134 amino acids), five additional peptides of 15, 18, 14, 59 and 20 amino acids on the second AT mRNA precursor (171 amino acids), and four additional peptides of 15, 68, 54 and 20 amino acids on the third AT mRNA precursor (200 amino acids) (Fig. 2). The cleavage site in the second mRNA at amino acid position 75 can also be RR instead of RK, which might be supported by the H just in front (HARR). This would imply a Lys residue as the first amino acid of the following peptide (15-residue peptide). The 14- or 15-residue peptide of the second isoform is of special interest as that peptide ends with a glycine, the signal for carboxy-terminal amidation, which is characteristic for many bioactive peptides. The peptide shares eight of 14 (15) identities with the Manse-AT-like III peptide predicted in *M. sexta* (Horodyski et al., 2001). The basic organisation of the Spofr-AT peptide precursor (134 amino acids) is similar to that of *M. sexta* (Taylor et al., 1996), *P. unipuncta* (Truesdell et al., 2000), *A. convolvuli* (H. Kataoka, unpublished), and *B. mori* (Park et al., 2002) with 84, 93, 85 and 83% amino acid sequence identity, respectively (Fig. 3).

3.2. S. frugiperda *allatostatin (AS) cDNA*

The complete Spofr-AS cDNA sequence consists of 699 nucleotides as shown in Fig. 4. It contains a 5′ untranslated region of 215 nucleotides upstream of an open reading frame of 125 amino acids. The assigned initiator codon is located at position 216–218. It is possible that the translation initiation occurs at methionines further downstream (Met^{16}, Met^{29}). Initiation at these sites would, however, yield a precursor without an appropriate signal peptide. The open reading frame is followed by a 106-nucleotide 3′ untranslated region including the poly (A) tail. A consensus polyadenylation signal (AATAAA) is found after position 648, which is 19 nucleotides upstream from the poly (A) tail.

The *S. frugiperda* neuropeptide precursor contains a peptide sequence identical to Manse-AS. This Spofr-AS is located at the carboxy-terminus between Arg^{108} and Arg^{124} and is flanked by potential Lys-Arg and Arg-Lys endoproteolytic cleavage sites. The Spofr-AS gene is

present as a single copy. Within the amino-terminus of the precursor a single potential signal cleavage site downstream Ala[26] (Von Heijne, 1986) could be found (Fig. 4). The signal peptide would then contain 18 out of 26 apolar residues and is followed by an 80 amino acids sequence (Ala[27] to Asp[106]). Endoproteolytic cleavage of this internal peptide at Arg[63]/Lys[64] could result in two peptides of 36 and 42 amino acids. These peptides share sequence similarity with those in the Pseun-AS precursor (Jansons et al., 1996). Alignment of the Spofr-AS precursor peptide with that for *P. unipuncta* (Jansons et al., 1996) shows that the C-termini, containing the AS sequence and the processing sites, are identical (Fig. 5). The two precursors are equally long and exhibit 85% amino acid sequence identity. Comparison between Spofr-AS precursor and the respective precursor from *D. melanogaster* (Williamson et al., 2001b) shows that the mature AS peptides differ in one amino acid (change of Phe to Tyr), whereas nearly no sequence homology exists in the other parts of the precursor.

3.3. Spofr-AT gene expression by RT-PCR

RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the three mRNAs of the Spofr-AT gene. The specific primers ATf7 and ATr9 were designed to yield a product of 225 bp, 323 bp, and 420 bp, respectively, of the three mRNAs. The probe of 225 bp, which was used for hybridisation, included the allatotropin encoding sequence (Fig. 1). The Spofr-AT gene was expressed in brains of all developmental stages of *S. frugiperda* studied here (Fig. 6B). The shortest (first Spofr-AT) mRNA was highly expressed in larvae of the 6th larval stage, in 2-day-old adult males and in 2-day-old adult females, whereas it was expressed at a lower level in the prepupal stage and in young pupae. The second Spofr-AT mRNA was highly expressed in the 6th larval stage, whereas no expression was detected in 10-day-old pupae. The expression of the third mRNA was found only in 2-day-old adult females and males and in young pupae. In summary, the RT-PCR analysis based on 20 ng mRNA revealed that the Spofr-AT gene is strongly

```
Spofr-AS    MKTSAYNVYLGVVAAMLALLFVTIN   25
Pseun-AS    ***NVC****AI***T**M*F***R   25
Drome-AS    *MKFVNILLCYGLLLT*FFALSEAR   25

Spofr-AS    A↓APMEADDETAENTLVAHPDGDMEL  50
Pseun-AS    ******E**Q*D***********M    50
Drome-AS    PSGA*TGPDSDGLDGQDAE*VRGAY   50

Spofr-AS    SGPWDAINTAALRKLLLQLDAEDRM   75
Pseun-AS    T****T****************E**   75
Drome-AS    G*GY*MPAN*IYPNIPMDRLNMLFA   75

Spofr-AS    GGVTRSWPQAEPRGWGLRALDSRLA   100
Pseun-AS    *R*S*****************G***   100
Drome-AS    NYRPTYSAYLRSPTY*NVNELY**P   100

Spofr-AS    RQWRADKRQVRFRQCYFNPISCFRK   125
Pseun-AS    ************************   125
Drome-AS    ES----*****Y************   121
```

Fig. 5. Alignment of the AS precursor peptides of *S. frugiperda* (Spofr-AS), *P. unipuncta* (Pseun-AS) (Jansons et al., 1996), and *D. melanogaster* (Drome-AS) (Williamson et al., 2001b). Asterisks represent amino acids identical to each of the precursors. The mature AS peptide is shown in bold type. A signal peptide cleavage site is indicated by a downward arrow.

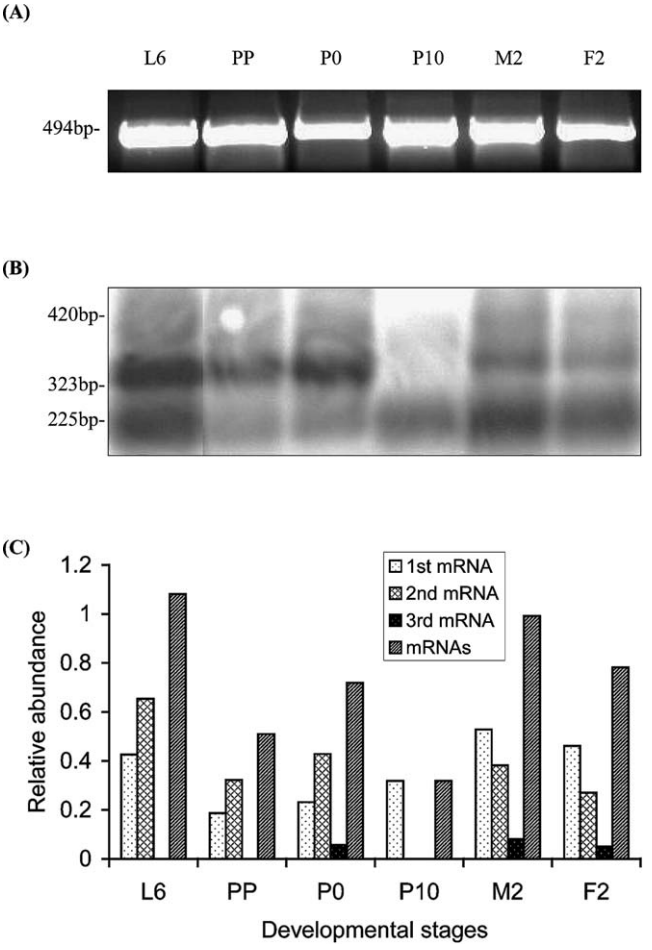

Fig. 6. Expression of Spofr-AT gene in the brains of the sixth larval stage (L6), prepupal stage (PP), young pupal stage (P0), 10-day-old pupae (P10), 2-day-old adult males (M2), and 2-day-old adult females (F2) as measured by RT-PCR. (A) Agarose gel separation of beta-actin control stained with ethidium bromide using 20 ng mRNA as a template in one step RT-PCR; (B) Blotting results for the expression of the three different mRNAs of Spofr-AT gene in the brains; (C) Densitometric quantification of the signals from (B).

expressed in the 6th larval stage (L6) and in 2-day-old adult males (M2) and females (F2), whereas expression is lower in pre-pupae (PP) and in 10-day-old pupae (Fig. 6C). No fragment was visualised in the controls without reverse transcriptase reaction. A beta-actin standard fragment of ACTf1-ACTr2 with 494 bp can be visualized with 20 ng mRNA from each of the developmental stages (Fig. 6A) and this expression strength was used to normalize the data.

3.4. Spofr-AS gene expression by RT-PCR

RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the Spofr-AS gene. The specific primers ASf6 and ASr9 were designed to yield a product of 127 bp of the mRNA corresponding to nucleotide positions 543–670 of Fig. 4. The digoxigenin labelled probe of 127 bp, which was used for hybridisation, proved to include the mature peptide sequence for Spofr-AS gene. RT-PCR analysis shows that the Spofr-AS gene is expressed in the brain of most of the development stages studied (Fig. 7B), with highest expression in 2-day-old adult females (F2). The gene was only slightly expressed in the young pupae (P0) and the 10-day-old pupae (P10) (Fig. 7C). Control reactions were done as described for Spofr-AT.

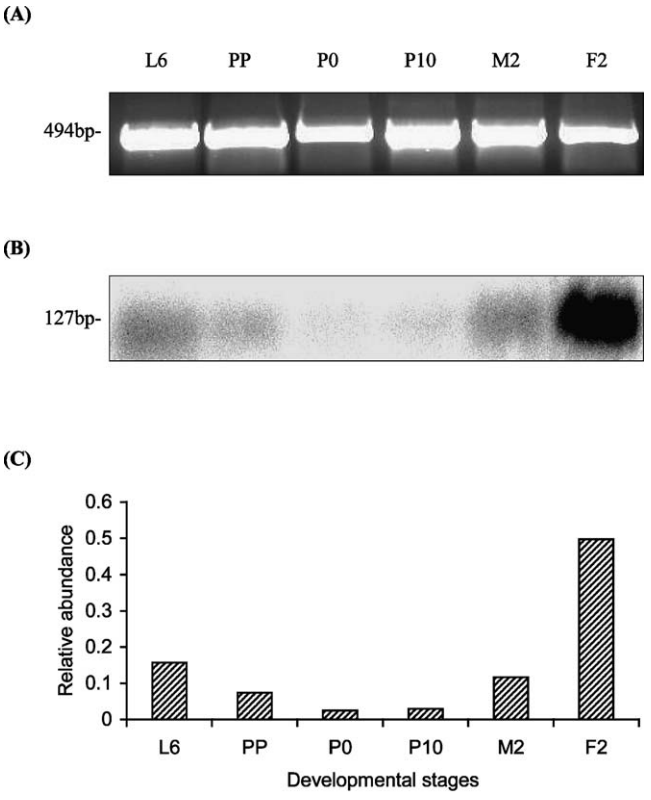

Fig. 7. Expression of Spofr-AS gene in the brains of the sixth larval stage (L6), prepupal stage (PP), young pupal stage (P0), 10-day-old pupae (P10), 2-day-old adult males (M2), and 2-day-old adult females (F2) as measured by RT-PCR. (A) Agarose gel separation of beta-actin control stained with ethidium bromide using 20 ng mRNA as a template in one step RT-PCR; (B) Blotting results for the expression of Spofr-AS gene in the brains; (C) Densitometric quantification of the signals from (B).

4. Discussion

Hitherto, the true armyworm, *P. unipuncta*, was the only lepidopteran species where the cDNAs encoding an allatostatin and an allatotropin had been characterised (Jansons et al., 1996; Truesdell et al., 2000). In this study a second cDNAs encoding *M. sexta* allatotropin and *M. sexta* allatostatin have been characterised for the fall armyworm, *S. frugiperda*, an important agricultural pest.

Factors with allatotropic activity exist in a number of insect species. A stimulating action of JH biosynthesis has been observed, for example, in larvae of the wax moth *Galleria mellonella* (Bogus and Scheller, 1996), in adult locusts *(Locusta migratoria;* Lehmberg et al., 1992 and *S. gregaria;* Veelaert et al., 1996), crickets (*G. bimaculatus;* Lorenz and Hoffmann, 1995) and cockroaches (*Diploptera punctata;* Unnithan et al., 1998), in adult females of the linden bug *Pyrrhocoris apterus* (Hodková et al., 1996), and in the brain of female *Phormia regina* (Tu et al., 2002). So far, however, none of these allatotropic factors has been identified and the *M. sexta* allatotropin (Manse-AT) remains the allatotropin isolated and characterised to date. Allatotropin related peptides have been isolated from the male accessory reproductive glands of *L. migratoria* (Paemen et al., 1991), from the brain of the Colorado potato beetle, *Leptinotarsa decemlineata* (Spittaels et al., 1996), and from abdominal ganglia of the mosquito *A. aegypti* (Veenstra and Costes, 1999). This indicates that allatotropin homologues may be generally present in insects, but have other functions than stimulating JH biosynthesis.

Recently, a peptide from methanolic brain extracts of adult *S. frugiperda*, which strongly stimulates JH biosynthesis in vitro by the CA of adult females was reported. Using HPLC separation, followed by Edman degradation and mass spectrometry, the peptide was identified as Manse-AT (Oeh et al., 2000). The present work confirms the structure of Manse-AT from brains of *S. frugiperda* by molecular techniques. As with many other neuropeptides (Sossin et al., 1989), Spofr-AT is translated from a rare transcript and derived from a polyprotein with a number of products contained on a single precursor. As in Manse-AT (Taylor et al., 1996; Horodyski et al., 2001), the Spofr-AT gene is expressed as at least three mRNA isoforms that differ from each other by alternative splicing. The second Spofr-AT mRNA contains (in addition to Manse-AT) a 14 or 15 amino acid residue peptide, which is flanked by potential endoproteolytic cleavage sites and ends with a glycine residue, the signal for carboxy-terminal amidation. This pep-

tide shows homology to the Manse-AT-like peptides I–III in *M. sexta* (Horodyski et al., 2001). Other than in Spofr-AT, there is no evidence that the cleavage sites flanking the Manse-AT-like peptide are used, and it is difficult to speculate as to the function of this peptide. The basic organisation of the Spofr-AT peptide precursor (134 amino acids) is similar to that in other lepidopteran species.

The results of our semi-quantitative one-step RT-PCR expression studies indicate that the three mRNA isoforms may be differently expressed in brains of larvae, pupae and adults of *S. frugiperda*. Generally, the Spofr-AT gene was strongly expressed in young last instar larvae and in 2-day-old adults, whereas expression was lower in the pupal stage. Efforts at an experimental proof of these results, using transcript-specific probes in in situ hybridisation, are in progress. Whether the Spofr-AT transcripts are alternatively spliced in a tissue-specific manner, as in *M. sexta* (Horodyski et al., 2001), is not yet known.

In a previous paper, it was demonstrated that synthetic Manse-AS may down-regulate higher rates of JH biosynthesis in the CA of adult females of *S. frugiperda* maintained by the allatotropin (Oeh et al., 2000). This inhibitory effect of Manse-AS on allatotropin activated glands was dose-dependent and reversible. However, allatostatin was not isolated from the brains of *S. frugiperda* females by HPLC separation. The current study strongly suggests the presence of Spofr-AS in the brains of adult females by molecular cloning of the preprohormone. The preprohormone is 125 amino acid residues long and contains one copy of a peptide sequence, which is identical to Manse-AS. The basic organisation of the precursor is similar to that of the only other known lepidopteran AS precursor from *P. unipuncta*, with 85% amino acid sequence identity. Williamson et al. (2001b) have demonstrated the existence of a C-type allatostatin preprohormone in *Drosophila*. The drostatin-C sequence differs in only one amino acid residue from the Manse-AS, although, the preprohormone shows little homology. Whether drostatin-C acts as an allatostatin in *Drosophila* itself, has yet to be determined. It is likely, however, that all three types of insect allatostatins may be regarded as generally inhibitory neurohormones and block specific endocrine organs, but they may also act on various muscle tissues in an insect (Williamson et al., 2001b). Our preliminary expression studies by RT-PCR indicate a developmentally regulated expression of Spofr-AS in the brain, but further studies are necessary to unequivocally demonstrate a time- and/or tissue-specific expression of the peptide in *S. frugiperda*. Our in vitro experiments on the regulation of JH biosynthesis in *S. frugiperda* have clearly shown an interaction between Spofr-AT and Spofr-AS, at least in adult females. Such a dual regulatory mechanism would allow a more precise control of hormone production than a single ‘on-off′ mechanism (McNeil and Tobe, 2001). Injections of Manse-AT and Manse-AS into adult females of *S. frugiperda* affected their life span, as well as the total number of eggs deposited. The oviposition rate was reduced with Manse-AT alone and Manse-AS plus Manse-AT, whereas egg deposition in Manse-AS injected females on a per day basis was not affected during their short life span (Oeh et al., 2001). These results indicate a rather sophisticated role of both hormones in adult development and reproduction of *S. frugiperda*. We hope that further experiments formulated within an ecological context, for example, on the life history strategy of populations (McNeil and Tobe, 2001), will yield a better understanding of the modes of actions of allatotropic and allatostatic neuropeptides.

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References

Audsley, N., Weaver, R.J., Edwards, J.P., 1998. Enzyme linked immunosorbent assay for Manduca sexta allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in Lacanobia oleracea. Insect Biochem. Mol. Biol. 28, 775–784.

Audsley, N., Weaver, R.J., Edwards, J.P., 1999. Juvenile hormone synthesis by corpora allata of tomato moth Lacanobia oleracea (Lepidoptera: Noctuidae), and the effects of allatostatins and allatotropin in vitro. Eur. J. Entomol. 96, 287–293.

Audsley, N., Weaver, R.J., Edwards, J.P., 2000. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, Lacanobia oleracea, and regulation by Manduca sexta allatostatin and allatotropin. Insect Biochem. Mol. Biol. 30, 681–689.

Bellés, X., Graham, L., Bendena, W.G., Ding, Q., Edwards, J.P., Weaver, R.J., Tobe, S.S., 1999. The molecular evolution of the allatostatin precursor in cockroaches. Peptides 20, 11–22.

Bendena, W.G., Garside, C.S., Yu, C.G., Tobe, S.S., 1997. Allatostatins: diversity in structure and function of an insect neuropeptide family. Ann. N.Y. Acad. Sci. 814, 53–66.

Bendena, W.G., Donley, B.C., Tobe, S.S., 1999. Allatostatins: a growing family of neuropeptides with structural and functional diversity. Ann. N.Y. Acad. Sci. 897, 311–329.

Bogus, M., Scheller, K., 1996. Allatotropin released by the brain controls larval moulting in Galleria mellonella by affecting juvenile hormone synthesis. Int. J. Dev. Biol. 40, 205–210.

Davey, M., Duve, H., Thorpe, A., East, P., 1999. Characterization of the helicostatin peptide precursor gene from Helicoverpa armigera (Lepidoptera: Noctuidae). Insect Biochem. Molec. Biol. 29, 1119–1127.

Ding, Q., Donly, B.C., Tobe, S.S., Bendena, W.G., 1995. Molecular cloning of the gene for the allatostatin family of neuropeptides from

the cockroach, Periplaneta americana. Eur. J. Biochem. 234, 737–746.

Donly, B.C., Ding, Q., Tobe, S.S., Bendena, W.G., 1993. Molecular cloning of the gene for allatostatin family of neuropeptides from the cockroach Diploptera punctata. Proc. Natl. Acad. Sci. USA 90, 8807–8811.

Duve, H., Thorpe, A., 1994. Distribution and functional significance of Leu-callatostatins in blowfly Calliphora vomitoria. Cell Tissue Res. 276, 367–379.

East, P.D., Tregenza, K., Duve, H., Thorpe, A., 1996. Identification of the dipteran leu-callatostatin peptide family: characterization of the prohormone gene from Calliphora vomitoria and Lucilia cuprina. Regul. Pept. 67, 1–9.

Edwards, J.P., Audsley, N., Marris, G.C., Cusson, M., Weaver, R.J., 2001. The role of allatostatic and allatotropic neuropeptides in the regulation of juvenile hormone biosynthesis in Lacanobia oleracea (Lepidoptera: Noctuidae). Peptides 22, 225–261.

Eipper, B.A., Stoffers, D.A., Mains, R.E., 1992. The biosynthesis of neuropeptides: peptide-amidation. Annu. Rev. Neurosci. 15, 57–85.

Gäde, G., Hoffmann, K.H., Spring, J., 1997. Hormonal regulation in insects: facts, gaps and future direction. Physiol. Rev. 77, 963–1032.

Hodková, M., Okuda, T., Wagner, R., 1996. Stimulation of corpora allata by extract from neuroendocrine complex; comparison of reproducing and diapausing Pyrrhocoris apterus (Homoptera: Pyrrhocoridae). Eur. J. Entomol. 93, 535–543.

Hoffmann, K.H., Meyering-Vos, M., Lorenz, M.W., 1999. Allatostatins and allatotropins: is regulation of corpora allata activity their primary function? Eur. J. Entomol. 96, 255–266.

Horodyski, F.M., Bhatt, S.R., Lee, K.Y., 2001. Alternative splicing of transcripts by Manduca sexta allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. Peptides 22, 263–269.

Jansons, I.S., Cusson, M., McNeil, J.W., Tobe, S.S., Bendena, W.G., 1996. Molecular characterisation of a cDNA from Pseudaletia unipuncta encoding the Manduca sexta allatostatin peptide (Mas-AST). Insect Biochem. Mol. Biol. 26, 767–773.

Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., Kramer, S.J., 1989. Identification of an allatotropin from adult Manduca sexta. Science 243, 1481–1483.

Koeppe, J.K., Fuchs, M., Chen, T.T., Hunt, L.M., Kovalick, G.E., Briers, T., 1985. The role of juvenile hormone in reproduction. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 8. Pergamon Press, Oxford, pp. 166–203.

Koladich, P.M., Cusson, M., Bendena, W.G., Tobe, S.S., McNeil, J.N., 2002. Cardioacceleratory effects of Manduca sexta allatotropin in the true armyworm moth, Pseudaletia unipuncta. Peptides 23, 645–651.

Kramer, S.J., Toschi, A., Miller, C.A., Kataoka, H., Quistad, G.B., Li, J.P., Carney, R.L., Schooley, D.A., 1991. Identification of an allatostatin from the tobacco hornworm Manduca sexta. Proc. Natl. Acad. Sci. USA 88, 9458–9462.

Lee, K.Y., Horodyski, F.M., Chamberlin, M.E., 1998. Inhibition of midgut ion transport by allatotropin (Mas-AT) and Manduca FLRFamides in the tobacco hornworm Manduca sexta. J. Exp. Biol. 201, 3067–3074.

Lehmberg, E., Ferenz, H.J., Applebaum, S.W., 1992. Maturation and responsiveness to extracts of corpora allata from male Locusta migratoria containing allatotropic factors. Z. Naturforsch. (C) 47, 191–196.

Lenz, C., Williamson, M., Grimmelikhuijzen, C.J.P., 2000. Molecular cloning and genomic organization of an allatostatin preprohormone from Drosophila melanogaster. Biochem. Biophys. Res. Commun. 273, 1126–1131.

Lorenz, M.W., Hoffmann, K.H., 1995. Allatotropic activity in the suboesophageal ganglia of crickets, Gryllus bimaculatus and Acheta domesticus (Ensifera: Gryllidae). J. Insect Physiol. 41, 191–196.

Lorenz, M.W., Kellner, R., Hoffmann, K.H., 1995. A family of neuropeptides that inhibit juvenile hormone biosynthesis in the cricket, Gryllus bimaculatus. J. Biol. Chem. 270, 21103–21108.

Lorenz, J.H., Lorenz, M.W., Hoffmann, K.H., 1997. Factors regulating juvenile hormone and ecdysteroid biosynthesis in Gryllus bimaculatus (Ensifera : Gryllidae). Eur. J. Entomol. 94, 369–379.

McNeil, J.N., Tobe, S.S., 2001. Flights of fancy: possible roles of allatostatin and allatotropin in migration and reproductive success of Pseudaletia unipuncta. Peptides 22, 271–277.

Meyering-Vos, M., Wu, X., Huang, J., Jindra, M., Hoffmann, K.H., Sehnal, F., 2001. The allatostatin gene of the cricket Gryllus bimaculatus (Ensifera : Gryllidae). Molec. Cell. Endocrinol. 184, 103–114.

Nijhout, H.F., 1994. Insect Hormones. Princeton University Press, Princeton, N.J.

Oeh, U., Lorenz, M.W., Dyker, H., Lösel, P., Hoffmann, K.H., 2000. Interaction between Manduca sexta allatotropin and Manduca sexta allatostatin in the fall armyworm Spodoptera frugiperda. Insect Biochem. Molec. Biol. 30, 719–727.

Oeh, U., Dyker, H., Lösel, P., Hoffmann, K.H., 2001. In vivo effects of Manduca sexta allatotropin and allatostatin on the development and reproduction in the fall armyworm Spodoptera frugiperda (Lepidoptera: Noctuidae). Invertebr. Reprod. Dev 39, 239–247.

Paemen, L., Tips, A., Schoofs, L., Proost, P., Van Damme, J., DeLoof, A., 1991. Lom-AG-myotropin: a novel myotropic peptide from the male accessory gland of Locusta migratoria. Peptides 12, 7–10.

Park, C., Hwang, J.S., Kang, S.W., Lee, B.H., 2002. Molecular characterization of a cDNA from the silk moth Bombyx mori encoding Manduca sexta allatotropin peptide. Zool. Sci. 19, 287–292.

Persson, M.G.B., Nässel, D.R., 1999. Neuropeptides in insect sensory neurones: Tachykinin-, FMRFamide- and allatotropin-related peptides in terminals of locust thoracic sensory afferents. Brain Res. 816, 131–141.

Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24, 213–274.

Rudwall, A.J., Sliwowska, J., Nässel, D.R., 2000. Allatotropin-like neuropeptide in the cockroach abdominal nervous system: myotropic actions, sexually dimorphic distribution and colocalization with serotonin. J. Comp. Neurol. 428, 159–173.

Sossin, F., Fisher, J.M., Sheller, R.H., 1989. Cellular and molecular biology of neuropeptides processing and packaging. Neuron 2, 1407–1417.

Spittaels, K., Van Keerberghen, A., Schoofs, L., Proost, P., Van Damme, J., DeLoof, A., 1996. Isolation and characterization of Locusta migratoria accessory gland myotropin I (Lom-Ag-MT-I) from the brain of the Colorado potato beetle, Leptinotarsa decemlineata. Arch. Insect Biochem. Physiol. 31, 149–155.

Stay, B., 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem. Molec. Biol. 30, 653–662.

Stay, B., Tobe, S.S., Bendena, W.G., 1994. Allatostatins: identification, primary structures, functions and distribution. Adv. Insect Physiol. 25, 267–337.

Taylor, P.A., Bhatt, T.R., Horodyski, F.M., 1996. Molecular characterization and expression analysis of Manduca sexta allatotropin. Eur. J. Biochem. 239, 588–596.

Teal, P.E.A., 2002. Effects of allatotropin and allatostatin on in vitro production of juvenile hormones by the corpora allata of virgin females of the moths of Heliothis virescens and Manduca sexta. Peptides 23, 663–669.

Truesdell, P.F., Koladich, P.M., Kataoka, H., Kojima, K., Suzuki, A., McNeil, J.N., Mizoguchi, A., Tobe, S.S., Bendena, W.G., 2000. Molecular characterization of a cDNA from the true armyworm

Pseudaletia unipuncta encoding Manduca sexta allatotropin peptide. Insect Biochem. Molec. Biol. 30, 691–702.

Tu, M.P., Kou, R., Wang, Z.S., Stoffolano, J.G., Yin, C.M., 2001. Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, Phormia regina (Diptera: Calliphoridae). J. Insect Physiol. 47, 233–244.

Tu, M.P., Kou, R., Remus, G.O., Yin, C.M., Stoffolano, J.G., 2002. Allatotropic activity in the brain of female Phormia regina (Diptera: Calliphoridae). J. Insect Physiol. 48, 733–741.

Unnithan, G.C., Sutherland, T.D., Cromey, D.W., Feyereisen, R., 1998. A factor causing stable stimulation of juvenile hormone synthesis by Diploptera punctata corpora allata in vitro. J. Insect Physiol. 44, 1027–1037.

Vanden Broeck, J., Veelaert, D., Bendena, W.G., Tobe, S.S., DeLoof, A., 1996. Molecular cloning of the precursor cDNA for schistostatins, locust allatostatin-like peptides with myoinhibiting properties. Molec. Cell. Endocrinol. 122, 191–198.

Veelaert, D., Devreese, B., Schoofs, L., Van Beeumen, J., Vanden Broeck, J., Tobe, S.S., DeLoof, A., 1996. Isolation and characterization of eight myoinhibiting peptides from the desert locust Schistocerca gregaria: new members of the cockroach allatostatin family. Molec. Cell. Endocrinol. 122, 183–190.

Veenstra, J.A., Hagedorn, H.H., 1993. A sensitive enzyme immunoassay for Manduca allatotropin and the existence of an allatotropin-immunoreactive peptide in Periplaneta americana. Arch. Insect Biochem. Physiol. 23, 99–109.

Veenstra, J.A., Lehman, H.K., Davis, N.T., 1994. Allatotropin is a cardioacceleratory peptide in Manduca sexta. J. Exp. Biol. 188, 347–354.

Veenstra, J.A., Noriega, F.G., Graf, R., Feyereisen, R., 1997. Identification of three allatostatins and their cDNA from the mosquito Aedes aegypti. Peptides 18, 937–942.

Veenstra, J.A., Costes, L., 1999. Isolation and identification of a peptide and its cDNA from the mosquito Aedes aegypti related to Manduca sexta allatotropin. Peptides 20, 1145–1151.

Von Heijne, G., 1986. A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. 20, 2111–2118.

Weaver, R.J., Edwards, J.P., Bendena, W.G., Tobe, S.S., 1998. Structures, functions and occurrence of insect allatostatic peptides. In: Coast, G.M., Webster, S.G. (Eds.), Recent Advances in Arthropod Endocrinology. Cambridge University Press, Cambridge, pp. 3–32.

Williamson, M., Lenz, C., Winther, M.E., Nässel, D.R., Grimmelikhuijzen, C.J.P., 2001a. Molecular cloning, genomic organization, and expression of a B-type (cricket-type) allatostatin preprohormone from Drosophila melanogaster. Biochem. Biophys. Res. Commun. 281, 544–550.

Williamson, M., Lenz, C., Winther, A.M., Nässel, D.R., Grimmelikhuijzen, C.J., 2001b. Molecular cloning, genomic organization, and expression of a C-type (Manduca sexta-type) allatostatin preprohormone from Drosophila melanogaster. Biochem. Biophys. Res. Commun. 282, 124–130.

Žitnan, D., Sehnal, F., Bryant, P.J., 1993. Neurons producing specific neuropeptides in the central nervous system of normal and pupariation-delayed Drosophila. Dev. Biol. 156, 117–135.

Žitnan, D., Kingan, T., Krame, S., Beckage, N., 1995. Accumulation of neuropeptides in the cerebral neurosecretory system of Manduca sexta larvae parasitized by the braconid wasp Cotesia congregata. J. Comp. Neurol. 365, 83–100.

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Expression and Localization of the *Spodoptera frugiperda* Allatotropin (Spofr-AT) and Allatostatin (Spofr-AS) Genes

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Manduca sexta allatotropin and allatostatin were the first corpora allata (CA) regulating neuropeptides identified from Lepidoptera. Recently, we cloned the allatotropin (Spofr-AT) and the allatostatin (Spofr-AS) genes from the fall armyworm *Spodoptera frugiperda*. Using one-step RT-PCR for semi-quantification of the gene expression, we now demonstrate that three mRNA isoforms of the Spofr-AT gene and the Spofr-AS gene are expressed in brain, digestive tract, and reproductive organs of larvae, pupae, and adults in a time- and tissue-specific manner. Expression rates in the brain and in various parts of the digestive tract prove the dual role of the peptides as brain/gut (neuro)peptides. The functional meaning of ovarian and testes expression of the genes is not yet clear, although myoregulatory properties of the peptides are probable. The tissue-specific localization of the prohormone expression, as demonstrated by whole mount in situ hybridization, confirms the overall distribution of the prohormones as shown by RT-PCR and supports the pleiotropic functions of the peptides. Arch. Insect Biochem. Physiol. 55:188–199, 2004.

KEYWORDS: allatotropin; allatostatin; one-step RT-PCR; in situ hybridization; *Spodoptera*

INTRODUCTION

The diversity of biological activities elicited by neuropeptides is often reflected in the unique expression of their genes in specific cells of the nervous system, and sometimes in other tissues such as the gut (Hökfelt et al., 2000; Strand, 1999). The expression pattern is precisely controlled and often modulated under certain physiological conditions or during the development of the organism in a tissue- and time-specific manner.

Juvenile hormone (JH) is known to be critical for regulation of larval development, metamorphosis, and reproduction in insects. First produced by the corpora allata (CA) in the late embryo, it appears to be important for normal dorsal closure, formation of the larval cuticle, and differentiation of the midgut (Bergot et al., 1981; Dorn 1982; Brüning and Lanzrein, 1987). The CA remain active until the adult stage, although there are fluctuations in activity depending on the stage (Tobe and Stay, 1985; Riddiford, 1994). In adult insects, JH is involved in the regulation of vitellogenesis in females and spermatogenesis in males (Nijhout, 1994; Gäde et al., 1997).

A number of neuropeptides have been isolated from several insect orders that may either stimulate (allatotropin; AT) or inhibit (allatostatin; AS) CA activity in vitro (for review see Gäde et al., 1997; Bendena et al., 1999; Hoffmann et al., 1999;

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Stay, 2000; Gäde, 2002). *Manduca sexta* allatotropin (Manse-AT; GFKNVEMMTARGF-NH_2) and *M. sexta* allatostatin (Manse-AS; pEVRFRQCYFNPISCF-OH) were the first allatoregulatory neuropeptides identified in Lepidoptera (Kataoka et al., 1989; Kramer et al., 1991). Both peptides have also been found in other lepidopteran species, where they seem to control JH biosynthesis (Audsley et al., 1998, 2000; Oeh et al., 2000). In addition to their role in the regulation of JH biosynthesis, both peptides exerted neuro- and myoregulatory activities (for review see Hoffmann et al., 1999; Stay, 2000; Gäde, 2002).

Insect neuropeptides are generally derived from proteolytic processing of a precursor protein, which may contain multiple structurally related or diverse peptides. Posttranscriptional control mechanisms, such as alternative splicing or proteolytic processing, can increase the diversity of the gene products. Alternative splicing of mRNA seems to occur in many genes of the endocrine system (Chew, 1997). The Manse-AT gene is expressed as three mRNA isoforms differing from each other by alternative splicing and the isoform pattern differs in a tissue- and developmental-specific manner (Horodyski et al., 2001; Lee and Horodyski, 2002).

Recently, we characterized cDNAs encoding Manse-AT and Manse-AS for the fall armyworm, *Spodoptera frugiperda* (Abdel-latief et al., 2003). The Spofr-AT gene is also expressed as three alternative mRNAs. Here we present a detailed study on the tissue- and time-specific expression (by one-step RT-PCR) of the Spofr-AT and Spofr-AS gene, respectively, during larval, pupal, and adult development of *S. frugiperda*. Moreover, we show some tissue-specific localization of the genes by whole mount in situ hybridization.

MATERIALS AND METHODS

Insect Rearing

Pupae and eggs of *S. frugiperda* were provided by Bayer AG (Leverkusen, Germany) and reared at 27°C and 70% relative humidity under a L 16:D 8 photoperiod as previously described (Oeh et al., 2000). Larvae and adults were reared as described by Oeh et al. (2000). Each pupa was individually kept in a separate compartment of assortment boxes (9 × 32 × 36 mm per compartment; Licefa, Bad Salzuflen, Germany) until emergence. Freshly emerged females were kept in 20 × 20 × 10 cm plastic boxes and fed with 10% sugar solution until dissection.

RNA and mRNA Extractions

Brains and midguts from 4th (L4), 5th (L5), and 6th instar larvae (L6) (<12 h after the respective moult), prepupae (PP), young pupae (P0), 10 day-old pupae (P10), and 1–5-day-old adult females and males as well as ovaries from 1–5-day-old adult virgin females and testes from 1–5-day-old adult males were dissected in modified cricket saline (Lorenz et al., 1997), frozen immediately in liquid nitrogen, and stored at –80°C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen GmbH, Hilden, Germany). The mRNA was quantified by spectrophotometric determination.

RT-PCR Analysis

For RT-PCR, 20 ng mRNA from each of the tissues was used with specific primers ATf7-ATr9 and primers ASf6-ASr9 as previously described (Abdel-latief et al., 2003), in 10 µl of the RT-PCR mixture (TITANIUM® One-Step RT-PCR Kit, Clontech Laboratories, Heidelberg, Germany). The PCR program for amplification of the fragment was 50°C for 1 h followed by 5 min at 94°C, 35 cycles of 94°C for 30 sec, 60°C for AT or 58°C for AS for 45 sec, 68°C for 1 min, and a final extension step of 68°C for 2 min. The DNA products were analyzed using 1.5% agarose gel at 8 V/cm for 1.5 h, transferred to Hybond-N^+ membrane (Amersham, Freiburg, Germany) and hybridized to the probe (ATf7-ATr9) 225 bp and probe (ASf6-ASr9) 127 bp (Abdel-latief et al., 2003), respectively, labeled by the PCR method with Dig dUTP (Roche, Mannheim, Germany). As a control, the same quantities (20 ng) of mRNA from different developmental stages were used as a template in combination with two specific primers ACTf1 corresponding to nucleotide positions 1–25 and ACTr2 corresponding to nucle-

otide positions 469–493 from the nucleotide sequence of *S. littoralis* mRNA for beta-actin (partial; EMBL Nucleotide Sequence Data Base, accession number Z46873), which yields a 494-bp amplified fragment. Expressions of AT and AS were normalized relatively to that of beta-actin. A negative control for genomic DNA has been done using RT-PCR program strategy 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 61°C for 45 sec, 68°C for 1 min, and a final extension step of 68°C for 2 min. Densitometry was performed using an ImageMaster® VDS (Pharmacia Biotech, Freiburg, Germany) and the ImageMaster 1D Database software. Values represent means of 2–3 determinations ± SD.

Synthesis of Digoxigenin (Dig-11-UTP)-Labelled RNA Probe

Antisense and sense Spofr-AT and Spofr-AS RNA probes were synthesized from the cDNA clones, ATf7-ATr9, and ASf6-ASr9, as previously described (Abdel-latief et al., 2003). One microgram ATf7-ATr9 (225 bp) and ASf6-ASr9 (127 bp) plasmids were linearized with ApaI or PstI restriction enzymes (about 30 U according to enzymes) (Promega, Mannheim, Germany) in a 20-µl volume to yield templates for the antisense and the sense probes, respectively. The templates were purified by phenol/chloroform/isoamyl alcohol extraction and then ethanol precipitated. The pellet was air-dried and adjusted to a concentration of 1 µg/µl in DEPC H_2O. Ten micrograms linearized DNA plasmide were labelled with Dig RNA labelling kit according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). The RNA probes were precipitated with ethanol dried in a speed vac concentrator, resuspended in 20–50 µl DEPC H_2O, and stored at –80°C until use.

Whole-Mount In Situ Hybridization

Whole tissues of brain, digestive tract, and ovary were dissected from 2-day-old adult females in modified cricket saline (Lorenz et al., 1997) and immediately placed in fixative. The tissues were fixed in 4% paraformaldehyde (PFA) in 1 × PBS buffer (7.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) overnight at 4°C. The solution was replaced by 1 ml 100% methanol in order to dehydrate the tissues and the tissues were stored for 3 weeks at –20°C until used for whole-mount in situ hybridization. Whole-mount in situ hybridization was performed as described by Harland (1991). All tissues were analyzed using a Leitz Diaplan microscope provided with a Wild Leitz MPS 46 photoautomate. Kodak Elite CHROME 200 film negatives were scanned with a HP Scan-Jet 5470C. Controls with sense-strand RNA probes were processed identically to the experimental tissues.

RESULTS

Brain Expression

Using one-step RT-PCR for semi-quantification of the gene expression, it was shown that three mRNA isoforms of the Spofr-AT gene (isoform 1, 225 bp; isoform 2, 323 bp; isoform 3, 420 bp) and the Spofr-AS gene are expressed in brains of all developmental stages with variable intensity (Figs. 1 and 2). During larval and pupal development, AT expression was relatively high in the 5th larval instar, but decreased thereafter and remained low until pupation (Fig. 1A). In adult females (Fig. 2A) and males (Fig. 2B), the three mRNA isoforms were expressed during the first 5 days after eclosion, and always in the order of isoform 1 > isoform 2 > isoform 3. No significant quantitative changes in total amounts of AT mRNA were observed.

The Spofr-AS expression studies demonstrated high expression rates in larvae (L4 to L6) (Fig. 1B), but low expression during the pupal phase (PP to P10) (Fig. 1B). A sex-specific expression of the Spofr-AS gene can be seen in older imagoes, with high expression rates in males (d4/d5) (Fig. 2D), but negligible values in females (d5) (Fig. 2C).

Midgut Expression

In the midgut of *S. frugiperda*, AT expression could be detected in all developmental stages, but with considerable qualitative and quantitative variability (Fig. 3A). In general, mRNA isoform 2 was

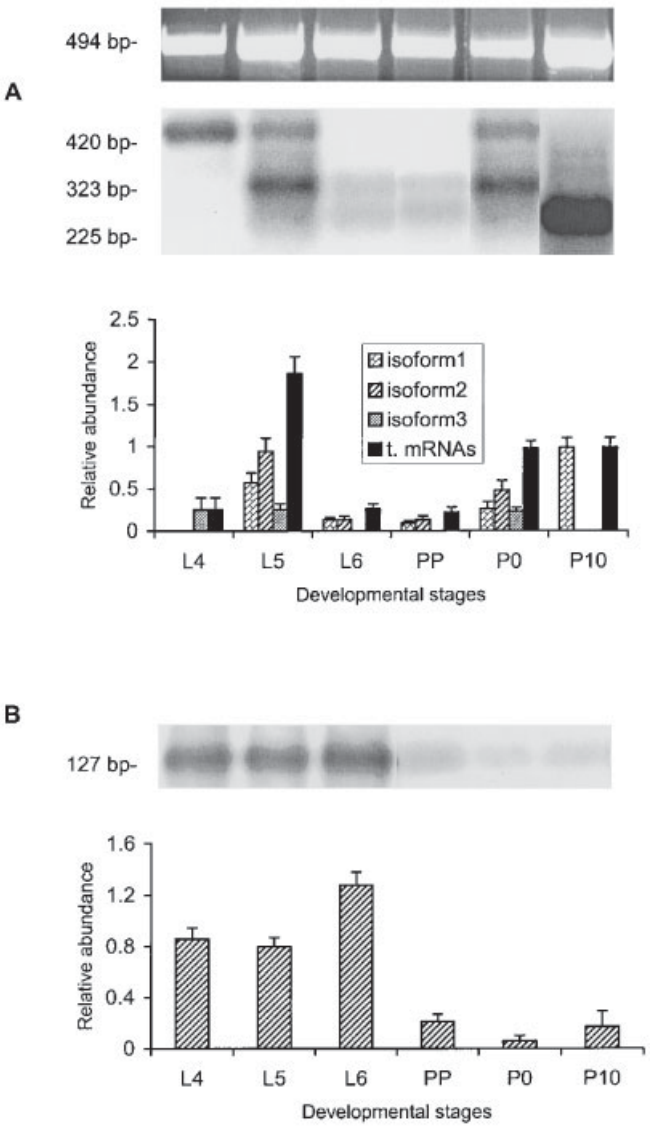

Fig. 1. Expression of Spofr-AT gene (**A**, three different mRNAs) and Spofr-AS gene (**B**) in the brains of the 4th (L4), 5th (L5), and 6th larval instar (L6), prepupal stage (PP), young pupal stage (P0), and 10-day-old pupae (P10) as measured by RT-PCR. Agarose gel separation of beta-actin control (494 bp) stained with ethidium bromide using 20 ng mRNA as a template in one-step RT-PCR. Densitometric quantification of the signals relative to that of beta-actin. Isoform 1 = 225 bp, isoform 2 = 323 bp, isoform 3 = 420 bp; t. mRNAs = total mRNA. Mean values of 2–3 determinations ± SD.

present in all stages and significant amounts of the mRNA isoform 3 were found in prepupae (PP) and in older pupae (P10). Total expression was lowest in 5-day-old adults.

Spofr-AS midgut expression was highest in L4 larvae, but low during the pupal stage (Fig. 3B). No clear time-dependent expression profile could be found for the imagoes, except for a peak value for 4-day-old adults

Ovary and Testis Expression

Our results demonstrate Spofr-AS and Spofr-AT expression in the reproductive tissues of both sexes. AT expression was high in ovaries and testes of young adults (day 1 to day 3), and dropped to negligible values thereafter (Figs. 4A, 5A). Within the testes, mRNA isoform 1 was most expressed, whereas the isoform 3 could not be detected. In the ovary, the 323-bp product represented the dominating one.

Spofr-AS expression rates in the ovary were high during the first 3 days after ecdysis and dropped thereafter (Fig. 4B), whereas for the testes a high precursor expression could be detected only during the first 24 h after ecdysis (Fig. 5B).

Whole-Mount In Situ Hybridization of Spofr-AT and Sporfr-AS mRNA

In situ hybridization (ISH) using digoxigenin-labelled cRNA revealed that Spofr-AT mRNA is distributed in several groups of cells in the brain from 2-day-old adult females. In *S. frugiperda*, the suboesophageal ganglion (SOG) lies directly below the cerebral ganglion and is fused to the ventral sides of the brain. In the anterior-lateral region of the SOG, we detected two large clusters of cells, which displayed strong expression of Spofr-AT mRNA (Fig. 6A). When using the Spofr-AT sense probe, no expression signals were found (Fig. 6B).

Spofr-AS was slightly expressed in a cluster of cells at the coxa region of the antenna in the deuterocerebrum. Another cluster of positive cells was found in the posterior-ventral region in each of the optical lobes adjacent to the protocerebrum (data not shown).

In situ hybridization of Spofr-AT mRNA in the digestive tract showed that the gene is expressed in various parts of the intestine. High expression

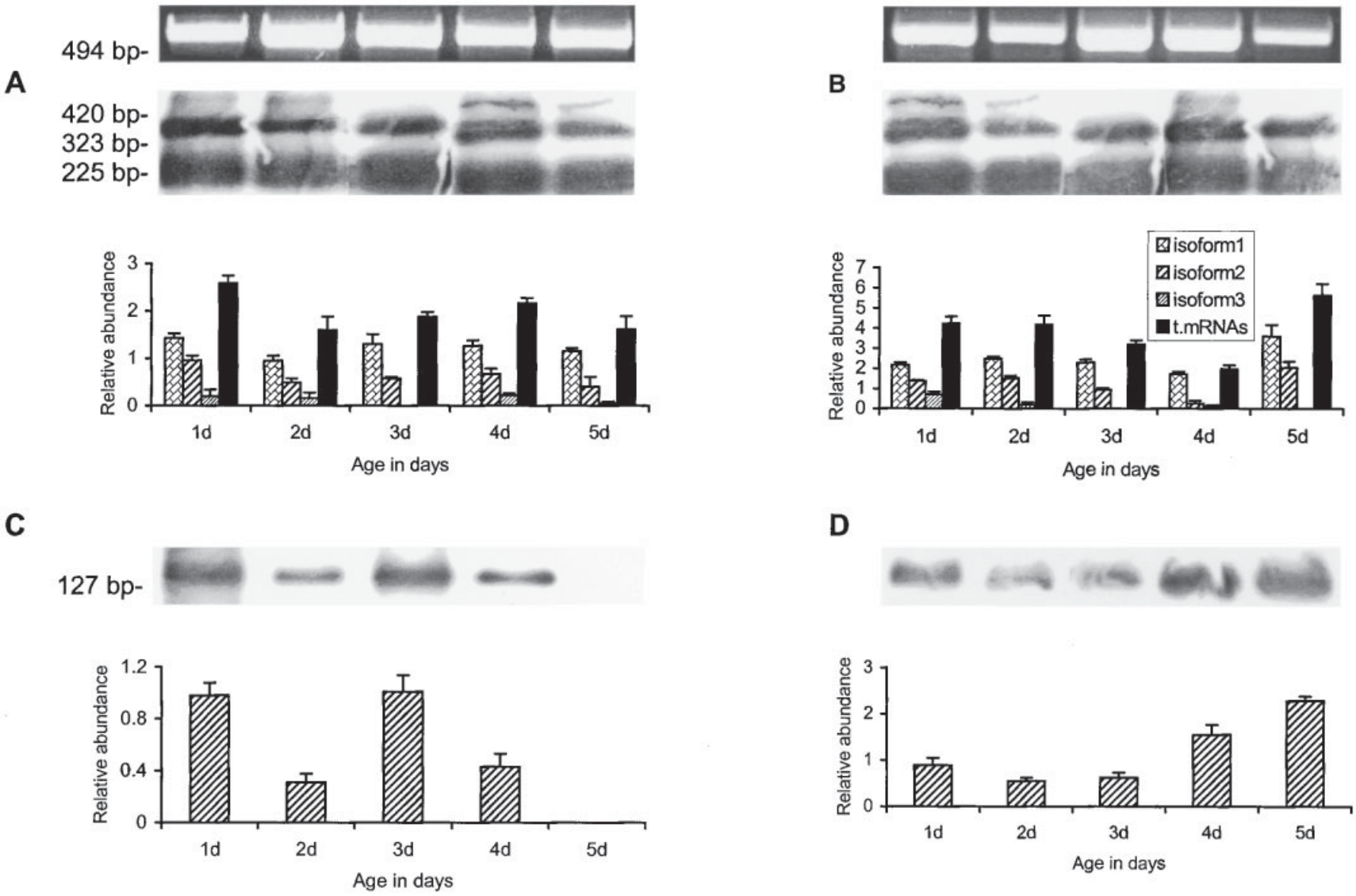

Fig. 2. Expression of Spofr-AT gene (**A,B**, three different mRNAs) and Spofr-AS gene (**C,D**) in the brains of 1- to 5-day-old (1d to 5d) adult females (A,C) and males (B,D) as measured by RT-PCR. Other details as in Figure 1.

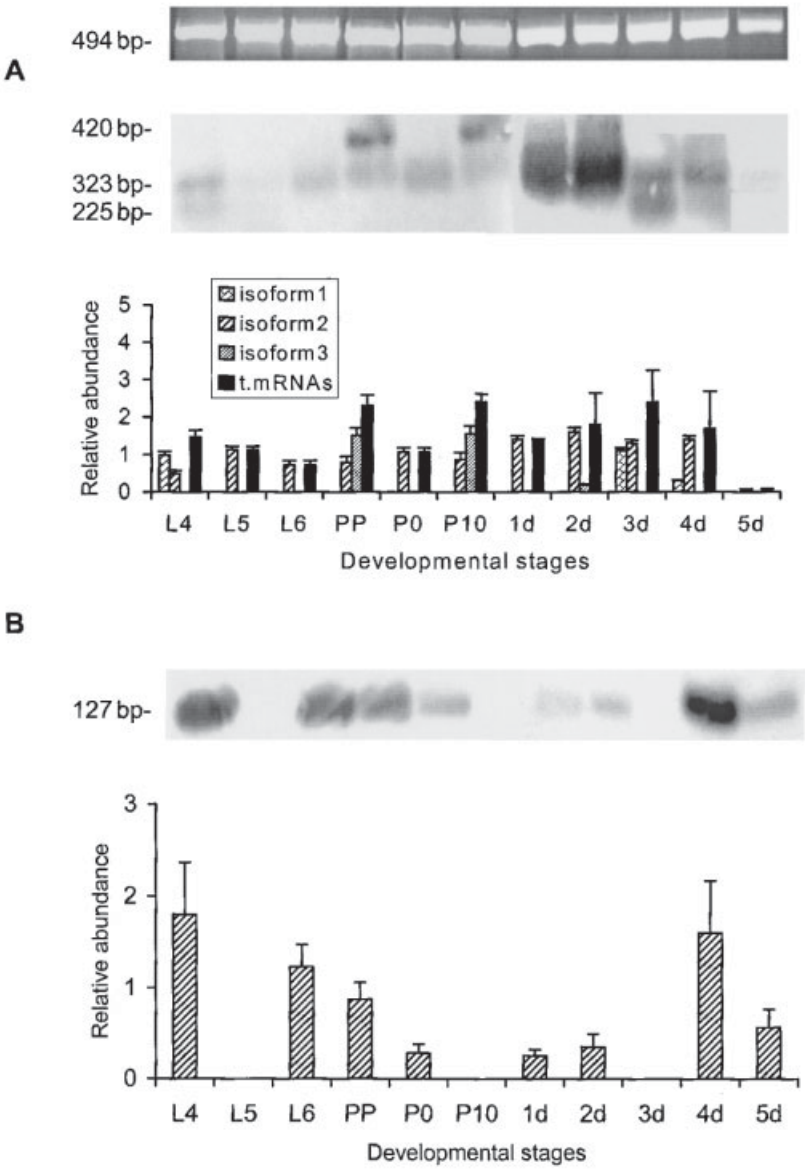

Fig. 3. Expression of Spofr-AT gene (**A**, three different mRNAs) and Spofr-AS gene (**B**) in the midgut of the 4th (L4), 5th (L5), and 6th larval instar (L6), prepupal stage (PP), young pupal stage (P0), 10-day-old pupae (P10), and 1- to 5-day-old (1d to 5d) adults of both sexes as measured by RT-PCR. Other details as in Figure 1.

was found in the posterior part of the crop and the anterior part of the midgut, whilst positive reaction was low in other parts of the midgut. No expression could be detected in the hindgut. Using the Spofr-AS antisense probe, we could show that this gene was expressed in the posterior part of the crop and the anterior part of the midgut (results not shown).

S. frugiperda ovarioles are of the polytrophic type. Spofr-AT gene expression was localized in the follicle cells between the oocytes (Fig. 7A). The Spofr-AS gene was localized in the outer region of the oocytes, and some slight hybridization could be seen between the oocytes (not shown). Using Spofr-AT and Spofr-AS sense probes, respectively, did not reveal any positive reactions in the ovary (Fig. 7B).

DISCUSSION

Allatostatins are structurally diverse peptides that were originally shown to inhibit JH biosynthesis in the CA of a variety of insect species (for review see Gäde et al., 1997; Hoffmann et al., 1999; Stay, 2000; Gäde, 2002). The allatostatins form three different peptide families as defined by their

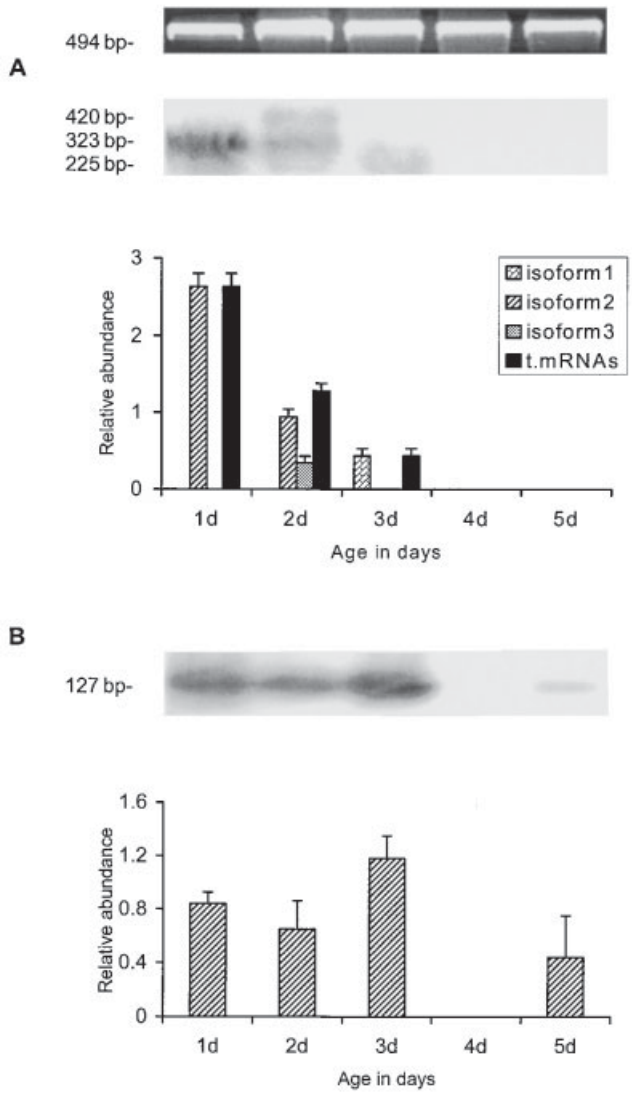

Fig. 4. Expression of Spofr-AT gene (**A**, three different mRNAs) and Spofr-AS gene (**B**) in the ovary of 1- to 5-day-old (1d to 5d) adult unmated females as measured by RT-PCR. Other details as in Figure 1.

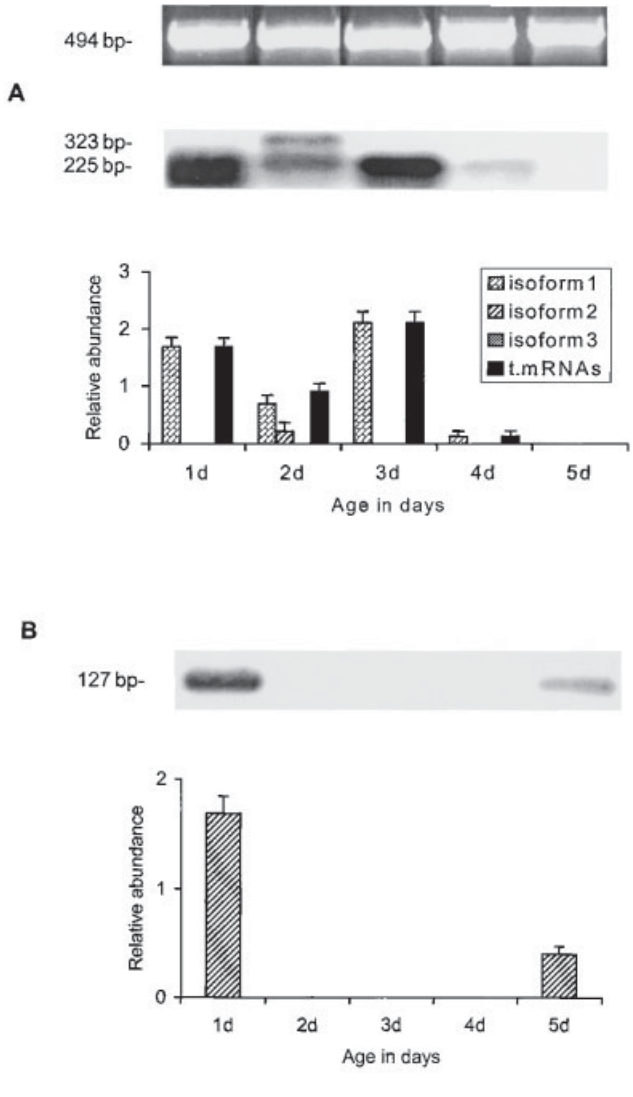

Fig. 5. Expression of Spofr-AT gene (A, three different mRNAs) and Spofr-AS gene (B) in the testes of 1- to 5-day-old (1d to 5d) adult males as measured by RT-PCR. Other details as in Figure 1.

structural homologies: type A allatostatins or cockroach type AS, type B allatostatins or cricket type AS, and the type C allatostatin of *M. sexta* (lepidopteran type AS). In contrast to the variability in the structure of allatostatic neuropeptides, only one allatotropin is known, the Manse-AT.

Members of the A-type allatostatin family inhibited JH biosynthesis in the CA from cockroaches and crickets. The allatostatic activity of the B-type peptides seems to be restricted to the crickets. Within the Lepidoptera, questions still remain about the universality of allatostatic function of Manse-AS throughout this order (Weaver et al., 1998). Members of the A- and B- type peptide families, in addition to their inhibiting action on JH biosynthesis, exerted neuro-and myomodulatory activities in many insect species and it was suggested, that inhibition of JH biosynthesis by CA may represent a secondary role for these neuropeptides. Other functions than allatoregulation have

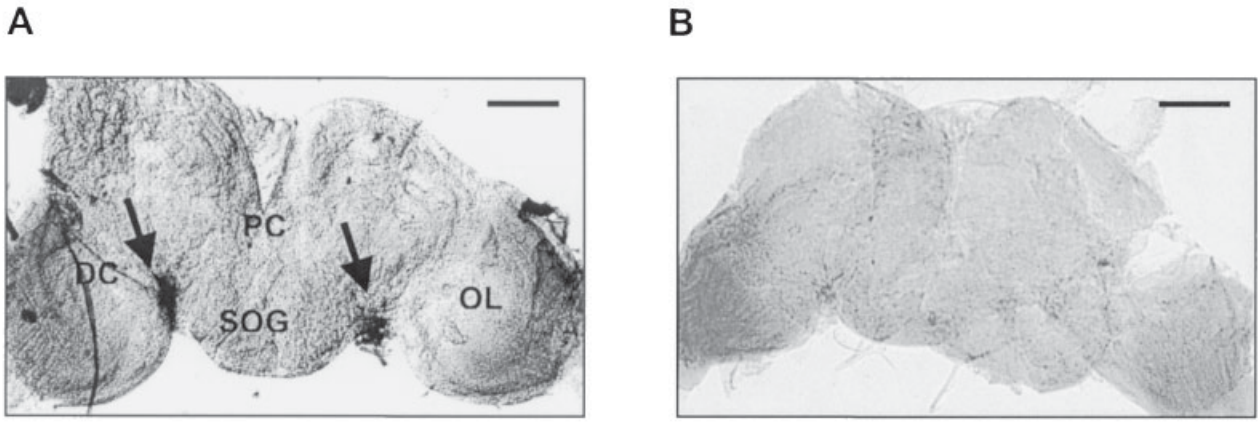

Fig. 6. Frontal view of the adult female brain (d 2) of *S. frugiperda*. A: Digoxigenin-labelled antisense probe transcribed from Spofr-AT cDNA was used to localize the expression of AT mRNA (arrows). B: Control with AT sense RNA probe. Deutocerebrum (DC), optic lobes (OL), protocerebrum (PC), and suboesophageal ganglion (SOG). Scale bar = 10 μm.

been presented also for Manse-AS and Manse-AT (Duve et al., 2000; Audsley et al., 2001; McNeil and Tobe, 2001; Koladich et al., 2002). In the search for other functions of Manse-AS and Manse AT, physiological (bioassays) and immunological methods were used to study the tissue-specific distribution of the peptides. The widespread distribution of Manse-AS and Manse-AT immunoreactivity (Veenstra et al., 1994; Zitnan et al., 1995; Audsley et al., 1998; Duve et al., 1999, 2000; Rudwall et al., 2000; Tu et al., 2001) supports their pleiotropic functions.

The availability of prohormone gene sequences provided the basis for measuring Manse-AS and Manse-AT expression by Northern blotting, (semi)-quantitative RT-PCR, or in situ hybridization. The results of such expression studies gave additional evidence that Manse-AT exhibits multiple functions (Park et al., 2002), some of which may be specific to a particular life stage (Bhatt and Horodyski, 1999). In *Pseudaletia unipuncta*, the pattern of Manse-AS prohormone gene expression suggested a function of this peptide associated with the migratory flight of that species (Jansons et al., 1996).

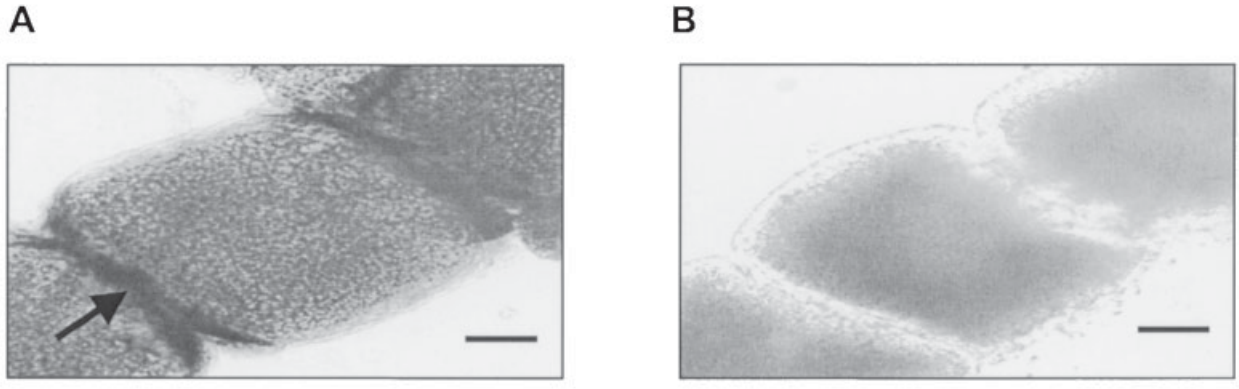

Fig. 7. Detection of Spofr-AT mRNA in the ovary of a 2-day-old adult female of *S. frugiperda* using whole-mount in situ hybridization. A: Photograph of oocytes that contained Spofr-AT mRNA (arrow). B: Control with AT sense RNA probe. Scale bar = 0.5 mm.

Recently we cloned the allatotropin gene from the fall armyworm *S. frugiperda* (Spofr-AT), which is expressed in three mRNA isoforms with 134, 171, and 200 amino acids, respectively. We also cloned a cDNA that encoded 125 amino acid residues including one copy of the *M. sexta* allatostatin (Spofr-AS) (Abdel-Latief et al., 2003). The basic organisation of the precursors is similar to those known from other Lepidoptera (83–93% amino acid sequence identity). The availability of the cDNA sequences allowed us to measure levels of Spofr-AT and Spofr-AS mRNA in tissues of the fall armyworm from various developmental stages by semi-quantitative one-step RT-PCR as well as to localize the distribution of the mRNAs in brain and ovarial tissues by in situ hybridization studies.

Using these techniques, we have shown that Spofr-AT and Spofr-AS are expressed in the brain, digestive tract, ovaries, and testes of both larval and adult females and males. The overall abundance of the mRNAs differs markedly between developmental stages and tissues. AT expression was high in the brains of 5th larval stage, but low in L6 larvae and during prepupation. Another increase in AT expression was observed towards the pupal phase. In contrast, AS expression remained high until the 6th larval instar and low during pupation. Although we do not know whether a correlation of JH biosynthesis in the CA and the presence of allatoregulating peptides in the brain exists, our results may be interpreted such that in the last larval stage (L6) high expression of the AS in combination with low expression of the AT could lead to reduction in CA activity at that time. The consistent ratio of expression of the three Spofr-AT mRNA isoforms in the brain of adult females and males suggests a sophisticated regulation of AT gene expression at that time. The time-dependent changes of AS gene expression in adult female brains (with fairly constant rate of AT expression) corresponds to previous measurements of the rate of JH biosynthesis by CA in vitro (Range et al., 2002), because in the brain the AS expression should be low when JH biosynthesis is high and vice versa.

Distinct changes in the rates of Spofr-AT and Spofr-AS expression in the midguts of larvae and adults suggest a role of lepidopteran AS and AT as brain/gut neuropeptides (Lee et al., 1998; Chamberlin and King, 1998; Duve et al., 1999, 2000). However, we can neither speculate on the functions of the peptides in the digestive tract of *S. frugiperda* nor on their cell-specific localization in the gut. Lee et al. (1998) demonstrated that Manse-AT inhibits midgut ion transport in larvae of *M. sexta*. Manse-AT also stimulated foregut contraction in *Helicoverpa armigera* (Duve et al., 1999) and *Lacanobia oleracea* (Duve et al., 2000), gut contraction in fifth instar larvae of *Heliothis virescens* (Oeh et al., 2003), and reduced weight gain and increased mortality when injected in *S. frugiperda* larvae (Oeh et al., 2001). Manse-AS inhibited spontaneous foregut contraction in *L. oleracea* (Duve et al., 2000) and inhibited feeding and hence growth, when injected into *L. oleracea* larvae (Audsley et al., 2001).

In several insect species, the ovary is required for the cycle of JH biosynthesis (Stay et al., 1983) and the ovaries contain extractable material that inhibits JH biosynthesis by CA in vitro (Applebaum et al., 1990; Ferenz and Aden, 1993; Hoffmann et al., 1996). Recently, we demonstrated the presence of A-type and B-type allatostatins in ovaries of the cricket *G. bimaculatus* by immunohistochemistry (Witek and Hoffmann, 2001). Garside et al. (2002), in studies on the cockroach *Diploptera punctata*, have provided evidence by quantitative competitive RT-PCR that A-type AS are synthesized not only in the ovaries but also in oviducts. The pattern of expression together with immunocytochemical staining suggests that the AS function, in part, to regulate the cycle of vitellogenesis in mated females. Meyering-Vos and Hoffmann (2003) demonstrated striking differences in the expression of A-type AS within the ovary of mated vs. virgin females of *G. bimaculatus*. In locusts, an inhibitory effect of A-type AS on lateral oviduct contraction was shown (Schoofs et al., 1997). To date, no evidence is available for the presence and function of C-type AS in reproductive tissues of a lepidopteran species. Our present results demonstrate a clear expression of Spofr-AT and Spofr-AS in ovaries and testes of young females and males. The in situ hybridization studies showed that the Spofr-AT

mRNA was restricted to the follicle cells between the oocytes whereas Spofr-AS mRNA was mainly found at the outer membrane of the oocytes. The function of these two peptides is unknown. It is not known whether the ovarian and testes peptides are released into the haemolymph, or if they act in a paracrine way. A homologue (Locmi-AG-MT 1) of Manse-AT was isolated from male accessory reproductive glands of the locust *L. migratoria*, which had a myotropic effect on the oviduct (Paemen et al., 1991). In *P. unipuncta*, males transfer a sex peptide in the ejaculate, which increases the haemolymph JH titre in the females and this peptide could potentially be AT or an AT-related peptide (McNeil et al., 2000; McNeil and Tobe, 2001).

In conclusion, our data strengthen the hypothesis that Spofr-AT and Spofr-AS exhibit multiple functions, which may be tissue-specific as well as specific to a particular developmental stage. Further quantitative experiments formulated in context of the life history of the animals will yield a more detailed understanding of the mode of action of these peptides in *S. frugiperda*.

ACKNOWLEDGMENTS

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LITERATURE CITED

Abdel-latief M, Meyering-Vos M, Hoffmann KH. 2003. Molecular characterisation of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides. Insect Biochem Mol Biol 33:467–476.

Applebaum SW, Hirsch J, El-Hadi FA, Moschitzki P. 1990. Trophic control of juvenile hormone biosynthesis and release in locusts. In: Epple E, Scanes CG, Stetson MH, editors. Progress in comparative endocrinology. New York: Wiley-Liss. p 186–192.

Audsley N, Weaver RJ, Edwards JP. 1998. Enzyme linked immunosorbent assay for *Manduca sexta* allatostatin (Mas-AS), isolation and measurement of Mas-AS immunoreactive peptide in *Lacanobia oleracea*. Insect Biochem Mol Biol 28:775–784.

Audsley N, Weaver RJ, Edwards JP. 2000. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. Insect Biochem Mol Biol 30:681–689.

Audsley N, Weaver RJ, Edwards JP. 2001. In vivo effects of *Manduca sexta* allatostatin and allatotropin on larvae of the tomato moth, *Lacanobia oleracea*. Physiol Entomol 26:181–188.

Bendena WG, Donley BC, Tobe SS. 1999. Allatostatins: a growing family of neuropeptides with structural and functional diversity. Ann NY Acad Sci 897:311–329.

Bergot BJ, Baker FC, Cerf DC, Jamieson G, Schooley D. 1981. Qualitative and quantitative aspects of juvenile hormone titres in developing embryos of several insect species: discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Pratt GE, Brooks GT, editors. Juvenile hormone biochemistry. Amsterdam: Elsevier. p 167–197.

Bhatt TR, Horodyski FM. 1999. Expression of the *Manduca sexta* allatotropin gene in the central and enteric nervous systems. J Comp Neurol 403:407–420.

Brüning C, Lanzrein B. 1987. Stage-dependent biosynthesis of methylfarnesoate and juvenile hormone III and metabolism of juvenile hormone III in embryos of the cockroach, *Nauphoeta cinerea*. Insect Biochem 18:3–9.

Chamberlin ME, King ME. 1998. Changes in midgut active ion transport and metabolism during the fifth instar of the tobacco hornworm (*Manduca sexta*). J Exp Zool 280:135–141.

Chew SL. 1997. Alternative splicing of mRNA as a mode of endocrine regulation. Trends Endocrinol Metab 8:405–413.

Dorn A. 1982. Precocene-induced effects and possible role of juvenile hormone during embryogensis of the milkweed bug, *Oncopeltus fasciatus*. Gen Comp Endocrinol 46:42–52.

Duve H, East PD, Thorpe A. 1999. Regulation of lepidopteran foregut movement by allatostatins and allatotropin from the frontal ganglion. J Comp Neurol 413:405–416.

Duve H, Audsley N, Weaver RJ, Thorpe A. 2000. Triple colocalisation of two types of allatostatin and an allatotropin in the frontal ganglion of the lepidopteran *Lacanobia oleracea* (Noctuidae): innervation and action on the foregut. Cell Tissue Res 300:153–163.

Ferenz H-J, Aden E. 1993. Ovarian control of juvenile hormone biosynthesis in *Locusta migratoria* (Orthoptera: Acrididae). Entomologia Generalis 18:9–17.

Gäde G. 2002. Allatoregulatory peptides: molecules with multiple functions. Invertebr Reprod Dev 41:127–135.

Gäde G, Hoffmann KH, Spring J. 1997. Hormonal regulation in insects: facts, gaps and future direction. Physiol Rev 77:963–1032.

Garside CS, Koladish PM, Bendena WG, Tobe SS. 2002. Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata.* Insect Biochem Mol Biol 32:1089–1099.

Harland RM. 1991. In situ hybridization and improved whole-mount methods for *Xenopus* embryos. Methods Cell Biol 36:685–695.

Hoffmann KH, Lorenz MW, Klein PM. 1996. Ovarian control of juvenile hormone III biosynthesis in *Gryllus bimaculatus* (Ensifera: Gryllidae). Entomologia Generalis 20:157–167.

Hoffmann KH, Meyering-Vos M, Lorenz MW. 1999. Allatostatins and allatotropins: is regulation of corpora allata activity their primary function? Eur J Entomol 96:255–266.

Hökfelt T, Broberger C, Xu Z-QD, Sergeyev V, Ubink R, Diez M. 2000. Neuropeptides: an overview. Neuropharmacology 39:1337–1356.

Horodyski FM, Bhatt SR, Lee K-Y. 2001. Alternative splicing of transcripts expressed by the *Manduca sexta* allatotropin (Mas-AT) gene is regulated in a tissue-specific manner. Peptides 22:263–269.

Jansons IS, Cusson M, McNeil JN, Tobe SS, Bendena WG. 1996. Molecular characterization of a DNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). Insect Biochem Mol Biol 26:767–773.

Kataoka H, Toschi A, Li JP, Carney RL, Schooley DA, Kramer SJ. 1989. Identification of an allatotropin from adult *Manduca sexta*. Science (Wash) 243:1481–1483.

Koladich PM, Cusson M, Bendena WG, Tobe SS, McNeil JN. 2002. Cardio-acceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth *Pseudaletia unipuncta*. Peptides 23:645–651.

Kramer SJ, Toschi A, Miller CA, Kataoka H, Quistad GB, Li JP, Carney RL, Schooley DA. 1991. Identification of an allatostatin from the tobacco hornworm *Manduca sexta.* Proc Natl Acad Sci USA 88:9458–9462.

Lee K-Y, Horodyski FM. 2002. Restriction of nutrient intake results in the increase of a specific *Manduca sexta* allatotropin (Manse-AT) mRNA in the larval nerve cord. Peptides 23:653–661.

Lee KY, Horodyski FM, Chamberlin ME. 1998. Inhibition of midgut ion transport by allatotropin (Mas-AT) and *Manduca* FLRFamides in the tobacco hornworm *Manduca sexta*. J Exp Biol 201:3067–3074.

Lorenz JH, Lorenz MW, Hoffmann KH. 1997. Factors regulating juvenile hormone and ecdysteroid biosynthesis in *Gryllus bimaculatus* (Ensifera:Gryllidae). Eur J Entomol 94:369–379.

McNeil JN, Miller D, Laforge M, Cusson M. 2000. Dynamics of juvenile hormone biosynthesis, degradation and titers in females of the true armyworm: a comparison of migratory and non-migratory populations. Physiol Entomol 25:103–111.

McNeil JN, Tobe SS. 2001. Flights of fancy: possible roles of allatostatin and allatotropin in migration and reproductive success of *Pseudaletia unipuncta.* Peptides 22:271–277.

Meyering-Vos M, Hoffmann KH. 2003. Expression of allatostatins in the Mediterranean field cricket, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae). Comp Biochem Physiol 136B:207–215.

Nijhout HF. 1994. Insect hormones. Princeton NJ: Princeton University Press. 280 p.

Oeh U, Lorenz MW, Dyker H, Lösel P, Hoffmann KH. 2000. Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda.* Insect Biochem Mol Biol 30:719–727.

Oeh U, Dyker H, Lösel P, Hoffmann KH. 2001. In vivo effects of *Manduca sexta* allatotropin and allatostatin on the development and reproduction in the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Invertebr Reprod Dev 39:239–247.

Oeh U, Antonicek H, Nauen R. 2003. Myotropic effect of helicokinins, tachykinin-related peptides and *Manduca sexta* allatotropin on the gut of *Heliothis virescens* (Lepidoptera: Noctuidae). J Insect Physiol 49:323–337.

Paemen L, Tips A, Schoofs L, Proost P, Van Damme J, De Loof A. 1991. Lom-AG-myotropin: a novel myotropic peptide from the main accessory reproductive glands of *Locusta migratoria*. Peptides 12:7–10.

Park C, Hwang JS, Kang SW, Lee BH. 2002. Molecular characterization of a cDNA from the moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. Zool Sci 19:287–292.

Range S, Oeh U, Lorenz MW, Etzel W, Nauen R, Hoffmann KH. 2002. Juvenile hormone biosynthesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). Comp Biochem Physiol 132 B:191–202.

Riddiford LM. 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv Insect Physiol 24:213–274.

Rudwall AJ, Sliwowska J, Nässel DR. 2000. Allatotropin-like neuropeptide in the cockroach abdominal nervous system: myotropic actions, sexually dimorphic distribution and colocalization with serotonin. J Comp Neurol 428:159–173.

Schoofs L, Veelaert D, Vanden Broeck J, De Loof A. 1997. Peptides in the locusts, *Locusta migratoria* and *Schistocerca gregaria*. Peptides 18:145–156.

Stay B. 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem Mol Biol 30:653–662.

Stay B, Tobe SS, Mundall ES, Ranking S. 1983. Ovarian stimulation of juvenile hormone biosynthesis in the viviparous cockroach, *Diploptera punctata*. Gen Comp Endocrinol 52:341–349.

Strand F. 1999. Neuropeptides: regulators of physiological processes. Cambridge MA: Massachusetts Institute of Technology Press. 658 p.

Tobe SS, Stay B. 1985. Structure and regulation of the corpus allatum. Adv Insect Physiol 18:305–432.

Tu MP, Kou R, Wang ZS, Stoffolano JG, Yin CM. 2001. Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, *Phormia regina* (Diptera: Calliphoridae). J Insect Physiol 47:233–244.

Veenstra JA, Lehman HK, Davis NT. 1994. Allatotropin is a cardioacceleratory peptide in *Manduca sexta*. J Exp Biol 188:347–354.

Weaver RJ, Edwards JP, Bendena WG, Tobe SS. 1998. Structures, functions and occurrence of insect allatostatic peptides. In: Coast GM, Webster SG, editors. Recent advances in anthropod endocrinology. Cambridge: Cambridge University Press. p 3–32.

Witek G, Hoffmann KH. 2001. Immunological evidence for FGL-amide and W^2W^9 allatostatins in the ovary of *Gryllus bimaculatus* (Ensifera Gryllidae). Physiol Entomol 26:49–57.

Zitnan D, Kingan T, Kramer S, Beckage N. 1995. Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. J Comp Neurol 365:83–100.

Archives of Insect Biochemistry and Physiology 56:120–132 (2004)

Type-A Allatostatins From the Fall Armyworm, *Spodoptera frugiperda*: Molecular Cloning, Expression and Tissue-Specific Localization

Mohatmed Abdel-Latief, Martina Meyering-Vos, and Klaus H. Hoffmann*

The gene encoding the *Spodoptera frugiperda* allatostatin type-A peptide family (Y/FXFGL-amides) was isolated from *S. frugiperda* brain cDNA. The gene encodes a precursor of 231 amino acids containing nine (or ten) Y/FXFGL-a peptides that are tandemly arranged in three blocks. The comparison of the Spofr-AST A precursor with the respective precursor genes from two other lepidopteran species, *Helicoverpa armigera* and *Bombyx mori*, shows high homology in size, sequence (84 and 57%, respectively), and organisation of the allatostatins. One-step RT-PCR analysis using a Spofr-AST A-6 to A-9 probe shows that the gene is not only expressed as one transcript in the brain and midgut of larvae and adults in a time- and tissue-specific manner, but also in the reproductive tissues of adult *S. frugiperda*. Data confirm the nature of the allatostatin type-A peptides as brain/gut myoregulatory hormones, whereas their function(s) in ovaries, oviduct, and testes still have to be resolved. The cell-specific localization of the preprohormone expression, as demonstrated by whole mount in situ hybridization, confirms the overall distribution of the Spofr-AST A preprohormone as shown by RT-PCR and supports the pleiotropic functions of the peptides. Arch. Insect Biochem. Physiol. 56:120–132, 2004.

KEYWORDS: allatostatin; *Spodoptera frugiperda*; preprohormone; hormone processing; one-step RT-PCR; whole-mount in situ hybridization.

INTRODUCTION

Originally, the term allatostatins (AS, AST) was introduced to define unknown regulating factors inhibiting juvenile hormone (JH) biosynthesis in vitro by the corpora allata (CA) of insects (Tobe, 1980; Weaver et al., 1998). In the Lepidoptera, a true allatostatin was first characterized from the tobacco hornworm, *Manduca sexta*. The *M. sexta* allatostatin (Manse-AS; C-type allatostatin) inhibited JH synthesis in vitro by CA of larvae and adult females of *M. sexta* (Kramer et al., 1991). The Manse-AS peptide was subsequently identified from other lepidopterans, such as *Pseudaletia unipuncta* and *Spodoptera frugiperda*, where its preprohormones were also cloned (Jansons et al., 1996; Abdel-latief et al., 2003).

Several allatostatins, structurally unrelated to Manse-AS, were identified in various insect orders including the lepidopterans (e.g., Duve et al., 1997a,c). The allatostatin A-type peptide family currently comprises more than 70 members from insects and some crustacean species (Duve et al., 1997b, 2002). The peptides share an amidated C-terminal pentapeptide sequence Y/FXFGL-a (A-type allatostatins; allatostatin superfamily). Their allatostatic function seems to be restricted to cockroaches and crickets (Hoffmann et al., 1999). However,

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The nucleotide sequence data published here have been submitted to EMBL Nucleotide Sequence Data Base, accession no. AJ555184.

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Y/FXFGL-a peptides have been shown to inhibit the spontaneous muscle contraction in foregut and/or hindgut preparations of all insect species tested. A-type allatostatins are predominant neuropeptides in the antennal pulsatile organ of cockroaches (Predel et al., 1999), but no myotropic effect on the antennal pulsatile organ muscle could be found.

Immunocytochemical studies have demonstrated a wide distribution of the Y/FXFGL-amides throughout the entire nervous system, in gut endocrine cells, and in the ovary, suggesting that these peptides are ubiquitous and multifunctional (for review see Bendena et al., 1999; Hoffmann et al., 1999; Stay, 2000; Gäde, 2002). A large number of Y/FXFGL-amides were deduced from prohormone gene sequences (Donly et al., 1993; Ding et al., 1995; East et al., 1996; Vanden Broeck et al., 1996; Veenstra et al., 1997; Bellés et al., 1999; Lenz et al., 2000; Meyering-Vos et al., 2001).

Duve et al. (1997c) isolated and purified members of the allatostatin type-A peptide family from two lepidopterans, the codling moth, *Cydia pomonella*, and the bollworm, *Helicoverpa armigera*. The peptides were designated cydiastatins and helicostatins, respectively. Eight peptides could be identified from each of the two species, with four identical in both species and three differing by a single amino acid. The gene that encodes the helicostatin peptide family was later isolated from a *H. armigera* genomic DNA library (Davey et al., 1999). The gene consists of three exons and encodes a precursor of 225 amino acids that contains three blocks of tandemly arranged helicostatins. A comparison of the helicostatin precursor with that of cockroaches, locusts, and flies revealed a variation in size, sequence, and organization of the allatostatin type-A precursors across different insect orders. In situ hybridization studies established that helicostatins are expressed in neurons of the central nervous system and in endocrine cells of the midgut, indicating that the peptides represent brain/gut peptides (Davey et al., 1999). Another lepidopteran gene precursor of the type-A allatostatins (bostatins) has been cloned recently for the silkworm *Bombyx mori* (Secher et al., 2001).

As an aid to identifying the physiological functions of type-A allatostatins in *S. frugiperda*, we here report on the identification of the *S. frugiperda* allatostatin type-A gene. Experiments were performed to demonstrate the mRNA expression profiles of the allatostatin A-type precursor in various tissues of larvae, pupae, and adult moths by one-step RT-PCR. Moreover, we show some tissue-specific localization of the allatostatin A-type mRNA by whole-mount in situ hybridization.

MATERIALS AND METHODS

Insect Rearing

Pupae and eggs of *S. frugiperda* were provided by Bayer AG (Leverkusen) and reared at 27°C and 70% relative humidity under a L 16: D 8 photoperiod as previously described (Abdel-Latief et al., 2003).

Genomic DNA Extraction

To isolate genomic DNA, individual *S. frugiperda* adult females were frozen and, in liquid nitrogen, ground to a fine powder using mortar and pestle. The powder was resuspended and the genomic DNA was extracted according to the protocol supplied by the manufacturer of QIAamp® DNA Mini kit (50) (Qiagen GmbH, Hilden, Germany).

Preparation of the cDNA by RACE and Genomic DNA

Brains (cerebral and suboesophageal ganglia) of 500 female adult fall armyworms, 2–3 days old, were dissected in modified cricket saline (Lorenz et al., 1997), frozen immediately in liquid nitrogen, and stored at –80°C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen). The Smart™ RACE cDNA amplification kit (Clontech Laboratories, Heidelberg, Germany) was used to amplify the cDNA. One microgram mRNA was added as a template for each RACE reaction. A 5′-RACE was performed by using degenerate oligonucleotide primer ASTar2 (5′-CNA RNC CRA ART TRT AYA TNG GNA R-3′; corresponding to nucleotide positions 625–649 of Fig. 2), in combination with the

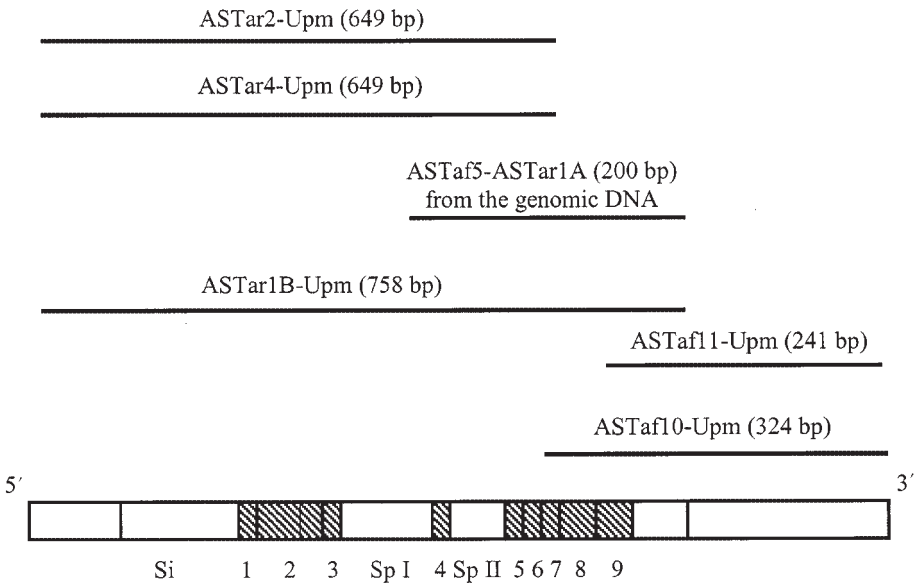

Fig. 1. Sequencing strategy of the Spofr-AST A cDNA. Horizontal lines represent the individual clones used for sequencing. **Bottom:** The structural organization of the Spofr-AST A gene. The positions of the allatostatin-related peptides 1-9 are marked with diagonal lines and the untranslated region, the signal peptide (Si), the intervening spacers (Sp I, II), and the poly A-tail are represented by white boxes. Sites of possible endoproteolytic cleavage are indicated by vertical lines.

universal primer (Upm) 5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3′ that recognizes the primer binding sequence, introduced by the Smart RACE kit components. Using the genomic DNA as a template in combination with the specific primer ASTaf5 and the degenerate primer ASTar1A (5′-AR NCC RAA NSW RAA NCC RTG CAT RTC-3′) corresponding to nucleotide positions 558–574 and 733–758, respectively, we obtained a fragment of 200 bp. The 5′ end of the cDNA was further amplified by using gene specific primers ASTar1B and ASTar4 corresponding to nucleotide positions 733–758 and 625–649, respectively. The specific primers ASTaf10 and ASTaf11 corresponding to nucleotide positions 651–672 and 736–760, respectively, were designed to obtain the 3′ end of the Spofr-AST A preprohormone (see Fig. 1). The PCR program was 95°C for 2 min, followed by 10 cycles of 94°C for 30 sec, 68°C for 45 sec decreased with 1°C per cycle, followed by 35 cycles of 94°C for 30 sec, 61°C for 45 sec, 68°C for 1.5 min, and a final extension step of 68°C for 10 min.

Cloning and Sequencing

The PCR products were eluted from low melting point agarose gel (Biozym Biotech, Oldendorf, Germany) with GFX™ purification kit (Pharmacia Biotech, Freiburg, Germany), and ligated into plasmids with the pGEM-T easy system kit supplied with *Escherichia coli* JM109 (Promega, Mannheim, Germany) for amplification. Plasmid DNA was purified using QIAprep® Spin Miniprep kit (Qiagen GmbH). The templates were sequenced by GATC (Konstanz, Germany). Sequences were analysed with the GCG software (Wisconsin Package).

RNA and mRNA Extractions for RT-PCR Analysis

Brains and midguts from 4th (L4), 5th (L5), and 6th instar larvae (L6) (<12 h after the respective moult), prepupae (PP), young pupae (P0), 10-day-old pupae (P10), and 1–5-day-old adult females and males as well as ovaries from 1–4-day-old adult virgin females and testes from 1–4-day-old adult males were dissected in modified cricket saline (Lorenz et al., 1997), frozen immediately in liquid nitrogen. and stored at −80°C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab Biotechnologie). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen). The mRNA was quantified by spectrophotometric determination.

RT-PCR analysis

For RT-PCR, 20 ng mRNA from each of the tissues was used with specific primers ASTaf5-ASTar1B, in 10 µl of the RT-PCR mixture (TITANIUM® One

Step RT-PCR Kit, Clontech Laboratories). The PCR program for amplification of the fragment was 50°C for 1 h followed by 5 min at 94°C, 35 cycles of 94°C for 30 sec, 61°C for 45 sec, 68°C for 1 min, and a final extension step of 68°C for 2 min. These reaction conditions were optimized as previously shown (Meyering-Vos and Hoffmann, 2003). The DNA products were analysed using 1.5% agarose gel at 8 V/cm for 1.5 h, transferred to Hybond-N^+ membrane (Amersham, Freiburg, Germany) and hybridized to the probe (ASTaf5-ASTar1B) 200 bp, labelled by the PCR method with Dig dUTP (Roche, Mannheim, Germany). As a control, the same quantities (20 ng) of mRNA from different developmental stages were used as a template in combination with two specific primers ACTf1 corresponding to nucleotide positions 1–25 and ACTr2 corresponding to nucleotide positions 469–493 from the nucleotide sequence of *S. littoralis* mRNA for beta-actin (partial; EMBL Nucleotide Sequence Data Base, accession no. Z46873), which yields a 494-bp amplified fragment. Expression of A-type allatostatins was normalized relative to that of beta-actin (mean values of three controls for each developmental stage). Negative controls for genomic DNA were done without the reverse transcriptase step, starting the one-step RT-PCR reaction at the level of the second step. These control experiments showed no contamination when using mRNA as a template (see Fig. 4; L4 C). Densitometry was performed using an ImageMaster® VDS (Pharmacia Biotech) and the ImageMaster 1D Database software.

Statistics

Data from RT-PCR analyses are means ± SD of three measurements. The Mann-Whitney U-test or the Wilcoxon-test were used in statistical treatment of the data.

Synthesis of Digoxigenin (Dig-11-UTP)-Labelled RNA Probe

Antisense and sense Spofr-AST A probes were synthesized from the cDNA clone ASTaf5-ASTar1B. One microgram ASTaf5-ASTar1B plasmid was linearized with ApaI and PstI restriction enzymes (about 30 U according to enzymes) (Promega) in a 20-µl volume to yield templates for the antisense and the sense probes, respectively. Purification of the template, synthesis, labelling, and precipitation of the RNA probes (200 nt) were carried out as described (Abdel-latief et al., 2004).

Whole-Mount In Situ Hybridization

Whole tissues of brain, digestive tract, and ovary were dissected from 2-day-old adult females in modified cricket saline (Lorenz et al., 1997) and immediately placed in fixative. Tissue fixation and in situ hybridization were performed as described (Abdel-latief et al., 2004), using a 200-nt-long digoxigenin-labeled sense and antisense Spofr-AST RNA probe.

RESULTS

Isolation of the Spofr-AST A Gene

The degenerate oligonucleotide primer ASTar2 was designed from the sequence of the peptide LPMYNFGL-amide (*H. armigera*; Davey et al., 1999). In combination of the primer ASTar2 with Upm primer and using the 5′ RACE reaction as a template, a product of 649 bp was obtained that contained a core region of the *S. frugiperda* allatostatin A-type gene. It included the untranslated region followed by a probable signal peptide and five copies of the Spofr-AST A like peptides (Fig. 1). Using this fragment, primers were designed to amplify the cDNA 5′ and 3′ ends, respectively. In a PCR reaction using specific primers ASTar4 and Upm and 5′ RACE cDNA as a template, we were able to amplify the same fragment (649 bp) (Fig. 1). Use of the genomic DNA of *S. frugiperda* as a template in a PCR reaction in combination with primers ASTaf5 and ASTar1A yielded a fragment of 200 bp, which included four copies of the Spofr-AST A peptides (A-6 to A-9). In order to amplify a fragment that included all the Spofr-AST A peptides, a 5′RACE reaction was preformed using the specific primers ASTar1B and Upm. The reaction

yielded a product of 758 bp that included the untranslated region followed by the signal peptide, two spacer regions, and all nine copies of the Spofr-AST A peptides. This fragment was used to design different specific primers to amplify the 3′ end of the Spofr-AST A cDNA. When the primer ASTaf11 was used in a PCR reaction with Upm primer and the 3′ RACE reaction as a template, a product of 241 bp was obtained, which included the ninth Spofr-AST A peptide, the stop codon signal, the polyadenylation signal, and the downstream poly A-tail. The primer ASTaf10 yielded a corresponding product of 324 bp (Fig.1).

Characterization of the Spofr-AST A cDNA

The complete sequence of the 976 nucleotides of the Spofr-AST A cDNA is shown in Figure 2. The cDNA contains 132 nucleotides of an untranslated sequence upstream of a single open reading

```
5′CTAATACGACTCACTATAGGGCAAGCAGTGGTAAACGCAGAGTACGCGGGG   51
  ACAGCTGTTAGCTGGCGGGCTTCAAGCACGCCGCATTAACATCGCGTGTGC  102
  CAAACCTTACGTGACTACGAACACATAAGAATGCTGTACCCATCAATTCCG  153
                                    M  L  Y  P  S  I  P    7
  GTTTGCTTCCTCGTGATTGGAGTAGCACTCTGCGCTCCAGAGAGGATGCAG  204
   V  C  F  L  V  I  G  V  A  L  C↓ A  P  E  R  M  Q   24
  AACGAACCAGACCCTCACGACACTCCGGTGCATGAGGGCACTGAGCCACAC  255
   N  E  P  D  P  H  D  T  P  V  H  E  G  T  E  P  H   41
  AGTGACCACATTGCCCCTCTTGAGAAGAGATCCCCTCACTACGACTTTGGG  306
   S  D  H  I  A  P  L  E  K  R  S  P  H  Y  D  F  G   58
                                  Spofr-AST A-1
  TTGGGCAAGAGGGCTTACAGCTACGTGTCAGAATATAAACGACTACCTGTC  357
   L  G  K  R  A  Y  S  Y  V  S  E  Y  K  R  L  P  V   75
                        Spofr-AST A-2
  TACAACTTTGGACTGGGCAAGAGATCCAGGCCCTACTCCTTTGGCCTGGGC  408
   Y  N  F  G  L  G  K  R  S  R  P  Y  S  F  G  L  G   92
                              Spofr-AST A-3
  AAACGTTCAGTTGACGAGGACCAGTCCAGCGAGAGCCAGCCTCTGACCAGC  459
   K  R  S  V  D  E  D  Q  S  S  E  S  Q  P  L  T  S  109

  GACCTGGACCAAGCTGCCTTAGCTGAATTCTTCGATCAGTATGATGATGCC  510
   D  L  D  Q  A  A  L  A  E  F  F  D  Q  Y  D  D  A  126
                     Spacer-I
  GGTTACGAGAAGCGCGCTCGACCTTACAGCTTTGGCCTCGGCAAACGCTTC  561
   G  Y  E  K  R  A  R  P  Y  S  F  G  L  G  K  R  F  143
                        Spofr-AST A-4
  GCTGACGACGAAACTTCCGAAGAAAAGCGGGCAAGGGCATACGACTTTGGA  612
   A  D  D  E  T  S  E  E  K  R  A  R  A  Y  D  F  G  160
      Spacer-II                  Spofr-AST A-5
  CTGGGCAAGCGGCTACCGATGTACAACTTTGGTTTGGGCAAGCGAGCGAGG  663
   L  G  K  R  L  P  M  Y  N  F  G  L  G  K  R  A  R  177
                     Spofr-AST A-6
  AGCTACAACTTTGGCTTGGGCAAGCGATTGAGCAGCAAATTCAACTTTGGT  714
   S  Y  N  F  G  L  G  K  R  L  S  S  K  F  N  F  G  194
    Spofr-AST A-7              Spofr-AST A-8
  TTAGGCAAAAGGGAGAGGGACATGCACGGTTTCAGTTTCGGCCTGGGCAAA  765
   L  G  K  R  E  R  D  M  H  G  F  S  F  G  L  G  K  211
                        Spofr-AST A-9
  AGGGTCCATAAGTTTACGGCCGAAATATGGACTTCTGTTGAGGTCTTAAAT  816
   R  V  H  K  F  T  A  E  I  W  T  S  V  E  V  L  N  228
  AAAATTCTATAATCGTCCTAATTGAATTTAATATGAATAAAGAATAACTTA  867
   K  I  L ---                                         231
  CTTAACAATGTTAATGTCCATGGGCGGCGCTGATCGCTTACCATCAGGTGA  918
  CTCGTTTGCTCGTTTGCCTCCTATTCCAGAAAAAAAAAAAAAAAAAAAAA  969
  AAAAAAA 3′                                           976
```

Fig. 2. Nucleotide and the deduced amino acid sequences of the *S. frugiperda* AST A-type cDNA. The sequences are numbered at the right. The allatostatin amino acid sequences (Spofr-AST A-1 to A-9) are shown in bold type. Possible proteolytic cleavage sites are in boxes and the glycine residues required for amidiation are underlined. The potential polyadenylation signal is shown in bold type and underlined. --- represents the stop code signal. A possible signal peptide cleavage site is indicated by a downward arrow.

frame beginning at position 133 and ending at position 826 with the translation stop codon TAA. The Spofr-AST A precursor begins with a hydrophobic domain that represents a probable signal peptide with the most likely site of cleavage between residues 18 and 19 (Von Heijne, 1986). The precursor contains the unprocessed sequences of nine allatostatin type-A peptides (Figs. 2 and 3), which are located at residues 286–312, 319–375, 382–408, 526–552, 592–618, 625–651, 658–684, 691–720, and 727–762, respectively. All of the putative peptides have a single glycine residue at the C-terminal end that provides a substrate for peptidyl-glycine-α-amidating monooxygenase (Eipper et al., 1992). Recognizing all the proteolytic cleavage sites would result in a tenth peptide (by representing of KR at amino acid positions 71/72). Comparison of the Spofr-AST A cDNA sequence with those for *H. armigera* and *B. mori* exhibited 84 and 57% identical amino acid residues, respectively. The general organisation of the three known lepidopteran allatostatin A-type peptide precursors is similar (Fig. 3).

Brain Expression

Semi-quantitative one-step RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the Spofr-AST A gene. Results in Figure 4 show that the Spofr-AST A gene is expressed as one transcript in the brains of 4th to 6th instar larvae as well as in the early (P0) and late (P10) pupal stage, whereas the expression signal was slightly lower ($P < 0.05$) in the brains of the prepupal stage (PP). In brains from adult moths, a sex-specific expression of the Spofr-AST A gene can be seen (Fig. 5A,B). Expression was high in the brain of adult females after eclosion (1 day) and decreased thereafter, whereas in males the gene expression was low

```
Spofr-AST A:MLYPSIPVCFLVIGVALCAPERMQNEPDPHDTPVHEGTEPHSDHIFTAPLEKRSPHYDFG  60
Helar-AST A:MLYSSLPVCFLVLGAALCAPERMQNEAEPHDLQPHE-AEPHSDHVAFTPLAKRSPHYDFG  59
Bommo-AST A:MLSACLPL-LLVLGAALAAEQVAEHG----------AEQVAEHA--APLEKRSPQYDFG  46
            **    *   ** * ** *                    *    *    ** **** ****

Spofr-AST A:LGKRAYSYVSEYKRLPVYNFGLGKRSRPYSFGLGKRSVDEDQSS-ESFTQPLTSDLDQAA 119
Helar-AST A:LGKRAYSYVSEYKRLPVYNFGLGKRSRPYSFGLGKRSVDEDQSNDEQFTQLTTSDLDQAA 119
Bommo-AST A:LGKRAYSYVSEYKRLPVYNFGLGKRSRPYLFGLGKRSAGA-----EQLDDDISNEADQNT 101
            ****************************** *******        *          **

Spofr-AST A:LAEFFDQYDDAG------YEKRARPYSFGLGKRFADDETSEEKRARAYDFGLGKRLPMYN 173
Helar-AST A:LAELFDQYDDA--------EKRARPYSFGLGKRFADDETSEEKRARAYDFGLGKRLPMYN 171
Bommo-AST A:LDELFDQYDDSAAVPTGYVEKRARPYSFGLGKRFA-EEPAED----------KRARMYS 147
            * * ******         ****************  *  *            **  **

Spofr-AST A:FGLGKRARSYNFGLGKRLSSKFNFGLGKRERDMHGFSFGLGKR-VHKFTAEIWT-FTSVE 231
Helar-AST A:FGLGKRARSYNFGLGKRYS-KFNFGLGKRERDMHRFSFGLGKRSGDDVSADDSDNYFTFD 230
Bommo-AST A:FGLGKRARSYSFGLGKRLSSKFNFGLGKRQRDMHRFSFGLGKRSEDDTSENYIDA----- 204
            ********** ****** * ********* **** ********

Spofr-AST A:VLNKIL 237
Helar-AST A:V----- 231
Bommo-AST A:------
```

Fig. 3. Alignment of the allatostatin A-type precursor peptides of *S. frugiperda* (Spofr-AST A), *H. armigera* (Helar-AST A) (Davey et al., 1999), and *B. mori* (Bommo-AST A) (Secher et al., 2001). Asterisks represent amino acids identical in each of the precursors. The AST A-type peptides are shown in bold type. Alignment was done by using T-COFFEE alignment program (Notredame et al., 2000).

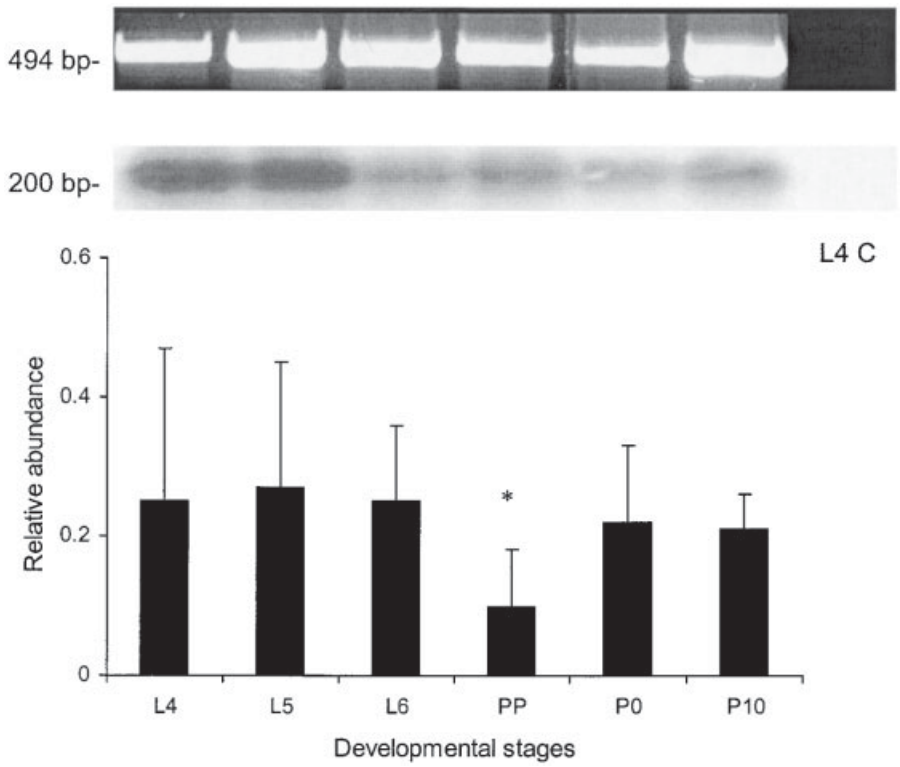

Fig. 4. Expression of Spofr-AST A gene in the brains of the 4th (L4), 5th (L5), and 6th larval stage (L6), prepupal stage (PP), young pupal stage (P0), and 10-day-old pupae (P10) as measured by RT-PCR. Agarose gel separation of beta-actin control (494 bp) stained with ethidium bromide using 20 ng mRNA as a template in one-step RT-PCR. Densitometric quantification of the signals relative to that of beta-actin. Mean values of 3 determinations ± SD. $*P < 0.05$ compared to the value for L4. The exterior right lane represents a negative genomic DNA control for the brain of a 4th instar larva (L4 C).

after eclosion but increased up to day 4 after moult ($P < 0.001$).

Midgut Expression

In the midgut of *S. frugiperda*, AST A expression could be detected in all developmental stages (from 4th instar larvae to 5-day-old adults of both sexes) (Fig. 6), but with considerable variability. Significantly higher expression rates were observed in last larval instars (L6) ($P < 0.01$) and in prepupae (PP) ($P < 0.001$), but also in the midgut of adult moths (day 3 to day 5) ($P < 0.01$).

Ovary and Testis Expression

Our results demonstrate Spofr-AST A gene expression in the reproductive tissues of both sexes (Figs. 7, 8). Expression rates in the ovary were high at days 2 and 3 after ecdysis and dropped to day 4 (Fig. 7) ($P < 0.01$), whereas for the testes, highest expression was detected at the first day after eclosion, but decreased thereafter (Fig. 8) ($P < 0.001$).

Whole-Mount In Situ Hybridization of Spofr-AST A

In situ hybridization (ISH) using a digoxigenin-labelled RNA probe was carried out with whole mounts of the brain, digestive tract (not shown), the ovary, and the oviduct from a 2-day-old adult female. *S. frugiperda* ovarioles are of the polytrophic type. In the ovaries, the Spofr-AST A gene transcript was localized within the oocytes with stronger labelling in older follicles (Fig. 9A). Using the Spofr-AST sense probe did not reveal a positive reaction in the ovary (Fig. 9B). Figure 10A shows a distinct expression of mRNA also in the lateral oviduct.

DISCUSSION

The Spofr-AST A precursor as deduced from the cDNA sequence includes nine clustered peptides interspaced by two acidic spacers and flanked N-terminally by a long leader peptide (including the signal peptide) and C-terminally by a peptide of 19 amino acid residues. All the peptides are characterized by the conserved C-terminal sequence Y/FXFGL-

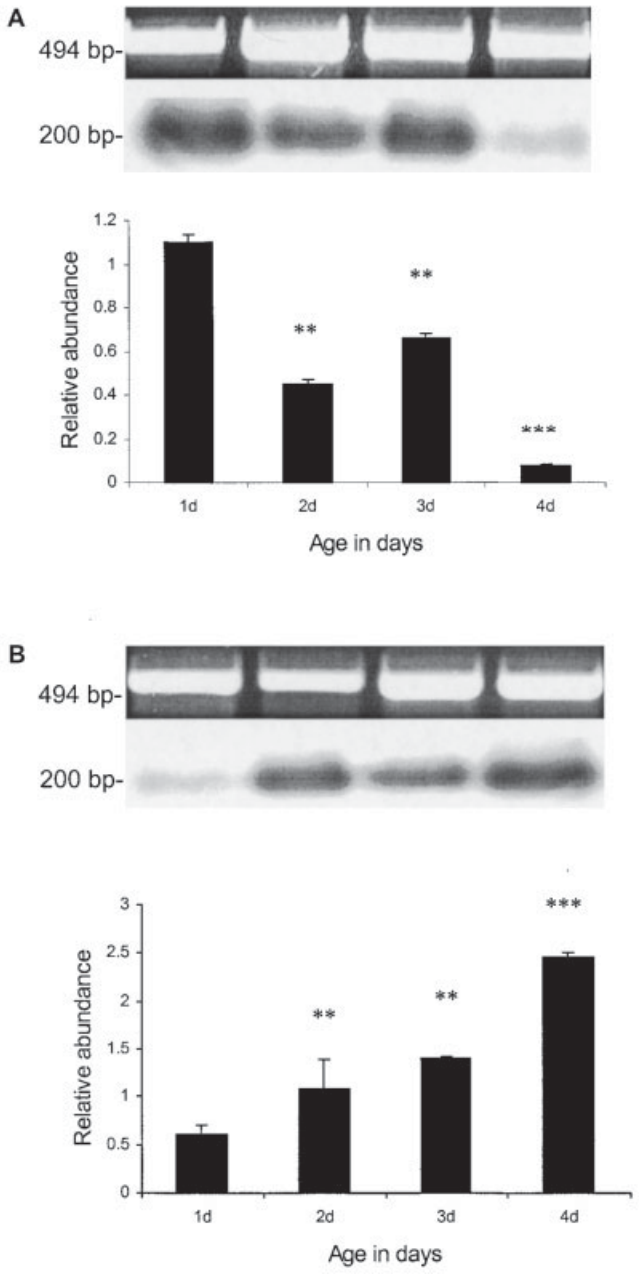

Fig. 5. Expression of Spofr-AST A gene in the brains of 1–4-day-old (1d–4d) adult females (**A**) and males (**B**) as measured by RT-PCR. Other details as in Figure 4. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the respective values for day 1.

a. All except one (Spofr-AST A-9) of the *S. frugiperda* peptides had previously been deduced from the cDNA sequences of two other lepidopteran species (Davey et al., 1999; Secher et al., 2001).

The internal cleavage site in Spofr-AST A-2 is present in the precursor of all examined insect species. It seems to be generally used in locusts, moths (*C. pomonella, H. armigera;* Duve et al., 1997c), and flies, in which either both the large and the shorter, or only the shorter form of AST A-2 were found. On the other hand, only the large version of the homologue was isolated from cockroach brain extracts (Pratt et al., 1991; for review see Stay, 2000), suggesting that the cleavage may not occur in these insects. Recently, however, Predel et al. (1999) demonstrated the occurrence of the cleavage product residues 11–18 of *Periplaneta americana* AST A-2 in the retrocerebral complex as well as in the antennal pulsatile organs of females and males by mass spectrometry. Nevertheless, there seems to be a correlation between the occurrence of the two versions of AST A-2 and its potential activities. The short form is a potent myoinhibitor but does not exert allatostatic activity (Veelaert et al., 1996; Duve et al., 1997c). It consistently occurs in insects whose CA are insensitive to A-type allatostatins. In contrast, the long form of AST A-2 is one of the most potent of all allatostatins in the CA standard cockroach assay (Weaver et al., 1998; Tobe et al., 2000). It represents the main, although not always the single, AST A-2 in the brain of cockroaches and crickets. Spofr AST A-9 represents a peptide structure that differs in one amino acid (Gly → Arg) at position 6 of the respective peptide in *H. armigera* (Davey et al., 1999) and in two amino acids (Glu → Gln at position 1 and Gly → Arg at position 6) from the homologous peptide found in *B. mori* (Secher et al., 2001).

The general organisation of the AST A-type precursor structure (total number of amino acids, number and arrangement of encoded peptides, number and localization of acidic spacers) seems to be conserved within the Order Lepidoptera, although some specific family differences may exist. This results in an 84% amino acid residue identity between the precursor proteins of *S. frugiperda* and *H. armigera* (both Noctuidae) but only a 57% identity between *S. frugiperda* and the silkworm *B. mori* (Bombycidae). Such family-specific differences have already been shown for other major neuropeptide families such as the adipokinetic hormones (see Gäde et al., 1994) and, more recently, for diapause

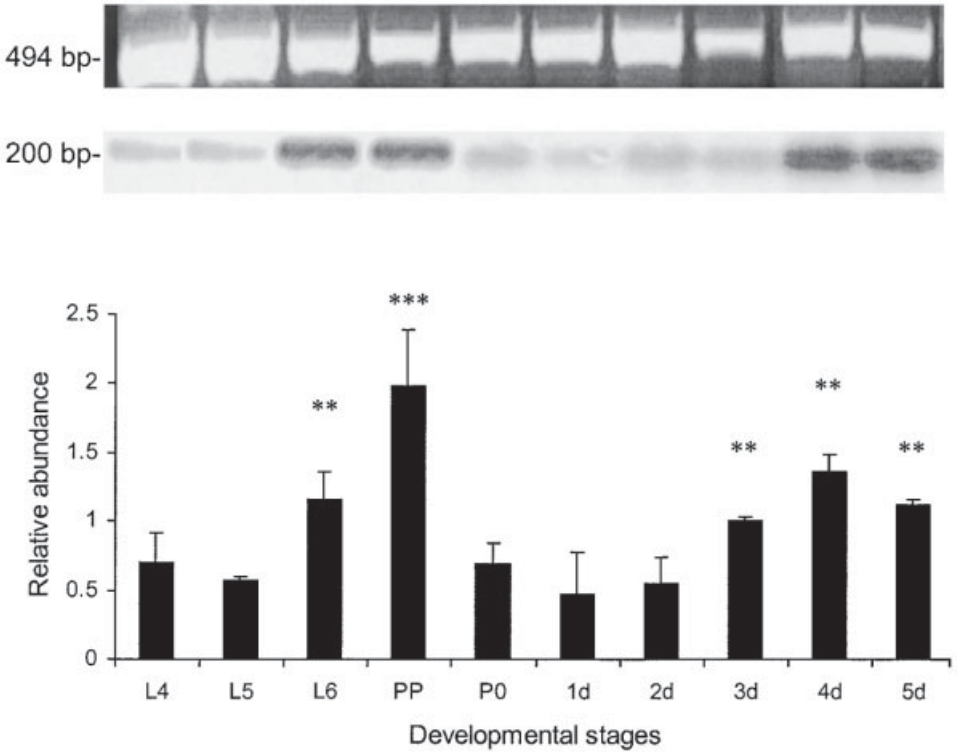

Fig. 6. Expression of Spofr-AST A gene in the midguts of the 4th (L4), 5th (L5), and 6th larval stage (L6), prepupal stage (PP), young pupal stage (P0), and 1–5-day-old (1d–5d) adults of both sexes as measured by RT-PCR. Other details as in Figure 4. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the value for L4.

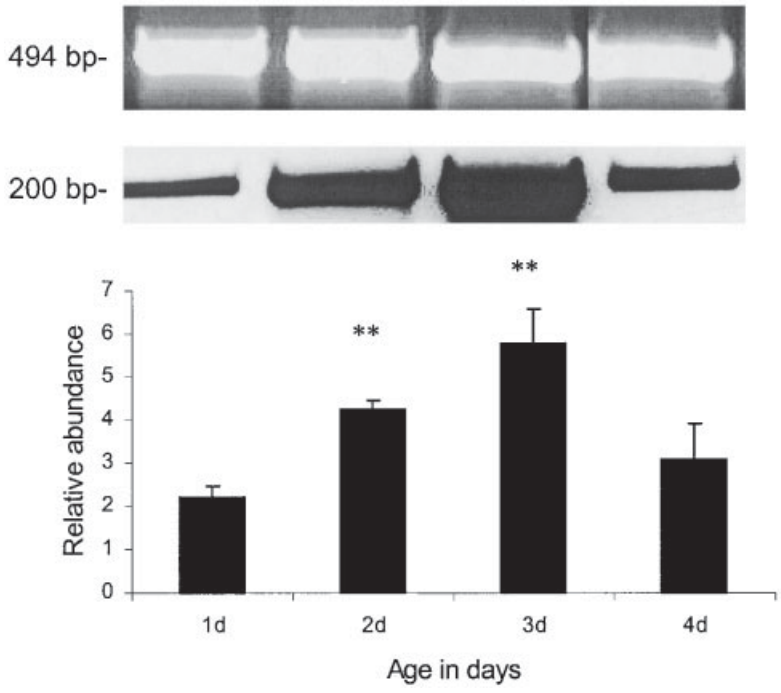

Fig. 7. Expression of Spofr-AST A gene in the ovary of 1–4-day-old (1d–4d) adult unmated females as measured by RT-PCR. Other details as in Figure 4. $^{**}P < 0.01$ compared to the value for day 1.

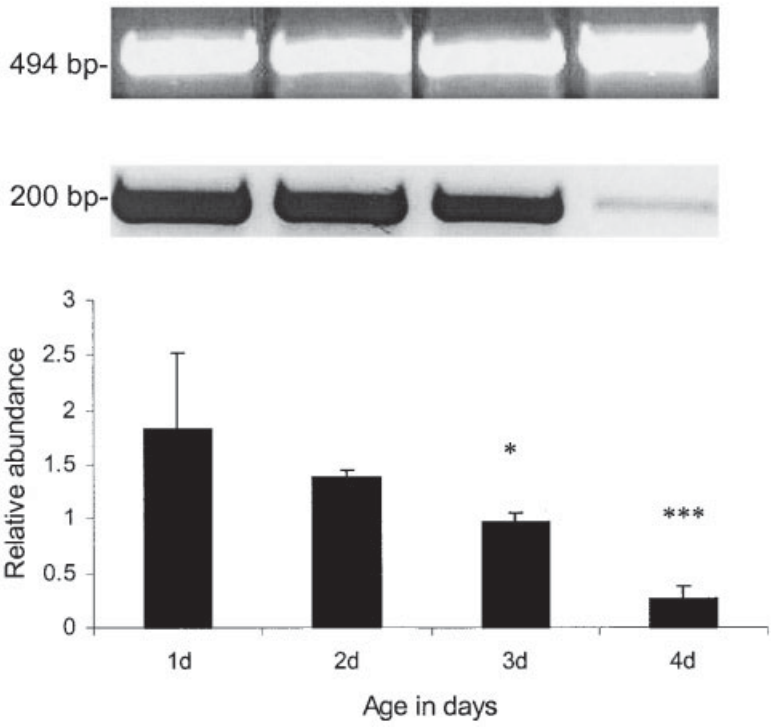

Fig. 8. Expression of Spofr-AST A gene in the testes of 1–4-day-old (1d–4d) adult males as measured by RT-PCR. Other details as in Figure 4. $^{*}P < 0.05$; $^{***}P < 0.001$ compared to the value for day 1.

hormones and pheromone biosynthesis-activating neuropeptides (PBAN) (Iglesias et al., 2002).

The availability of the Spofr-AST A cDNA sequence allowed us to measure mRNA levels in tissues of the fall armyworm for various developmental stages by the semi-quantitative one-step RT-PCR as well as to localize the distribution of the mRNA in brain, gut, and ovary by in situ hybridization. Our results from the one-step RT-PCR analyses indicate that the Spofr-AST A gene is expressed in different tissues of the moth in a developmental- and sex-specific manner. The distinct changes of expression of the gene in brain and midgut suggest a function of the allatostatin A-type peptides as insect

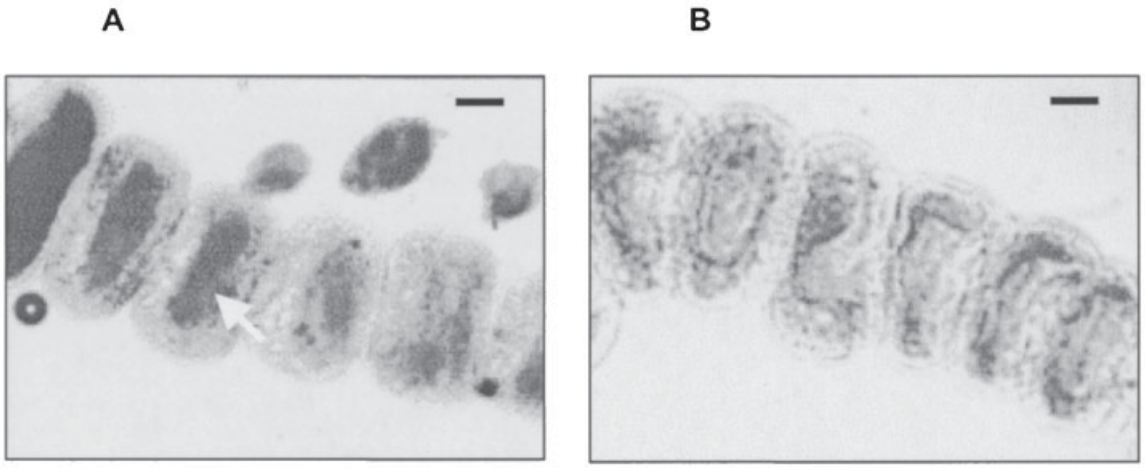

Fig. 9. Detection of Spofr-AST A mRNA in the ovary of a 2-day-old adult unmated female of *S. frugiperda* using whole-mount in situ hybridization. **A:** Photograph of oocytes that contained Spofr-AST A mRNA (arrow). **B:** Control with Spofr-AST A sense RNA probe. Scale bar = 0.5 mm.

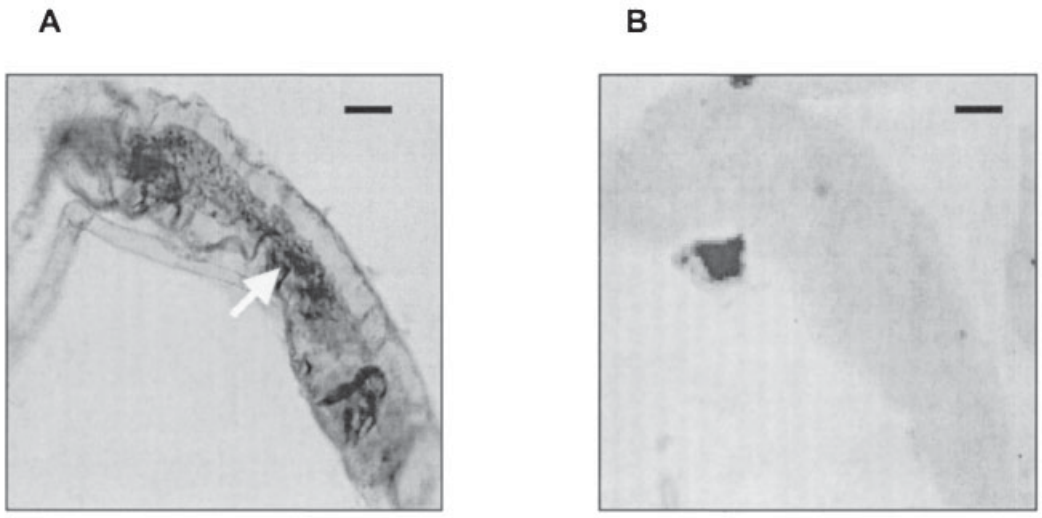

Fig. 10. **A:** Detection of Spofr-AST A mRNA in the oviduct (arrow) of a 2-day-old adult female of *S. frugiperda* using whole-mount in situ hybridization. **B:** Control with Spofr-AST A sense RNA probe. Scale bar = 0.1 mm.

brain/gut myomodulatory hormones. The sex-specific brain expression in adult animals during the first 4 days after eclosion is similar to that found for Spofr-AS (C-type allatostatin) in a previous study (Abdel-latief et al., 2004). However, the physiological meaning of the sex-specific brain expression of Spofr-AST A is not yet clear. We also do not know whether the gene expression in ovaries and testes of adult females and males, respectively, will be related to a regulatory function of the peptides in these tissues. Garside et al. (2002) and Meyering-Vos and Hoffmann (2003) have demonstrated expression of A-type allatostatins in the oviduct of the cockroach *Diploptera punctata* and the cricket *Gryllus bimaculatus*, respectively, but so far only in the locust *Schistocerca gregaria* has a direct effect of allatostatins on oviduct contraction been demonstrated (Schoofs et al., 1997). The Spofr-AST A mRNA localization within the oocytes might suggest a role of the peptides during embryonic development. Transcript localization in the brain and midgut of adult *S. frugiperda* was similar to that described for the helicostatin gene expression in *H. armigera* larvae (Davey et al., 1999).

Knowledge of the allatostatin type-A peptide precursor sequence has opened the way for synthesis of all members of the peptide family in *S. frugiperda* for detailed physiological and functional studies. Other major challenges in the future will be to use the probe for detailed tissue expression studies in context of the life history of the animals by quantitative RT-PCR, to clone the receptor(s) for these peptides, and to study the receptor distribution in *S. frugiperda*.

ACKNOWLEDGMENTS

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LITERATURE CITED

Abdel-latief M, Meyering-Vos M, Hoffmann, KH. 2003. Molecular characterization of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides. Insect Biochem Mol Biol 33:467–476.

Abdel-latief M, Meyering-Vos M, Hoffmann, KH. 2004. Expression and localization of the *Spodoptera frugiperda* allatotropin (Spofr-AT) and allatostatin (Spofr-AS) genes. Arch Insect Biochem Physiol 55:188–199.

Bellés X, Graham L, Bendena WG, Ding Q, Edwards JP, Weaver RJ, Tobe SS. 1999. The molecular evolution of the allatostatin precursor in cockroaches. Peptides 20:11–22.

Bendena WG, Donley BC, Tobe SS. 1999. Allatostatins: a growing family of neuropeptides with structural and functional diversity. Ann NY Acad Sci 897:311–329.

Davey M, Duve H, Thorpe A, East P. 1999. Characterization of the helicostatin peptide precursor gene from *Helicoverpa armigera* (Lepidoptera: Noctuidae). Insect Biochem Mol Biol 29:1119–1127.

Ding Q, Donly BC, Tobe SS, Bendena WG. 1995. Molecular cloning of the gene for the allatostatin family of neuropeptides from the cockroach, *Periplaneta americana*. Eur J Biochem 234:737–746.

Donly BC, Ding Q, Tobe SS, Bendena WG. 1993. Molecular cloning of the gene for allatostatin family of neuropeptides from the cockroach *Diploptera punctata*. Proc Natl Acad Sci USA 90:8807–8811.

Duve H, Johnsen AH, Maestro J-H, Scott AG, Crook N, Winstanley D. 1997a. Identification, tissue localization and physiological effect in vitro of a neuroendocrine peptide identical to a dipteran leu-callatostatin in the codling moth *Cydia pomonella* (Tortricidae: Lepidoptera). Cell Tissue Res 289:73–83.

Duve H, Johnsen, AH, Maestro JL, Scott AG, Jaros PP, Thorpe A. 1997b. Isolation and identification of multiple neuropeptides of the allatostatin superfamily in the shore crab *Carcinus maenas.* Eur J Biochem 250:727–734.

Duve H, Johnsen AH, Maestro JL, Scott AG, Jaros PP, Winstanley D, Davey M, East PB, Thorpe A. 1997c. Lepidopteran peptides of the allatostatin superfamily. Peptides 18:1301–1309.

Duve H, Johnsen AH, Scott AG, Thorpe A. 2002. Allatostatins of the tiger prawn, *Penaeus monodon* (Crustacea: Penaeidea). Peptides 23:1039–1051.

East PD, Tregenza K, Duve H, Thorpe A. 1996. Identification of the dipteran leu-callatostatin peptide family: characterization of the prohormone gene from *Calliphora vomitoria* and *Lucilia cuprina*. Regul Pept 67:1–9.

Eipper BA, Stoffers DA, Mains RE. 1992. The biosynthesis of neuropeptides: peptide-amidation. Annu Rev Neurosci 15:57–85.

Gäde G. 2002. Allatoregulatory peptides: molecules with multiple functions. Invertebr Reprod Dev 41:127–135.

Gäde G, Reynolds SE, Beeching JR. 1994. Phylogenetic aspects of peptides of the AKH-RPCH family. In: Davey KG, Peter RE, Tobe SS, editors. Perspectives in comparative endocrinology. National Research Council of Canada, Ottawa; p 119–128.

Garside CS, Koladish PM, Bendena WG, Tobe SS. 2002. Expression of allatostatin in the oviducts of the cockroach *Diploptera punctata*. Insect Biochem Mol Biol 32:1089–1099.

Hoffmann KH, Meyering-Vos M, Lorenz MW. 1999. Allatostatins and allatotropins: is regulation of corpora allata activity their primary function? Eur J Entomol 96:255–266.

Iglesias F, Marco P, François M-C, Camps F, Fabrias G, Jaquin-Joly G. 2002. A new member of the PBAN family in *Spodoptera littoralis*: molecular cloning and immunovisualization in scotophase hemolymph. Insect Biochem Mol Biol 32:901–908.

Jansons IS, Cusson M, McNeil JW, Tobe SS, Bendena WG. 1996. Molecular characterisation of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). Insect Biochem Mol Biol 26:767–773.

Kramer SJ, Toschi A, Miller CA, Kataoka H, Quistad GB, Li JP, Carney RL, Schooley DA. 1991. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. Proc Natl Acad Sci USA 88:9458–9462.

Lenz C, Williamson M, Grimmelikhuijzen CJP. 2000. Molecular cloning and genomic organization of an allatostatin preprohormone from *Drosophila melanogaster*. Biochem Biophys Res Commun 273:1126–1131.

Lorenz JH, Lorenz MW, Hoffmann KH. 1997. Factors regulating juvenile hormone and ecdysteroid biosynthesis in *Gryllus bimaculatus* (Ensifera: Gryllidae). Eur J Entomol 94:369–379.

Meyering-Vos M, Hoffmann KH. 2003. Expression of allatostatins in the Mediterranean field cricket, *Gryllus bimaculatus* (Ensifera, Gryllidae). Comp Biochem Physiol B 136:207–215.

Meyering-Vos M, Wu X, Huang J, Jindra M, Hoffmann KH, Sehnal F. 2001. The allatostatin gene of the cricket *Gryllus bimaculatus* (Ensifera:Gryllidae). Mol Cell Endocrinol 184:103–114.

Notredame C, Higgins D, Heringa J. 2000. T-Coffee: a novel method for multiple sequence alignments. J Mol Biol 302:205–217.

Pratt GE, Farnsworth DE, Fok KF, Siegel NR, McCormack AL, Shabanowitz J, Hunt DS, Feyereisen R. 1991. Identity of a second type of allatostatin from cockroach brains: an octadecapeptide amide with a tyrosine-rich address sequence. Proc Natl Acad Sci USA 88:2412–2416.

Predel R, Kellner R, Rapus J, Gäde G. 1999. Allatostatins from the retrocerebral complex and antennal pulsatile organ of the American cockroach: structural elucidation aided by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Regul Pept 82:81–89.

Schoofs L, Veelaert D, Vanden Broeck J, De Loof A. 1997. Peptides in the locusts *Locusta migratoria* and *Schistocerca gregaria*. Peptides 18:145–156.

Secher T, Lenz C, Cazzamali G, Sorensen G, Williamson M, Hansen GN, Svane P, Grimmelikhuijzen CJP. 2001. Molecular cloning of the functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. J Biol Chem 276:47052–47060.

Stay B. 2000. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem Mol Biol 30:653–662.

Tobe SS. 1980. Regulation of the corpora allata in the adult female insects. In: Locke M, Smith DS, editors. Insect biology in the future. New York: Academic Press. p 345–367.

Tobe SS, Zhang JR, Bowser PRF, Donly PBC, Bendena WG. 2000. Biological activities of the allatostatin family of peptides in the cockroach *Diploptera punctata*, and potential interactions with receptors. J Insect Physiol 46:231–242.

Vanden Broeck J, Veelaert D, Bendena WG, Tobe SS, De Loof A. 1996. Molecular cloning of the precursor cDNA for schistostatins, locust allatostatin-like peptides with myoinhibiting properties. Mol Cell Endocrinol 122:191–198.

Veelaert D, Devreese B, Schoofs L, Van Beeumen J, Vanden Broeck J, Tobe SS, De Loof A. 1996. Isolation and characterization of eight major inhibiting peptides from the desert locust, *Schistocerca gregaria*: new members of the cockroach allatostatin family. Mol Cell Endocrinol 122:183–190.

Veenstra JA, Noriega FG, Graf R, Feyereisen R. 1997. Identification of three allatostatins and their cDNA from the mosquito *Aedes aegypti*. Peptides 18:937–942.

Von Heijne G. 1986. A new method for predicting signal sequence cleavage sites. Nucl Acids Res 14:4683–4690.

Weaver RJ, Edwards JP, Bendena WG, Tobe SS. 1998. Structures, functions and occurrence of insect allatostatic peptides. In: Coast GM, Webster SG, editors. Recent advances in arthropod endocrinology. Cambridge: Cambridge University Press. p 3–32.

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Characterization of a novel peptide with allatotropic activity in the fall armyworm *Spodoptera frugiperda*☆

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Abstract

A cDNA that encodes 53 amino acids, including one copy of the RVRGNPISCF–OH peptide, was cloned from *Spodoptera frugiperda*. This peptide strongly stimulates the synthesis and release of juvenile hormone (JH) in vitro by the corpora allata (CA) of *S. frugiperda* and was code-named Spofr-AT 2. Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR) analyses revealed that the preprohormone is expressed as one transcript in the brain, midgut (Mg) and ovary (Ov) in a tissue- and developmental-specific manner. Whole-mount in situ hybridization confirmed the gene expression in the suboesophageal ganglion (SOG) and in the ovary of adult females. Treating the CA with the synthetic peptide caused an up to tenfold increase in the release of JH. The stimulation was dose-dependent with an apparent EC_{50} of ca. 10^{-7} M. CA that were activated with Spofr-AT 2 could be inhibited by the addition of synthetic allatostatin type-C from *Manduca sexta* (Manse-AS). This is the first report on the presence and function of two different peptides with allatotropic activity in an insect species.

Keywords: Neuropeptide; Allatotropin; Corpora allata; Juvenile hormone biosynthesis; Gene expression; RT-PCR; In situ hybridization

1. Introduction

Juvenile hormones (JHs) are unique hormones, sesquiterpenoids, which play a crucial role in almost every aspect of insect development and reproduction. The biosynthesis of JH by the corpora allata (CA) can be either stimulated or inhibited by neuropeptides termed allatotropin (AT) or allatostatin (AS), respectively (reviewed in Refs. [1–4]). Allatostatins are structurally diverse peptides which can be divided into three groups. These families are now designated type-A allatostatins (Y/FXFGL/I-amides; cockroach type; allatostatin superfamily), type-B allatostatins [W(X_6)W-amides; cricket type], and type-C allatostatin(s) (*Manduca sexta*-type). Their allatoregulating functions seem to be restricted to a limited number of species, but all three types of peptides appear to be myoinhibiting on visceral muscles in a number of insects. The type-C allatostatin group is represented by only one peptide, called Manse-AS (pEVRFRQCYFNPISCF–OH), at first isolated from the tobacco hornworm, *M. sexta* [5]. Genes encoding the type-C allatostatin have been identified in the moths *Pseudaletia unipuncta* [6], *Spodoptera frugiperda* [7] and in *Drosophila melanogaster* (Drome-AS) [8]. Drome-AS differs in one amino acid residue from Manse-AS (F → Y in position 4).

To date, only one allatotropin (Manse-AT; GFKNVEMMTARGF $-NH_2$) is known to stimulate the CA. It was first isolated from the head of pharate adults of *M. sexta* [9], for which the gene encoding the peptide has also been cloned [10]. The gene is expressed in three mRNA isoforms that differ from each other by alternative splicing [11]. Manse-AT has also been isolated from larval and adult brains of *Lacanobia oleracea* [12] and from brain extracts of adult *S. frugiperda* [13]. The gene encoding Manse-AT has been cloned from *S. frugiperda* [7], *Bombyx mori* [14], *P. unipuncta* [15] and from *Agrius convolvuli* (Kataoka, unpublished). An allatotropin immunoreactive peptide has been isolated from the mosquito *Aedes aegypti* and its structure was determined as APFRNSEMMTARGF–NH_2. Further-

☆ The nucleotide sequence data published here have been submitted to EMBL Nucleotide Sequence Data Base, accession no. AJ566903.

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more, the cDNA encoding this peptide was identified [16]. No allatotropin precursor has been found in the *Drosophila* genome [17,18]. Manse-AT was shown to stimulate JH biosynthesis in the CA of various lepidopteran species, but it seems that Manse-AT and/or AT-related peptides may have functions other than regulation of JH biosynthesis in some insects [19] (for review, see Ref. [20]).

Here we report the identification of a novel neuropeptide with allatotropic activity from the brain of the fall armyworm, *S. frugiperda*, by molecular techniques. One-step reverse transcriptase polymerase chain reaction (RT-PCR) and Northern blotting revealed that the gene is expressed as one transcript in brains of larvae and adults, but also in the midgut (Mg) and the ovary (Ov) of adult females. To elucidate its biological activity, the synthetic peptide was tested in a radiochemical assay for measuring CA activity in vitro.

2. Materials and methods

2.1. Insects

Pupae and eggs of *S. frugiperda* were provided by Bayer CropScience AG (Leverkusen) and were reared at 27 °C and 70% relative humidity under a L 16:D 8 photoperiod as previously described [7].

2.2. RNA and mRNA extractions

Brains of 4th (L4), 5th (L5) and 6th instar larvae (L6; <12 h after the respective moult), prepupae (PP), young pupae (P0), 10-day-old pupae (P10) and 0- to 5-day-old adult females, as well as midguts and ovaries of 0- to 5-day-old females were dissected, frozen immediately in liquid nitrogen and were stored at −80 °C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen) and was quantified by spectrophotometric determination.

2.3. Genomic DNA extraction

To isolate genomic DNA, individual *S. frugiperda* adult females were frozen and ground, under liquid nitrogen, to a fine powder using mortar and pestle. The powder was resuspended and the genomic DNA was extracted following the instructions of QIAamp® DNA Mini kit (Qiagen).

2.4. Cloning of the cDNA by RACE and genomic DNA

The Smart™ RACE cDNA amplification kit (Clontech) was used to obtain the full length of the cDNA using 1-μg mRNA of the brain which was added as a template for each RACE reaction. Primers for 5′–RACE were the degenerate oligonucleotide primer (d.AT2r1; 5′–YAY TTY CTR AAR CAN GAD ATN G–3′), and the specific primer AT2r2 corresponding to nucleotide positions 140–161 in Fig. 2. Primers for 3′–RACE were AT2f1 and AT2f3 corresponding to nucleotide positions 132–156 and 94–115, respectively. Primers were used in combination with the universal primer (Upm) 5′–CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT–3′ which recognizes the primer binding sequence, introduced by the Smart RACE kit components. All the RACE PCR reactions were performed in the presence of 1 μl of the RACE reaction as a template and a final concentration of 0.2 μM of each primer. An amount, 100 ng, of genomic DNA were used as a template in a reaction volume of 50 μl, combined with 0.2 μM specific primers AT2f1 or AT2f2, corresponding to nucleotide positions 132–156 and 22–40, respectively, and the specific primer AT2r3, corresponding to nucleotide positions 199–226, to amplify the encoded region of the peptide. The PCR program used for amplification of the products was 95 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 68 °C for 45 s decreased by 1 °C/cycle, followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s, 68 °C for 1.5 min and a final extension step of 68 °C for 10 min. The PCR products were subjected to electrophoresis on 1.5% low melting point agarose gel (Biozym), eluted with GFX™ purification kit (Pharmacia), and ligated into plasmids with the pGEM-T easy system kit supplied with *Escherichia coli* JM109 (Promega) for transformation. Plasmid DNA was purified using the QIAprep® Spin Miniprep kit (Qiagen). The templates were sequenced by

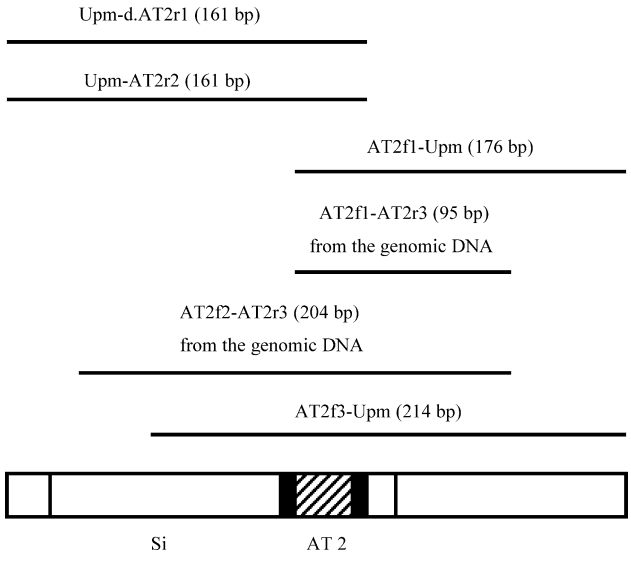

Fig. 1. Sequencing strategy of the Spofr-AT 2 cDNA. Horizontal lines represent the individual clones used for sequencing. The primers and the length (bp) of the fragments are given above the lines. The lower part of the graph represents the structural organization of the Spofr-AT 2 preprohormone. The position of the peptide (AT 2) is marked with diagonal lines and the untranslated region, the signal peptide (Si) and the poly A-tail are represented by white boxes. Sites of possible endoproteolytic cleavage are indicated by black boxes.

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5′TAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTC  51
                  M  H  P  T  R  W  E  L  S  H  M  V   12
GACCTGCAGGCGGCCGCGAATTCACTAGTGATTCTAATACGACTCACTATA 102
 D  L  Q  A  A  A  N  S  L  V  I  L  I  R  L  T  I   29
GGGCAAGCAGTGGTAAAACGCAGAGTACGCGGGAACCCAATTTCCTGCTTC 153
 G  Q  A↓ V  V  K  R  R  V  R  G  N  P  I  S  C  F   46
CGCAAGAAGAAGCTGAAGGATTAAATTATTCTATTTCTTTCAATTGCATAG 204
 R  K  K  K  L  K  D ---                              53
CATTACCAATTTACCATCTTACATTTTGCTATTCATTTAATTAAGATGACA 255

AAATTAATAAAAATGTAACAGCCAAAAAAAAAAAAAAAAAAAAAAAAAAA 306
A3′                                                   307
```

Fig. 2. The nucleotide and the deduced amino acid sequences of the Spofr-AT 2 cDNA. The sequences are numbered at the right. The Spofr-AT 2 amino acid sequence is shown in bold type. Potential cleavage sites are in boxes. The polyadenylation signal is shown in bold type and is underlined—represents the stop code signal. (↓) A possible signal peptide cleavage site.

GATC (Konstanz). Sequences were analysed with the GCG software (Wisconsin Package).

2.5. One-step RT-PCR analysis

For RT-PCR, 20 ng mRNA from each of the tissues were used with the specific primers AT2f1 and AT2r3 in 10 μl of the RT-PCR mixture (TITANIUM® One-Step RT-PCR Kit, Clontech). The PCR program for the amplification of the fragment was as previously described [7]. The DNA products were analyzed using 1.5% agarose gel at 8 V cm^{-1} for 1.5 h, transferred to Hybond–N^+ membrane (Amersham) and hybridized to the probe (AT2f1–AT2r3) 95 bp, labeled by the PCR method with Dig dUTP (Roche). As a control, the same quantities (20 ng) of mRNA from different developmental stages were used as a template in combination with two specific primers ACTf1 corresponding to nucleotide positions 1–25 and ACTr2 corresponding to nucleotide positions 469–493 from the nucleotide sequence of *Spodoptera littoralis* mRNA for beta-actin (partial; EMBL Nucleotide Sequence Data Base, accession no. Z46873), which yields a 494-bp amplified fragment. Expression of the *Spofr-AT 2* gene was normalized relative to that of beta-actin. Negative controls for genomic DNA were done without the reverse transcriptase step [7]. Densitometry was performed using an Image Master® VDS (Pharmacia) and the Image Master 1D Database software.

2.6. Synthesis of Dig-RNA probes

Antisense and sense Spofr-AT 2 probes were synthesized from the cDNA clones AT2f1–AT2r3 (95 bp) for the

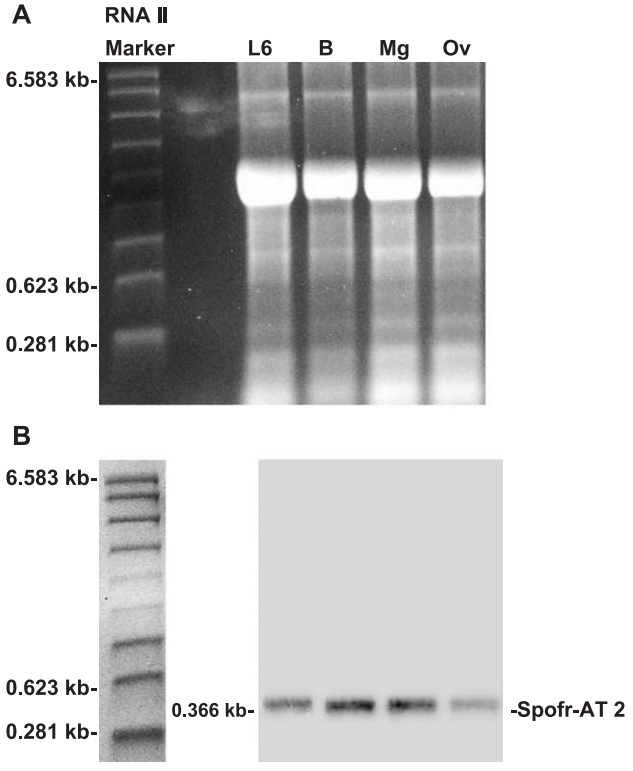

Fig. 3. Northern blot hybridization of the brains of 6th instar larvae (L6) and 2-day-old adult females (B), and of midguts (Mg) and ovaries (Ov) of 2-day-old females of *S. frugiperda*. (A) Agarose gel separation of the mRNAs in the presence of 6% formaldehyde. Each lane contains about 20 μg mRNA from each tissue (corresponding to 300 brains, 30 midguts and 10 ovaries, respectively). RNA marker II is shown on the left lane (kb). (B) Northern blotting using the digoxigenin antisense (AT2f2–AT2r3) RNA probe. The transcript size (0.366 kb) is given at the left.

whole-mount in situ hybridization experiments and the antisense probe from the clone AT2f2–AT2r3 (204 bp) for the Northern blot. A total of 1 μg from each plasmid type was linearized with *ApaI* restriction enzyme (about 30 units μg^{-1}; Promega) in a 20-μl volume to yield templates for the antisense and the sense probes, respectively. The templates were purified by phenol/chloroform/isoamyl alcohol extraction and then ethanol precipitated. The pellet was air-dried and adjusted to a concentration of 1 μg μl^{-1} in DEPC H_2O. A total of 1-μg linearized DNA plasmid was transcribed and a label was introduced with the Dig-RNA labelling kit according to the manufacturer protocol (Roche Diagnostics). The RNA probes were precipitated with ethanol, dried in a speed vac concentrator, resuspended in 20–50 μl DEPC H_2O and was stored at −80 °C until use.

2.7. Northern analysis

Total RNA was isolated from selected tissues and 20 μg mRNA samples were separated on a 1% agarose gel system containing 6% formaldehyde in the presence of RNA Marker II (Roche). The RNA was transferred to a positively charged nylon membrane (Roche) prior to hybridization at 60 °C with a digoxigenin-labelled antisense RNA probe of 204 bp (see above), followed by detection according to the instructions of the Dig-RNA labelling kit (Roche).

2.8. Whole-mount in situ hybridization

Brain, midgut and ovaries were dissected from 2-day-old adult females and were immediately fixed as previously described [21]. Whole-mount in situ hybridization was performed as described [22] with the digoxigenin-labelled antisense AT2f1–AT2r3 (95 bp) probe. Tissues were observed using a Leitz Diaplan microscope with a Wild Leitz MPS 46 photoautomate. The resulting Kodak Elite CHROME 200 film negatives were scanned with a HP Scan-Jet 5470C. Controls with the sense–strand Dig-RNA probes were processed identically to the experimental tissues.

2.9. Synthetic peptides

The Spofr-AT 2 peptide (RVRGNPISCF–OH) was custom-synthesized by Thermo Hybaid (Ulm). Synthetic Manse-AT was purchased from Bachem (Bubendorf) and Manse-AS was a generous gift of Bayer (Leverkusen). All peptides were assayed as 97% pure by analytical reversed-phase HPLC and mass spectroscopy.

2.10. Radiochemical assay for allatoregulating activity

Rates of JH biosynthesis and release from the single CA, dissected from 2- to 3-day-old adult females were measured

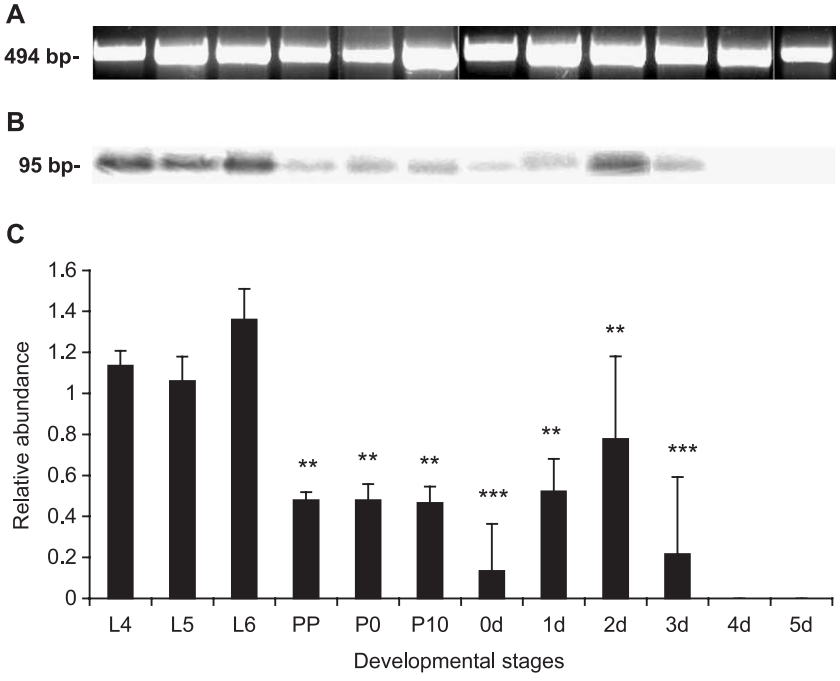

Fig. 4. Expression of the *Spofr-AT 2* gene in brains of *S. frugiperda* as measured by RT-PCR. (A) Representatives of agarose gel separation of beta-actin control (494 bp) stained with ethidium bromide using 20 ng mRNA as a template in one-step RT-PCR. (B) Representatives of blotting results for the expression of the *Spofr-AT 2* gene in the brains of 4th (L4), 5th (L5) and 6th instar larvae (L6), prepupae (PP), young pupae (P0) and 10-day-old pupae (P10) and in the brains of 0- to 5-day-old adult females (0d to 5d). (C) Densitometric quantification of the signals relative to that of beta-actin. Mean values of 2–3 determinations ± S.D. ** $p < 0.01$; *** $p < 0.001$ compared to the value for L4.

according to the rapid partition assay [23] with some modifications [13,24]: TC 199 incubation medium (Sigma) with Hanks' salts and sodium bicarbonate, without L-glutamine, buffered with 25 mM HEPES, supplemented with $CaCl_2$, to a final concentration of 10 mM, and NaCl, to a final concentration of 120 mM, and fortified with 1% Ficoll 400, was adjusted to pH 6.8 and sterilized by suction through a 0.2-μm filter. [1-^{14}C]Acetic acid, sodium salt (Moravek; initial specific activity 2.04 GBq mmol^{-1}) was used as the radiolabelled precursor. The final specific activity of the radiolabelled precursor was set to 0.59 GBq mmol^{-1} (final acetate concentration ca. 0.6 mM). Following preincubation in radioactive medium for 2 h, the CA were incubated in the presence or absence (controls) of synthetic peptides for 2 h in the dark with gentle shaking at 27 °C. After this time, the CA were removed and the medium was analysed for JHs and diols [24]. In another set of experiments, following preincubation, the CA were subjected to two successive incubations (each for 2 h) in the presence or absence (control I, 2–4 h; control II, 4–6 h) of peptides, according to the details in the figure legends. Media following the first and/or second incubation were analysed. Because in recent years problems have arisen resulting from contaminated [*methyl*-^{3}H]- methionine (high blank values) or inaccurate specific radioactivity [25], we decided to use [^{14}C]-acetic acid, sodium salt as radioactive precursor for the radiochemical assay (RCA).

2.11. Statistical analysis

All values are means ± S.D. or S.E.M. of measurements indicated in the figure legends. The Mann–Whitney *U*-test or the Wilcoxon-test were used in the statistical treatment of the data.

3. Results

3.1. The coding sequence of the Spofr-AT 2 gene

The degenerate oligonucleotide primer d.AT2r1 was originally synthesized to amplify the cDNA for the *S. frugiperda* type-C allatostatin, as published in a previous paper [7]. Using this primer in combination with the Upm primer and the 5′–RACE reaction as a template yielded a product of 161 bp that proved to contain a core region of the gene for the novel peptide. It included the untranslated region followed by a probable signal peptide and one copy of the peptide (Spofr-AT 2; Fig. 1). Using this fragment, primers were designed to amplify the cDNA 5′ and 3′ ends, respectively. In a PCR reaction, using specific primer AT2r2 in combination with Upm primer and 5′–RACE cDNA as a template, we were able to amplify the same fragment of 161 bp (Fig. 1). This fragment was used to design different specific primers to amplify the 3′ end of the Spofr-AT 2 cDNA. When the specific primer AT2f1 was used in a PCR reaction, with Upm primer and the 3′ RACE reaction as a template, a product of 176 bp was yielded which included the Spofr-AT 2 peptide, the stop codon signal, the polyadenylation signal and the downstream poly A-tail (Figs. 1 and 2). Using the genomic DNA of *S. frugiperda* as a template in a PCR reaction in combination with primers AT2f1–AT2r3 and AT2f2–AT2r3 yielded fragments of 95 bp and 204 bp, respectively, which also included one copy of the Spofr-AT 2 peptide. Finally, using the specific primer AT2f3 in combination with Upm primer and the 3′–

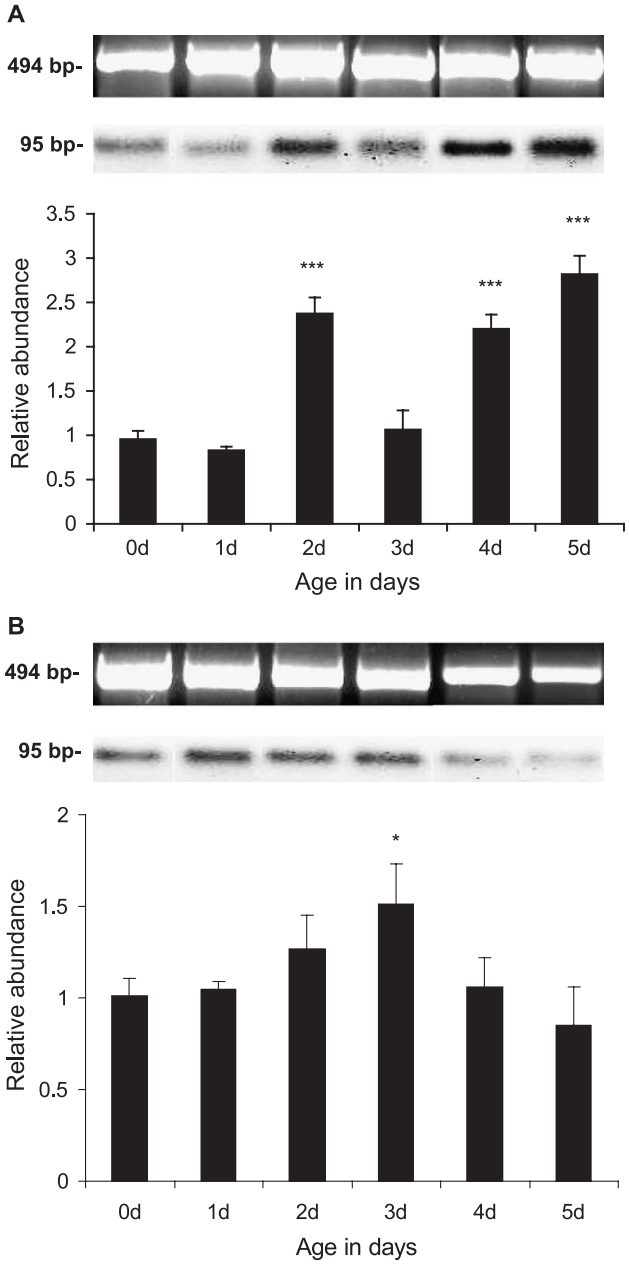

Fig. 5. Expression of the *Spofr-AT 2* gene in midgut and ovary of *S. frugiperda* as measured by RT-PCR. (A) Gene expression in the midgut of 0- to 5-day-old females. (B) Gene expression in the ovary of 0- to 5-day-old females. Other details as in Fig. 4. Mean values of 2–3 determinations ± S.D. * $p < 0.05$; *** $p < 0.001$ compared to the respective values for day 0.

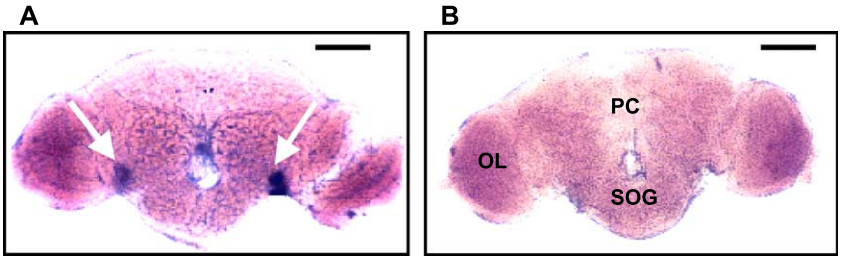

Fig. 6. Whole-mount in situ hybridization of the brain from a 2-day-old adult female of *S. frugiperda* using a RNA probe corresponding to AT2f1–AT2r3 (see Materials and methods). (A) Frontal view of the brain showing two large clusters of cells in the anterior–lateral region of the suboesophageal ganglion which showed strong expression with the antisense RNA probe. (B) Control with the sense RNA probe. OL, optic lobes; PC, protocerebrum; SOG, suboesophageal ganglion. Arrows indicate the expression sites. Scale bar 10 μm applies to A and B.

RACE reaction as a template in a PCR reaction, a product of 214 bp was amplified which included a part of the signal peptide, the Spofr-AT 2 peptide, the stop codon signal, the polyadenylation signal and the downstream poly A-tail (Figs. 1 and 2).

Combination of all our data yielded a cDNA sequence consisting of 307 nucleotides (Fig. 2). It contains a 5′ untranslated region of 15 nucleotides upstream of an open reading frame of 53 amino acids. The assigned initiation codon is located at nucleotide positions 16–18. Within the amino-terminus of the precursor, a single-potential signal-peptide cleavage site downstream Ala^{32} was found [26]. The open reading frame is followed by a 130-nucleotide 3′ untranslated region including the poly A-tail. A consensus polyadenylation signal (AATAAA) was found after position 260, which is 12 nucleotides upstream from the poly A-tail. The neuropeptide precursor contains one copy of a novel decapeptide sequence RVRGNPISCF–OH (Spofr-AT 2). The peptide is located between amino acid residues Arg^{36} and Arg^{47} and is flanked by potential Lys–Arg and Arg–Lys endoproteolytic cleavage sites. The amino acid sequence of the peptide is identical at its C-terminus (–NPISCF) with the *M. sexta* type-C allatostatin (Manse-AS).

3.2. Northern blot

Northern blot analysis demonstrated that the gene is expressed in only one transcript of ca. 0.366 kb in the brains of 6th instar larvae and of 2-day-old adult females and in the midgut and ovary of 2-day-old females (Fig. 3). Expression was strong in brain and midgut of adult females but weaker in larval brains and in the ovary of adult females.

3.3. RT-PCR analyses

Semiquantitative one-step RT-PCR analyses confirmed the expression of the gene as demonstrated by Northern blotting. A clear expression signal was found in the brains of 4th (L4), 5th (L5) and 6th instar larvae (L6), and in prepupae (PP), young pupae (P0), 10-day-old pupae (P10) and 0- to 3-day-old adult females (Fig. 4). The gene signal was undetectable in the brains of 4- and 5-day-old adult females when using 20 ng mRNA as a template. Furthermore, no expression signal could be seen for the brains of 0- to 5-day-old adult males (not shown). A distinct gene expression was found in the midgut (Fig. 5A) and in the ovary (Fig. 5B) of the females during the first 5 days of adult lifespan, but no signal could be detected in the midgut of the preimaginal stages and of adult males (not shown). No fragment was visualized in the controls without reverse transcriptase reaction. A beta-actin 494 bp standard fragment could be visualized with 20 ng mRNA from each of the developmental stages in both sexes (Figs. 4 and 5A,B).

3.4. Whole-mount in situ hybridization

In situ hybridization using digoxigenin-labelled RNA probe was carried out with whole mounts of the brain,

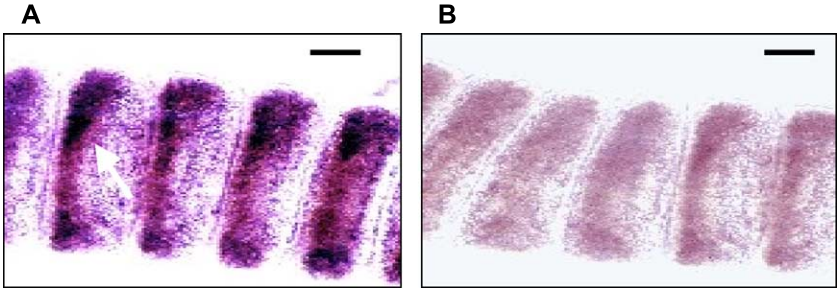

Fig. 7. Detection of Spofr-AT 2 mRNA in the ovary of a 2-day-old adult virgin female of *S. frugiperda* using whole-mount in situ hybridization. (A) Photograph of an ovariole which contains the Spofr-AT 2 mRNA in the oocytes (arrow). (B) Control with the sense RNA probe. Scale bar 0.5 mm applies to A and B.

midgut (not shown) and of the ovary. In *S. frugiperda* females, the suboesophageal ganglion (SOG) is fused to the ventral sides of the brain. Fig. 6A shows two large clusters of cells in the anterior–lateral region of the SOG, which displayed strong expression of the prohormone mRNA. When using the sense probe, no expression signal was found (Fig. 6B). The ovarioles in *S. frugiperda* females are of the polytrophic type. In the ovary of a 2-day-old adult female, the *Spofr-AT 2* gene transcript was localized within the oocytes (Fig. 7A). Using the sense probe did not reveal a positive reaction in the ovary (Fig. 7B).

3.5. The biological activity of the synthetic peptide

According to the C-terminal hexapeptide sequence—NPISCF—which the novel peptide shares with the *M. sexta* type-C allatostatin, we expected an allatostatic activity when incubating the CA from adult females of *S. frugiperda* in the presence of the peptide. Unexpectedly, however, when the CA from 2- to 3-day-old adult females (at that age of females, we have measured high rates of JH release from the CA in a previous study Ref. [24]), after a 2-h preincubation in medium without peptide, were transferred into medium containing the synthetic peptide at concentrations ranging from 10^{-8} to 10^{-4} M, a strong and dose-dependent allatotropic effect was observed (Fig. 8A). The peptide caused an approximate tenfold rise in JH release at a concentration of 10^{-4} M, from about 4 pmol h^{-1} CA^{-1} in the control to ca. 40 pmol h^{-1} CA^{-1}. From the dose–response graph, the apparent EC_{50} for its allatotropic activity was calculated to be ca. 10^{-7} M. Due to its strong allatotropic activity, we code-named the novel peptide *S. frugiperda* allatotropin 2 (Spofr-AT 2).

Results from further experiments on the mechanism of action of Spofr-AT 2 on the CA from 2-day-old females are presented in Fig. 8B, C and D. At a concentration of 10^{-6} M, the allatotropic effect of Spofr-AT 2 was slightly higher than that of Manse-AT (4.4- to 3.1-fold; $p < 0.05$), whereas Manse-AS at the same concentration did not affect the rate of JH release by the CA (Fig. 8B). In other set of experiments, following the preincubation, the CA were subjected

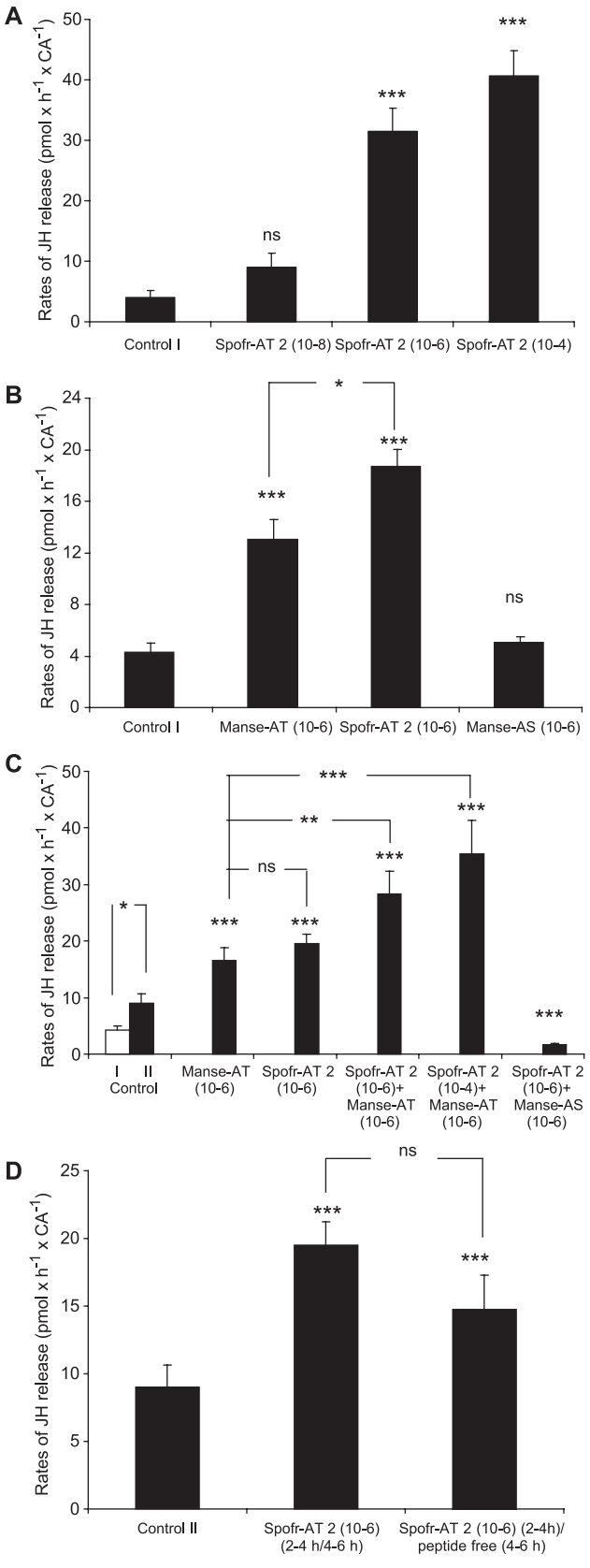

Fig. 8. Biological activity (in vitro) of the synthetic peptide. (A) Dose–response for stimulation of the CA (rates of JH release in pmol × h^{-1} × CA^{-1}) from 2- to 3-day-old females of *S. frugiperda* by Spofr-AT 2 during a 2-h incubation period. Mean values ± S.E.M. of 20 determinations. ns, not significant; *** $p < 0.001$ (different from control I). (B) Effects of Manse-AT, Spofr-AT 2 and Manse-AS (in 10^{-6} M concentration) on JH release from the CA of 2-day-old females of *S. frugiperda* during a 2-h incubation period. Mean values ± S.E.M. of 20 determinations. ns, not significant; * $p < 0.05$; *** $p < 0.001$ (different from control I). (C) Effects of Manse-AT, Spofr-AT 2, Manse-AS and mixtures of peptides during a second 2-h incubation period (4–6 h). Control I represents JH release during the first 2-h incubation (2–4 h) in the absence of peptide, control II represents JH release during the second 2-h incubation (4–6 h) in the absence of peptide. Mean values ± S.E.M. of 20 determinations. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (for black columns different from control II). (D) Reversibility of activation of JH release by Spofr-AT 2 for CA from 2-day-old females. The second incubation was carried out in peptide-free medium. Mean values ± S.E.M. of 20 determinations. ns, not significant; *** $p < 0.001$ (different from control II).

to two successive incubations, each for 2 h. Control CA (control II; without peptide in the medium) increased their rates of JH release to about twice of that during the first 2-h incubation (control I; $p<0.05$; Fig. 8C). Such "higher active" glands were further activated by the addition of 10^{-6} M Manse-AT (1.9-fold) and Spofr-AT 2 (2.2-fold), respectively ($p<0.001$). Simultaneous addition of Spofr-AT 2 (10^{-6} or 10^{-4} M) and Manse-AT (10^{-6} M) during the second incubation resulted in a cumulative effect—the glands released significantly more JH ($p<0.01$ and $p<0.001$, respectively) than when incubated in the presence of only one of the peptides. When CA were incubated in medium containing Spofr-AT 2 and Manse-AS (10^{-6} M each), JH release was even lower than in the untreated controls ($p<0.001$). This indicates that the stimulating effect of Spofr-AT 2 was completely abolished by the addition of Manse-AS. Glands that had been activated with 10^{-6} M Spofr-AT 2 in the first incubation and then transferred to peptide-free medium for the second 2-h incubation still produced and released significantly more JH ($p<0.001$) than the untreated controls (Fig. 8D).

4. Discussion

Brain allatotropic control of JH biosynthesis was observed in adults of the migratory locust *Locusta migratoria* [27–30], larvae of the greater wax moth *Galleria mellonella* [31], adults of the firebug *Pyrrhocoris apterus* [32] and female adults of the fly *Phormia regina* [33]. Allatotropic control of JH biosynthesis by the suboesophageal ganglion (SOG) was first observed in the cockroach *Leucophaea maderae* [34]. Allatotropic activity was also reported to be present in the SOG of crickets (*Gryllus bimaculatus* and *Acheta domesticus* Ref. [35]) and the adult male Loreyi leafworm, *Mythimna loreyi* [36]. In addition to the brain and SOG, ovarian factors were considered as potential stimulatory regulators [37,38]. So far, however, none of these allatotropic factors has been identified. Recently [39], it was shown that the *Drosophila* sex peptide, a 36 amino acid residue peptide produced in the male accessory glands [40] can stimulate the biosynthesis of JH, in vitro, by the CA of *D. melanogaster* and of the noctuid moth *Helicoverpa armigera*. The only known authentic allatotropin, Manse-AT, activates JH biosynthesis in adult tobacco hornworms, *M. sexta*, but had no effect on CA activity of larvae or pupae. CA from the beetle *Tenebrio molitor*, the locust *Schistocerca gregaria* and the cockroach *Periplaneta americana* were also not activated by the synthetic peptide, whereas CA of the noctuid moths *Heliothis virescens* and *S. frugiperda* were stimulated, suggesting some Order specificity [9–13]. Synthetic Manse-AT, however, had no significant effect on the CA from adult males of *M. loreyi* [36].

Our present data suggest that we have isolated a cDNA encoding a novel *S. frugiperda* preproallatotropin. The first 32 amino acids of the deduced preprohormone compose a sequence with properties strongly reminiscent of a signal peptide [26]. The precursor is small and contains one copy of the putative peptide, located between the only two existing dibasic endoproteolytic cleavage sites. Northern hybridization demonstrated the site for peptide synthesis (brain, midgut and ovary) and the approximate size of the prohormone RNA transcript (~ 0.366 kb). Semiquantitative RT-PCR analyses proved that the peptide is synthesized in brains of larval, pupal and adult animals and in the midgut and the ovary of adult females. The peptide thus seems to represent a brain/gut neuropeptide which is also expressed in the female reproductive organs. The tissue distribution as well as its clear expression in the anterior–lateral region of the SOG and in the oocytes corresponds to that recently demonstrated for Manse-AT and Manse-AS in *S. frugiperda* [7,21]. To our knowledge, this is the first report on the presence and function of two different neuropeptides with allatotropic activity in an insect species.

The amino acid sequence of Spofr-AT 2 is identical at its C-terminus with that of Manse/Spofr-AS, whereas the N-terminus as well as the total number of amino acids differ. In order to reveal a physiological function of the peptide, we used the radiochemical assay (RCA) for JH biosynthesis and release to determine rates of JH release by CA in vitro. Our current measurements of the rates of JH release by CA from 2-day-old females of *S. frugiperda* as well as the effect of Manse-AT, Manse-AS and Manse-AT plus Manse-AS (not shown here) do not differ from the previous values [13,24] where L-[methyl-^{3}H]-methionine was used as the marker. In a previous study, we have also shown that acetate/methionine incorporation into the release products is stoichiometric [24]. In the present results, control CA released about twice as much JH during a second 2-h incubation (control II) than during the first 2-h period (control I). Such an increase in JH release was also observed in the previous study. The present data clearly show that the Spofr-AT 2 acts as an allatotropin. Treating CA with the synthetic peptide caused an up to tenfold increase in JH release. The apparent EC_{50} was about one order of magnitude higher than for Manse-AT [13]. The allatotropic effect of Spofr-AT 2 lasted for at least 2 h in peptide-free medium. When CA were incubated in the presence of both Spofr-AT 2 and Manse-AS, a previously demonstrated novel mechanism of allatostatin action [13] could be confirmed. JH release was inhibited only in those glands, which had been activated by an allatotropin. Finally, we could show that the two allatotropins of *S. frugiperda* may act in an additive manner.

The similarity in the primary structures of Manse-AS and Spofr-AT 2 is striking. In a former paper [41], it was reported that in Manse-AS the C^7 to F^{15} C-terminal sequence may define the active core of the peptide (disulfide bridge between C^7 and C^{14}) and that complete inactivation of Manse-AS may occur after cleavage beyond position C^7. In Manse-AT, the octapeptide AT 6–13 (EMMTARGF–a) seems to represent the biologically active core and the free

acid form is much less active than the amidated one [9]. Further experiments using deletion analogs of Spofr-AT 2 will provide insight into the active core for this peptide. More experiments will also be necessary to explain the physiological role of Spofr-AT 2 as well as its possible interaction with Manse-AT and Manse-AS in the regulation of JH biosynthesis by the CA of *S. frugiperda*. Pleiotropic functions are expected also for this peptide, and it is likely that the regulation of CA activity may not represent the solely or primary function of the peptide [2]. Finally, we shall try to isolate the Spofr-AT 2 peptide from *S. frugiperda* brain extracts and we will look for the occurrence and function of this peptide in other lepidopteran and non-lepidopteran species. Screening the ''*Drosophila melanogaster* and *Anopheles gambiae* genome projects'' database revealed that the Spofr-AT 2 preprohormone sequence is not present there.

Acknowledgements

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References

[1] Bendena WG, Donley BC, Tobe SS. Allatostatins: a growing family of neuropeptides with structural and functional diversity. Ann N Y Acad Sci 1999;897:311–29.

[2] Hoffmann KH, Meyering-Vos M, Lorenz MW. Allatostatins and allatotropins: is regulation of corpora allata activity their primary function? Eur J Entomol 1999;96:255–66.

[3] Stay B. A review of the role of neurosecretion in the control of juvenile hormone synthesis: a tribute to Berta Scharrer. Insect Biochem Mol Biol 2000;30:653–62.

[4] Gäde G. Allatoregulatory peptides—molecules with multiple functions. Invertebr Reprod Dev 2002;41:127–35.

[5] Kramer SJ, Toschi A, Miller CA, Kataoka H, Quistad GB, Li JP, et al. Identification of an allatostatin from the tobacco hornworm *Manduca sexta*. Proc Natl Acad Sci U S A 1991;88:9458–62.

[6] Jansons IS, Cusson M, McNeil JN, Tobe SS, Bendena WG. Molecular characterisation of a cDNA from *Pseudaletia unipuncta* encoding the *Manduca sexta* allatostatin peptide (Mas-AST). Insect Biochem Mol Biol 1996;26:767–73.

[7] Abdel-latief M, Meyering-Vos M, Hoffmann KH. Molecular characterization of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides. Insect Biochem Mol Biol 2003;33:467–76.

[8] Williamson M, Lenz C, Winther AM, Nässel DR, Grimmelikhuizen CJP. Molecular cloning, genomic organization, and expression of a C-type (*Manduca sexta*-type) allatostatin preprohormone from *Drosophila melanogaster*. Biochem Biophys Res Commun 2001;282:124–30.

[9] Kataoka H, Toschi A, Li JP, Carney RL, Schooley DA, Kramer SJ. Identification of an allatotropin from adult *Manduca sexta*. Science 1989;243:1481–3.

[10] Taylor PA, Bhatt TR, Horodyski FM. Molecular characterization and expression analysis of *Manduca sexta* allatotropin. Eur J Biochem 1996;239:588–96.

[11] Horodyski FM, Bhatt SR, Lee KY. Alternative splicing of transcripts by *Manduca sexta* allatotropin (*Mas-AT*) gene is regulated in a tissue-specific manner. Peptides 2001;22:263–9.

[12] Audsley N, Weaver RJ, Edwards JP. Juvenile hormone biosynthesis by corpora allata of larval tomato moth, *Lacanobia oleracea*, and regulation by *Manduca sexta* allatostatin and allatotropin. Insect Biochem Mol Biol 2000;30:681–9.

[13] Oeh U, Lorenz MW, Dyker H, Lösel P, Hoffmann KH. Interaction between *Manduca sexta* allatotropin and *Manduca sexta* allatostatin in the fall armyworm *Spodoptera frugiperda*. Insect Biochem Mol Biol 2000;30:719–27.

[14] Park C, Hwang JS, Kang SW, Lee BH. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Manduca sexta* allatotropin peptide. Zool Sci 2002;19:287–92.

[15] Truesdell PF, Koladich PM, Kataoka H, Kojima K, Suzuki A, McNeil JN, et al. Molecular characterization of a cDNA from the true armyworm *Pseudaletia unipuncta* encoding *Manduca sexta* allatotropin peptide. Insect Biochem Mol Biol 2000;30:691–702.

[16] Veenstra JA, Costes L. Isolation and identification of a peptide and its cDNA from the mosquito *Aedes aegypti* related to *Manduca sexta* allatotropin. Peptides 1999;20:1145–51.

[17] Hewes RS, Taghert PH. Neuropeptides and neuropeptide receptors in the *Drosophila melanogaster* genome. Genome Res 2001;11:1126–42.

[18] Vanden Broeck J. Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. Peptides 2001;22:241–54.

[19] Koladich PM, Cusson M, Bendena WG, Tobe SS, McNeil JN. Cardioacceleratory effects of *Manduca sexta* allatotropin in the true armyworm moth, *Pseudaletia unipuncta*. Peptides 2002;23:645–51.

[20] Nässel DR. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Prog Neurobiol 2002;86:1–84.

[21] Abdel-latief M, Meyering-Vos M, Hoffmann KH. Expression and tissue-specific localization of the *Spodoptera frugiperda* allatotropin (*Spofr-AT*) and allatostatin (*Spofr-AS*) genes. Arch Insect Biochem Physiol 2004;55:188–99.

[22] Harland RM. In situ hybridization and improved whole-mount methods for *Xenopus* embryos. Methods Cell Biol 1991;36:685–95.

[23] Feyereisen R, Tobe SS. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Anal Biochem 1981;111:372–5.

[24] Range S, Oeh U, Lorenz MW, Etzel W, Nauen R, Hoffmann KH. Juvenile hormone biosynthesis in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae). Comp. Biochem. Physiol. B 2002;132:191–202.

[25] Yagi KJ, Tobe SS. The radiochemical assay for juvenile hormone biosynthesis in insects: problems and solutions. J Insect Physiol 2001;47:1227–34.

[26] Von Heijne G. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14:4683–90.

[27] Ferenz HJ, Diehl I. Stimulation of juvenile hormone biosynthesis in vitro by locust allatotropin. Z Naturforsch, C 1983;38:856–8.

[28] Gadot M, Applebaum SW. Rapid in vitro activation of corpora allata by extracted locust brain allatotropic factor. Arch Insect Biochem Physiol 1985;2:117–29.

[29] Couillaud F, Girardie A. Disconnected corpora allata provide a novel assay for allatostimulating factors. J Exp Zool 1990;253:330–3.

[30] Lehmberg E, Ferenz HJ, Applebaum SE. Maturation and responsiveness to extracts of corpora allata from male *Locusta migratoria* containing allatotropic factors. Z Naturforsch, C 1992;47:449–52.

[31] Bogus M, Scheller K. Allatotropin released by the brain controls larval moulting in *Galleria mellonella* by affecting juvenile hormone synthesis. Int J Dev Biol 1996;40:205–10.

[32] Hodkova M, Okuda T, Wagner R. Stimulation of corpora allata by

extract from neuroendocrine complex: comparison of reproducing and diapausing *Pyrrhocoris apterus* (Homoptera: Pyrrhocoridae). Eur J Entomol 1996;93:535–43.

[33] Tu MP, Kou R, Remus GO, Yin CM, Steffolano Jr JG. Allatotropic activity in the brain of female *Phormia regina* (Diptera: Calliphoridae). J Insect Physiol 2002;48:733–41.

[34] Engelmann F. Die Steuerung der Ovarfunktion bei der Ovoviviparen schabe *Leucophaea maderae*. J Insect Physiol 1957;1:257–78.

[35] Lorenz MW, Hoffmann KH. Allatotropic activity in the suboesophageal ganglia of crickets, *Gryllus bimaculatus* and *Acheta domesticus* (Ensifera: Gryllidae). J Insect Physiol 1995;41:191–6.

[36] Kou R, Chen S-J. Allatotropic activity in the suboesophageal ganglia and corpora cardiaca of the adult male Loreyi leafworm, *Mythimna loreyi*. Arch Insect Biochem Physiol 2000;43:78–86.

[37] Stay B, Tobe SS, Mundall ES, Rankin S. Ovarian stimulation of juvenile hormone biosynthesis in the viviparous cockroach, *Diploptera punctata*. Gen Comp Endocrinol 1983;52:341–9.

[38] Hoffmann KH, Lorenz MW, Klein PM. Ovarian control of juvenile hormone III biosynthesis in *Gryllus bimaculatus* (Ensifera: Gryllidae). Entomol Gen 1996;20:157–67.

[39] Fan Y, Rafaeli A, Gileadi C, Kubli E, Applebaum SW. *Drosophila melanogaster* sex peptide stimulates JH-synthesis and depresses sex-pheromone production in *Helicoverpa armigera*. J Insect Physiol 1999;45:127–33.

[40] Chen PS, Stumm-Zollinger E, Aigaki T, Balmer J, Bienz M, Bohlen P. A male accessory gland peptide that regulates reproductive behavior of female *D. melanogaster*. Cell 1988;54:291–8.

[41] Audsley N, Weaver RJ, Edwards JP. Metabolism of *Manduca sexta* allatostatin by hemolymph of larvae of the tomato moth, *Lacanobia oleracea*. Peptides 2002;23:717–23.

Summary

This thesis investigates the allatoregulatory neuropeptides and their genes in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae).

1. Two genes encoding *S. frugiperda* allatotropin and allatostatin preprohormone peptides (Spofr-AT and Spofr-AST) have been cloned which are identical to the respective peptides from another lepidopteran species, *Manduca sexta* (Manse-AT and Manse-AST). The *Spofr-AT* gene is expressed in three mRNA isoforms with 134, 171, and 200 amino acids, differing from each other by alternative splicing. Each isoform includes one copy of the Manse-AT mature peptide. Another 14- or 15-residues peptide from the second isoform shows eight identities in its primary sequence with the Manse-AT-like III peptide predicted in *M. sexta*. The *Spofr-AST* cDNA encodes 125 amino acid residues including one copy of the Manse-AST mature peptide (type-C allatostatin).

2. Using one-step RT-PCR for semi-quantification of the gene expression, it is demonstrated that both genes (*Spofr-AT* and *Spofr-AST*) are expressed in brain, digestive tract, and reproductive organs of larvae, pupae, and adults of *S. frugiperda* in a time-, tissue-, and sex-specific manner. The tissue-specific localization of the prohormone expression, as demonstrated by whole-mount *in situ* hybridization, confirms the overall tissue distribution of the prohormones as shown by RT-PCR and supports the pleiotropic functions of the peptides.

3. The gene encoding the *S. frugiperda* allatostatin type-A (Spofr-AST A) peptide family (Y/FXFGL-amides) was isolated from *S. frugiperda* brain cDNA. The deduced precursor sequence contains 231 amino acids and allowed unambiguous identification of nine (or ten) peptides which are tandemly arranged in three blocks. All except one of the peptides had previously been deduced from the cDNA sequences of two other lepidopteran species. The *Spofr-AST* type-A gene is expressed in the brain, midgut, and reproductive organs of *S. frugiperda* larvae and adults in a time- and tissue-specific manner. Data confirm the nature of the allatostatin type-A peptides as brain/gut myoregulatory hormones, whereas their function (s) in ovaries and testes have still to be resolved.

4. In *S. frugiperda,* a cDNA that encodes 53 amino acids was cloned, including one copy of a non-amidated decapeptide (Arg-Val-Arg-Gly-Asn-Pro-Ile-Ser-Cys-Phe-OH). This peptide strongly stimulates the synthesis and release of juvenile hormone (JH) *in vitro* by the corpora

allata (CA) of *S. frugiperda* adult females and was code-named Spofr-AT 2. The primary structure of Spofr-AT 2 is identical at its C-terminus (-NPISCF) with the *M. sexta* type-C allatostatin (Manse-AST). Northern blotting and RT-PCR analyses revealed that the preprohormone is expressed as one transcript in the brain, midgut, and ovary in a tissue- and developmental-specific manner. Whole-mount *in situ* hybridization studies with brain and ovaries from 2 day-old adult females showed that the gene is expressed in two large clusters of cells in the suboesophageal ganglion and within the oocytes. Treating the CA with the synthetic peptide caused an up to tenfold increase in the release of JH. The stimulation of JH release was dose-dependent with an apparent EC_{50} of ca. 10^{-7} M. CA that were activated with Spofr-AT 2 could be inhibited by the addition of synthetic Manse-AST.

5. In conclusion, the presented date strengthen the hypothesis that "allatoregulating" neuropeptides are diverse in structure, widely distributed and exhibit multiple functions. The functions may be tissue-specific as well as specific to particular developmental stages of insects. Knowledge of the various peptide precursor sequences has opened the way for synthesis of these peptides for detailed physiological and functional studies. Further quantitative experiments formulated in context of the life history of the animals will certainly yield a more detailed understanding of the mode of action of these peptides in *S. frugiperda*. Other major challenges in the future will be to clone the receptors for these peptides and to study the receptor distribution in the fall armyworm.

Zusammenfassung

In dieser Arbeit wurden die allatoregulierenden Neuropeptide und ihre Gene beim Heerwurm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) untersucht.

1. Zunächst wurden zwei Gene kloniert, die für die Präprohormone des *S. frugiperda* Allatotropins und des *S. frugiperda* Allatostatins (Spofr-AT und Spofr-AST) kodieren. Die entsprechenden Peptide sind identisch mit dem schon bekannten Allatotropin bzw. Allatostatin aus einer anderen Lepidopteren-Art, *Manduca sexta* (Manse-AT und Manse-AST). Das *Spofr-AT* Gen wurde in drei mRNA Isoformen mit 134, 171 und 200 Aminosäuren exprimiert, die sich durch alternatives Splicing voneinander unterscheiden. Jede Isoform enthält eine Kopie des Manse-AT Neuropeptid. Die Isoform 2 enthält ein weiteres 14 bzw. 15 Aminosäuren langes Peptid, das in acht seiner Aminosäuren mit dem aus *M. sexta* bekannten Manse-AT-ähnlichen Peptide III identisch ist. Die *Spofr-AST* cDNA kodiert für eine Folge von 125 Aminosäuren, die eine Kopie des Manse-AST Neuropeptids (C-Typ Allatostatin) einschließt.

2. Mittels Einschritt-RT-PCR zur Semiquantifizierung der Genexpression konnte gezeigt werden, dass beide Gene (*Spofr-AT* und *Spofr-AST*) im Gehirn, Verdauungstrakt und in den Fortpflanzungsorganen von Larven, Puppen und adulten Tieren in einer zeit-, gewebe- und geschlechtsspezifischen Weise exprimiert werden. Die gewebsspezifische Lokalisation der Prohormonexpression mittels Ganzgewebe *in situ*-Hybridisierung bestätigte die durch RT-PCR gezeigte Verbreitung der Prohormone in den Geweben und unterstützt die Annahme, dass die Peptide pleiotrope Funktionen aufweisen.

3. Aus der cDNA von *S. frugiperda* wurde ein Gen isoliert das für die Familie der Allatostatin Typ-A Neuropeptide (Spofr-AST A) kodiert. Die abgeleitete Precursorsequenz enthält 231 Aminosäuren und ermöglichte die Identifizierung von 9 bzw. 10 Allatostatin Typ-A Peptiden, die in drei Blöcken zusammengefasst sind. Bis auf ein Peptid waren alle Strukturen aus den entsprechenden Prohormonen von zwei anderen Lepidopteren-Arten bereits bekannt. Das *Spofr-AST* Typ-A Gen wird im Gehirn, Mitteldarm und den Reproduktionsorganen von larvalen und adulten *S. frugiperda* zeit- und gewebespezifisch exprimiert. Die Ergebnisse bestätigen die Funktion der Allatostatin Typ-A Neuropeptide als myoregulatorisch aktive Hormone im Gehirn und Mitteldarm, während ihre Funktion(en) in den Ovarien und Hoden noch ungeklärt ist/sind.

4. Aus der cDNA von *S. frugiperda* wurde ein weiteres Gen isoliert, das eine Kopie eines nicht-amidierten Decapeptids (Arg-Val-Arg-Gly-Asn-Pro-Ile-Ser-Cys-Phe-OH) enthält. Dieses Peptid stimuliert die Biosynthese und Freisetzung von Juvenilhormon (JH) *in vitro* aus den Corpora allata adulter Weibchen von *S. frugiperda* und wurde als Spofr-AT 2 bezeichnet. Die Primärstruktur des Spofr-AT 2 ist am C-Terminus (-NPISCF) identisch mit jener des C-Typ Allatostatins aus *M. sexta* (Manse-AST). Northern Blot und RT-PCR Analysen ergaben, dass das Präprohormon als ein Transkript im Gehirn, Mitteldarm und in den Ovarien in einer gewebs- und entwicklungsspezifischen Weise exprimiert wird. Mittels *in situ*-Hybridisierung an Gehirn- und Ovarpräparationen konnte gezeigt werden, dass das Gen in Form von zwei großen Zellclustern im Suboesophagealganglion sowie innerhalb der Oocyten exprimiert wird. Wurden die CA adulter Weibchen mit dem synthetischen Peptid behandelt, so stieg die JH-Biosynthese bis zum zehnfachen des Ausgangswertes an. Die Stimulierung der JH-Biosynthese verläuft dosisanhängig mit einem apparenten EC_{50}-Wert von ca. 10^{-7} M. Corpora allata, die mit Spofr-AT 2 aktiviert worden waren, konnten durch Zugabe von synthetischem Manse-AST gehemmt werden.

5. Zusammenfassend bestätigen die vorliegenden Ergebnisse die Hypothese, dass „allatoregulierende“ Neuropeptide in ihrer Struktur sehr vielseitig sind, weit verbreitet gefunden werden und vielfältige Funktionen aufweisen. Die Funktionen sind vermutlich gewebs- und entwicklungsspezifisch unterschiedlich. Die Kenntnis der Peptidhormon-strukturen ermöglicht die Verwendung von synthetischen Peptiden bei weiteren Untersuchungen zu ihrer Physiologie bzw. Funktion. Weitere quantitative Experimente sollten unter Berücksichtigung der biologischen Lebensweise der Tiere durchgeführt werden und werden sicher genauere Erkenntnisse zur Wirkungsweise der Peptide bei *S. frugiperda* erbringen. Ein weiteres Ziel ist die Klonierung der Rezeptoren für diese Hormone sowie Studien zur Verteilung der Rezeptoren beim Heerwurm.

List of publications

Publications in peer reviewd journals

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2003): Molecular characterisation of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides. *Insect Biochem. Molec. Biol.* 33, 467-476.

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2004): Expression and localization of the *Spodoptera frugiperda* allatotropin (Spofr-AT) and allatostatin (Spofr-AS) genes. *Arch. Insect Biochem. Physiol*. 55,188-199.

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2004): Type-A allatostatins from the fall armyworm, *Spodoptera frugiperda*: molecular cloning, expression and tissue-specific localization. *Arch. Insect Biochem. Physiol*., 56, 120-132.

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2004): Characterization of a novel peptide with allatotropic activity in the fall armyworm *Spodoptera frugiperda. Reg. Pept.* (accepted for publication at 24th of May, 2004)

Published conference abstracts

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2002): Molecular characterization of the *Manduca sexta* allatotropin neuropeptide precursor gene in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *21st Conference of European Comparative Endocrinologists. Bonn, Germany.* Abstract Book, p. 62.

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2002): Molecular characterization of the *Manduca sexta* allatostatin neuropeptide precursor gene in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *21st Conference of European Comparative Endocrinologists. Bonn, Germany.* Abstract Book, p. 62.

Abdel-latief M., Meyering-Vos M., Hoffmann K.H. (2003): Expression of allatoregulatory neuropeptides in a lepidopteran pest species, the fall armyworm, *Spodoptera frugiperda*. *Jahrestagung der Deutschen Gesellschaft für allgemeine und angewandte Entomologie. Halle, Germany*. Abstract Book, p. 292.

Abbreviations

ACT	actin
AS or AST	allatostatin
AT	allatotropin
ATL	allatotropin-like
bp	basepair
CA	corpora allata
CC	corpora cardiaca
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
DNA	deoxyribonucleic acid
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidine triphosphate
dGTP	2'-deoxyguanosine triphosphate
dTTP	2'-deoxythymidine triphosphate
dNTPs	mixture of dATP, dCTP, dGTP and dTTP
DEPC	diethyl pyrocarbonate
d.H_2O	deionized water
DC	deutocerebrum
Dig-dUTP	digoxigenin-dUTP
E. coli	*Escherichia coli*
HPLC	high- performance liquid chromatography
***IS*H**	*in situ* hybridization
JH	juvenile hormone
mRNA(s)	messenger RNA(s)
mM	millimolar
mmol	millimoles
OL	optical lobe
PC	protocerebrum
PCR	polymerase chain reaction
RNA	ribonucleic acid
SOG	suboesophageal ganglion
RT-PCR	reverse transcriptase-PCR
V	Volt
ApaI	*Acetobacter pasteurianus* restriction enzyme
PstI	*Pronidencia stuartii* restriction enzyme

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine andern als die angegebenen Hilfsmittel verwendet habe.
Ferner erkläre ich, dass ich diese Arbeit weder einer anderen Prüfungsbehörde vorgelegt noch anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, den

Mohatmed Abdel-Latief

Erklärung

Hiermit erkläre ich, dass ich selbständig und ohne fremde Hilfe alle Experimente aus den vier Publikationen in meiner Doktorarbeit durchgeführt habe. In die molekularbiologischen Arbeitsmethoden wurde ich zu Beginn meiner Doktorarbeit von Frau Dr. Martina Meyering-Vos eingewiesen, die auch meine Experimente während der Doktorarbeit überwachte.

In Artikel 1 habe ich die Strategie für das Klonieren der Allatotropin- und der Allatostatin-cDNAs, die Sequenzierungsanalysen für jedes Plasmid und den Aufbau der cDNA Bibliotheken selbst entworfen. Ferner habe ich die Primer für die Synthese der Proben selbst konstruiert und die RT-PCR Expressionsstudien selbständig durchgeführt. Die Sequenzierungen wurden bei einer auswärtigen Firma durchgeführt. Auch alle anderen Experimente, einschließlich der Insekentaufzucht, Gewebevorbereitungen, RNA Extraktion, und mRNA Extraktionen, habe ich selbst durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet und die Abbildungen angefertigt.
Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Frau Dr. Martina Meyering-Vos und Herrn Prof. Dr. Klaus Hubert Hoffmann fertiggestellt wurde.

In Artikel 2 habe ich die Strategie für die Einschritt-RT-PCR zur Semiquantifizierung der Genexpression selbständig geplant und durchgeführt, ebenso wie die Ganzgewebe *in situ*-Hybridisierungsstudien. Ich habe die Sense- und Antisense-Proben der Allatotropin- und Allatostatin-Gene synthetisiert. Auch die anderen Experimente, einschließlich der Insektaufzucht, Gewebevorbereitungen, RNA Extraktion, und mRNA Extraktionen, habe ich selbständig durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statistischen Analysen durchgeführt.
Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Frau Dr. Martina Meyering-Vos und Herrn Prof. Dr. Klaus Hubert Hoffmann fertiggestellt wurde.

In Artikel 3 habe ich die Strategie für das Klonieren der Allatostatin Typ-A cDNA, die Sequenzierungsanalysen für jedes Plasmid und den Aufbau der cDNA Bibliothek selbst entworfen. Ich habe die RT-PCR Expressionsexperimente, die Ganzgewebe *in situ*-Hybridisierungsstudien und die Synthese der Sense- und Antisense-Proben für das Allatostatin Typ-A Gen selbst durchgeführt. Auch die anderen Experimente, einschließlich der

Insektaufzucht, Gewebevorbereitungen, RNA Extraktion und mRNA Extraktionen, habe ich selbständig durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statistischen Analysen durchgeführt.
Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Frau Dr. Martina Meyering-Vos und Herrn Prof. Dr. Klaus Hubert Hoffmann fertiggestellt wurde.

In Artikel 4 habe ich ebenfalls alle molekularbiologischen Arbeiten (Extraktion der genomischen DNA, der Gesamt-RNA und der mRNA, cDNA-Klonierung, RT-PCR, Northern-Blot, Ganzgewebe *in situ*-Hybridisierung) selbständig durchgeführt. Die physiologischen Experimente zur Messung der Synthese von Juvenilhormon *in vitro* habe ich nach einer Einführung durch Herrn PD Dr. Matthias Lorenz ebenfalls selbst durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statistischen Analysen durchgeführt.
Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Frau Dr. Martina Meyering-Vos und Herrn Prof. Dr. Klaus Hubert Hoffmann fertiggestellt wurde.

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