B-Type Allatostatins and Sex Peptides

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ABSTRACT

In many species, mating induces many behavioral changes in the female. For *Drosophila melanogaster*, the sex peptide (SP) has been identified as the main molecular factor behind these responses. Recently, the sex peptide receptor (SPR), a GPCR activated by SP, has also been characterized as responsive to *Drosophila* myoinhibiting peptides, a conserved peptide family that is also known as B-type allatostatins. These peptides bear the C-terminal hallmark of $-WX_6Wamide$. Given this unexpected link between these peptides, they will be covered together in the following chapter.

DISCOVERY

Sex Peptides

Sex peptide (SP, also known as Accessory gland peptide 70A), was characterized in 1988 as a factor synthesized in the *Drosophila* male accessory glands and transferred to the female on copulation. The observed effects were an increase in egg laying and a loss of receptivity of the female toward new mating partners. The sequencing of the peptide and cDNA revealed a 36-aa long peptide, preceded in its precursor by a 19-aa hydrophobic signal sequence.¹⁵

The second of the sex peptides, ductus ejaculatorius peptide 99B (DUP99B), was not characterized until 2002. As its name implies, DUP99B is produced in the ejaculatory duct from a precursor that gives rise to a 31-aa mature peptide. The sequence of the peptide is almost identical to that of SP with regard to its C-terminal region. DUP99B was shown to elicit the same postmating responses as SP when injected into a female fly's hemolymph.²³

Although both peptides have only been identified in *Drosophila* species, they also elicit physiological responses (see Biological Action) in the lepidopteran, *Helicoverpa armigera*. Therefore, it has been suggested that a similar sex peptide could be also be present in this species.^{7,15} Because these sex peptides have only been characterized in

various species of fruit flies, the following chapter on the sex peptides will be largely focused on these dipterans.

B-type Allatostatins

The B-type—or cricket type—allostatin family is characterized by a C-terminal $WX_6Wamide$ sequence. The first identified B-type allatostatin was the myoinhibiting peptide from *Locusta migratoria*.²⁴ *Lom-MIP* was purified from an extract of brains, corpora cardiaca, corpora allata, and subesophageal ganglia. It was determined to repress spontaneous contractions of the hindgut and the oviduct in *L. migratoria*, as well as the hindgut of *Leucophaea maderae*. In the locust, however, the peptide does not display an inhibitory effect on juvenile hormone (JH) synthesis by the corpora allata. It was not until the discovery of the four peptide family members (*Grb-AST1-4*) from the cricket, *Gryllus bimaculatus*, that they were characterized as allatostatins.^{16,17} The names “B-type allatostatin” and “myoinhibiting peptide” are synonymous to each other.

Although most results on this peptide family have been generated in insects, members of this peptide family have also been identified in a number of crustacean species. Most of these were discovered in decapod species, but a B-type allatostatin was also found in *Daphnia pulex*.^{3,5}

STRUCTURE OF THE mRNA/GENE

Sex Peptides

Both genes encoding SP and DUP99B have been cloned and sequenced in *Drosophila melanogaster*, and both display an intron insertion at the same site. The highly similar C-terminal parts of the peptides are encoded by the second exon, whereas the first encodes the signal sequence and the N-terminal part of the peptide. It has been proposed that both genes have arisen by gene duplication. This is supported by the aa sequence similarities in not only the C-terminal regions of the peptides but also in the signal sequences.^{15,23}

B-type Allatostatins

Knowledge of the gene structure for the B-type allatostatins was not generated until the publication of the *Drosophila* genome. The fruit fly *myoinhibitory peptide* (*mip*) gene encodes a precursor in two exons, spaced by an intron. This preprohormone gives rise to five MIPs.³¹ In *Rodnius prolixus*, the *Ast-B* gene shows more complexity, having four exons.¹⁸ With five exons, the *AstB* gene features more complexity in *D. pulex*.⁵

PROCESSING OF THE PRECURSOR

Sex Peptides

SP synthesis occurs via a precursor, 55 aa in length, from which a 19-aa signal peptide is released. Amino acid sequence analysis of the SP from various *Drosophila* species has revealed two regions of high similarity. The five most N-terminal aa (WEWPW) are identical and the C-terminal region, encoded by the second exon, shows strong conservation.¹⁵

Like SP, DUP99B is synthesized from a precursor of 54 aa, from which 21 are cleaved off N-terminally as well as the two C-terminal aa (RK). The peptide displays a pyroglutamic acid modification in the N-terminus. Further, the N-terminal region was shown to undergo glycosylation, consisting of a difucosylated oligosaccharide structure. This latter modification decreases the amount of peptide required to induce ovulation, although this effect has not been observed in receptivity assays.²³

The processed SP and DUP99B peptides are, respectively, 36 and 31 aa long and display a linear N-terminal region and a circular C-terminal part, resulting from a disulfide bridge formation. In between the two bridge-forming Cys residues, 9 out of the 11 aa are identical.

B-type Allatostatins

In *Drosophila*, the B-type allatostatin gene encodes a precursor of 211 aa that starts with a signal sequence and harbors five WX₆W₆ peptides, all of which are flanked by dibasic or tribasic cleavage sites.³¹ Results from *Rhodnius* indicate the presence of an *AST-B* gene encoding at least nine peptides.¹⁸ For *Bombyx mori*, the precursor gives rise to eight peptides.³² In the tick, *Ixodes scapularis*, the 132 aa MIP precursor likely gives rise to three peptides, of which two show the hallmarks for C-terminal amidation.²⁶

DISTRIBUTION OF THE mRNAs AND PEPTIDES

Sex Peptides

The results generated in *D. melanogaster* reveal very similar temporal expression profiles for both the *sp* and the *dup99B*

gene. Little to no transcript can be detected during development until the late pupal stages. Not surprisingly, expression levels are by far the highest in male adults. By contrast, female adults show no expression of *sp* or *dup99B*.²⁰

The spatial expression patterns of *sp* and *dup99B* are equally constricted but notably different. Although *sp* transcripts are only found in the male accessory gland, the expression of *dup99B* is limited to the upper ductus ejaculatorius.^{15,20,23} In addition, *dup99B* is also expressed in the cardia of both male and female flies.¹⁵

B-type Allatostatins

In contrast to the strictly limited expression profile of the sex peptides, the transcript for the B-type allatostatin is much more widely distributed. In *D. melanogaster*, the expression of the precursor has been found throughout all developmental stages.²⁰ The expression of *mip* is by far the greatest in the central nervous system (CNS). These results correspond with those obtained from immunohistochemical analyses, which point out the presence of MIP in the CNS of *Drosophila* and *Aedes aegypti*.¹¹

Detailed immunohistological studies on *D. melanogaster* have revealed additional sites of strong MIP immunoreactivity in the larval midgut in the region before the copper cells and much weaker signals from between the copper cells. Also, a group of cells in the posterior part of the larval midgut is weakly MIP immunoreactive, and these decrease in density toward the middle of the posterior midgut. By contrast, MIP immunoreactivity is hard to pinpoint on the adult fly's midgut.²⁹ Immunohistochemistry in combination with Gal4 enhancers has allowed for the identification of MIP expression sites in the central complex of the *Drosophila* brain. Immunoreactivity appears in the ventral, central, and dorsal layers, with the latter showing strong labeling.¹⁰

Using *in situ* hybridization, the transcript for the *B. mori* prothoracicostatic peptides (PTSP) has been mapped to the CNS with the most intense signals in a pair of brain medial neurosecretory cells and also strong signals from the interneuron 704 and epiproctodeal glands. In the brain, the expression is detected during the feeding period but decreases before the time of each ecdysis. Transcript levels in the terminal ganglia, however, remained constant during the fourth and fifth instar stages. In the epiproctodeal glands, the expression is strong in pharate fifth instar larvae and just after ecdysis. It is at a low level during the first 5 days of the fifth instar stage, then rises. Immunohistological results are also available from *B. mori* and concur with the findings from the expression results.³² These results generally correspond with what is known from *Manduca sexta*.⁴

Further, in *L. migratoria*, the presence of the peptide has been traced to approximately 12 neurons, including those innervating the glandular cells of the corpora cardiaca and the corpora allata.²⁴

Detection of the myoinhibiting peptide resulted in very similar patterns for the tick species *I. scapularis* and *Rhipicephalus appendiculatus*. In the synganglion, immunoreactivity could be detected in the neurons of the protocerebrum, palpal lobe, cheliceral lobe and the pedal lobes 2 and 3 (and 4 in the case of *I. scapularis*) and on the ventral side, the opistosomal lobe and a number of small neurons that were not detected in *R. appendiculatus*. Further, it was shown that the salivary gland in the tick is innervated by MIP-positive axonal projections.²⁵

In decapods, the B-type allatostatins are widely distributed in the CNS as indicated by mass spectrometry analyses. In these animals, they are likely to serve as both autocrine or paracrine factors and as circulating hormones.³ Using an antibody raised against *Cancer borealis* AST-B1, immunostaining reveals the distribution of this peptide throughout the stomatogastric nervous system of the Jonah crab. Immunoreactivity is detected in neurons and neuropil in the commissural ganglia, in somata in the esophageal ganglion, in fibers in the stomatogastric nerve and in neuropilar processes in the stomatogastric ganglion.²⁷

RECEPTORS AND SIGNALING CASCADES

Studies in *Drosophila* show that SP activates the G-protein-coupled receptor encoded by CG16752, the sex peptide receptor, of which putative homologs have also been identified in various other insect species.^{11,20,34} Transcript and protein presence of this receptor has been shown in the female reproductive organs, especially in the spermatheca, and in the CNS of both females and males in very similar patterns.^{20,34} An in-depth study focusing on the neurons

in which *spr* is expressed reveals that the presence of SPR in neurons expressing both *fruitless (fru)* and *pickpocket (ppk)* is necessary and sufficient for the mating-induced behavioral changes. This conclusion is warranted as *spr* knockdown in *ppk*⁺ or *fru*⁺ neurons is enough to eliminate postmating responses. In addition, specifically expressing *spr* in either of these neuron populations rescues the phenotype of *spr* null mutants in assays of egg laying, virgin receptivity, and remating frequency. Further, silencing the *ppk*⁺ neuron signaling by means of specifically blocking the synaptic transmission of these neurons reveals a significant decrease in virgin receptivity and an increase in virgin egg laying. The sensory neurons innervating the uterus are thus far the only ones identified as expressing both *ppk* and *fru*.⁸ Independently, other data support the importance of these neurons in SP signaling. Inhibition of PKA in these neurons raises the rate of egg laying by virgins and reduces the virgin's receptivity. Therefore, signaling through *Gai* followed by PKA inhibition has been proposed as the *in vivo* path of action.³³

Most interestingly, SPR from *Drosophila* has also been identified as the cognate receptor for MIPs, with MIP4 showing a lower EC₅₀ value than SP (Fig. 1). As the C-terminal sequence similarity between MIPs and SP is limited to the -WX₆W motif in MIPs compared with that of -WX₈W in SP, it was hypothesized that the Trp residues present in these peptides might be essential for receptor activation. Indeed, truncated and Ala-substituted analogs of the peptides in which the Trp residues were eliminated show a strong loss of agonist activity.^{11,20} Given the conserved nature of the receptor, in contrast to the limited number of species in which SP has been identified, it is sensible

FIGURE 1 *Drosophila* SPR is a receptor for both SP and MIPs. CHO cells coexpressing aequorin, Gα16, and *Drosophila* SPR dose dependently increase intracellular Ca²⁺-concentration when challenged with *Drosophila* SP and MIPs 1–5. Data points represent mean ± SD of three independent measurements performed in triplicate and are given as a percentage of the maximal response. The zero response level corresponds to the treatment with buffer only. (Figure reproduced from Ref. 20 with the publisher's permission.)

to put forward the MIP family as the ancestral ligands for SPR. Further evidence from *B. mori* supports this theory. In this organism too, the SPR homolog has been identified as a cognate receptor for the *Bom*PTSPs (B-type allatostatins).³²

BIOLOGICAL ACTIONS

Sex Peptides

The biological effects initially attributed to SP have been witnessed in both mated female flies and virgin females that were injected with SP. First, there is dramatic loss of receptivity of mated females compared with virgins. Once fruit fly females have copulated, they will actively reject new males that present themselves as additional mating partners. Second, after mating, the *Drosophila* female will display a strong increase in egg laying.

Further, it has been shown that SP induces the progression of oocytes to stage 10. This effect is likely mediated by juvenile hormone (JH) as SP is known to have allatotrophic effects and because the oocyte progression can also be stimulated by methoprene, a JH mimic. Like SP, methoprene also induces the uptake of yolk protein (YP) into the ovary, in addition to strong increase of yp transcription in the ovary. However, the stimulation of egg laying and loss of receptivity cannot be mimicked by methoprene treatment.¹⁵

Further, female flies, having mated, significantly increase their food uptake. This effect has also been attributed to SP-transfer during copulation, without substantial involvement of sperm or the act of copulation.² A closer look at this aspect has made clear that this effect is probably an indirect consequence of the increased egg laying mentioned above. This is supported by the lack of feeding increase in sterile *ovo^{D1}* mutant females and the triggering of the effect in virgin females by silencing *fru* neurons rather than exposing them to SP.¹

The observation that mated female's life spans are significantly shorter in comparison with their virgin counterparts raises the question as to whether this could result from adverse effects coupled with an increase in feeding. This has been refuted based on sterile *ovo^{D1}* mutant females displaying the same survival cost linked with mating, despite the observation that their food intake is not elevated.¹

Results on feeding in *Drosophila* indicate that not only the quantity of the female's food intake will differ after copulation, but also its preference. In contrast to virgin females, mated females that have been deprived of a protein rich food source (i.e. yeast) for 3 days will show a strong preference for food containing yeast. SPR activity in the *ppk+* neurons is essential for this effect because expression of SPR in these neurons in an *SPR* mutant background largely restores the wildtype phenotype. Further, evidence suggests the involvement of TOR/S6K signaling and serotonin in the modulation of food preference.^{22,28}

The presence of an intact C-terminal region of SP, including its disulfide bridge, is key to the biological activity of the peptide in receptivity and oviposition assays but it is not sufficient. In contrast, deletion of the 10 most N-terminal aa residues does not affect the peptide's activity in these assays.¹⁵ However, the N-terminal part of the peptide has been shown to induce JH biosynthesis in the corpora allata of both *Drosophila melanogaster* and the moth, *H. armigera*. In concurrence with these results, DUP99B, which does not show strong similarity with SP in the N-terminal region, is not capable of eliciting allatal stimulation. For *Helicoverpa*, the C-terminal region of SP can reduce PBAN-induced pheromone production, which has been interpreted as a mechanism to lower the chance of a female participating in multiple copulations, thus increasing a male's chance for successful reproduction.^{7,15}

Based on female postmating responses after mating with males that either produce no sperm-bound SP or SP that lacks the dibasic cleavage site at its N-terminal region, for the postmating responses to linger longer than 1 day (short-term postmating response), sperm-bound SP needs to be present and the long term postmating response requires the possibility of SP being cleaved from the sperm at the aforementioned dibasic cleavage site. The short-term postmating response, however, does not require this and is most likely primarily the result of unbound SP present in the seminal fluid.¹⁹

Although SP alone is capable of inducing a transient increase in egg production and laying, the presence of many conserved classes of seminal proteins is also required to reach the witnessed long term postmating effects.¹⁵ A cascade unfolds in which the protease CG9997 is necessary for the transfer of lectins CG1652 and CG1656 to the female on mating. These lectins also require the presence of cysteine-rich secretory protein CG17575 to efficiently accumulate in the seminal receptacle. A lack of any of these factors will lead to a quick deficiency of SP in the female seminal receptacle.²¹

In addition to the postmating effects described above, copulation induces a number of other responses that have been attributed to the action of SP. Mating induces the expression of an array of antimicrobial peptide genes. Using *SP⁰* males and males lacking germline, it has been demonstrated that SP is the principal factor underlying this response. *SP⁰* males fail to induce transcription of the antimicrobial peptide genes *Metchnikowin*, *Drosomycin*, and *Diptericin*. However, the effect on the last two is weaker in comparison with the first. Further, continuous expression of SP in the fat body elevates the transcript levels of these genes above the level of mating-induced expression. SP is therefore believed to regulate the transcription of antimicrobial peptide genes in a dose-dependent manner.¹⁹ Looking further into this aspect of SP functionality, it was discovered that the hydroxyproline-rich region in the middle of the peptide is the eliciting factor of the immune response. Given the particular pattern formed by the five hydroxyprolines,

the hypothesis was raised that these motifs' actions result from its mimicking, on a molecular level, of sugar components within the microbial cell wall. In this case, pattern recognition receptors would be responsible for the elevated immune response on challenging by SP.⁶

A sexual dimorphism exists in *Drosophila* in relation to sleep-wake behavior. Male flies show an increased activity concentrated around the period of lights-on or lights-off, interspaced with periods of quiescence. A similar pattern is seen in virgin females. In contrast, females that have copulated only display a period of quiescence during the nighttime. These females are significantly more active during the daytime than males or virgin females. This effect is not sperm dependent, as mating of wildtype females with males lacking sperm does not abolish it. It is, however, also an effect of SP as this behavioral change is not found in females that are mated to SP⁰ males. This loss of daytime rest has been proposed as one of the factors contributing to the decrease of lifespan observed in mated females.⁹

The link between *Drosophila* male reproductive success and SP has been studied in more detail, resulting in the notion that more SP is transferred to the female during mating in the presence of a competitor male. Additionally, males that have larger accessory glands, and therefore produce more SP, will have a greater reproductive success.³⁰

B-type Allatostatins

The first characterized biological function of these peptides is the ability of *L. migratoria* MIP to inhibit spontaneous muscle contractions in the hindgut and ovaries of the locust.²⁴ Further, juvenile hormone biosynthesis in the cricket *G. bimaculatus* is inhibited by Grb-ASTs and, to a lesser extent, by Lom-MIP.¹⁶

In both *D. melanogaster* and *M. sexta*, the CCAP/MIP neurons have been determined as responsible for the initiation and/or execution of the ecdysis behavior. Specifically, MIP was proposed to silence neurons that are not required during the ecdysis program.^{12,13}

In *Drosophila*, a novel type of MIP-producing neuron was identified, which has cell bodies anterior to the base of the optic lobe medulla. Because branches of these neurons are closely associated with branches of clock neurons a putative link between the circadian clock and MIP has been proposed.¹⁴

In vitro evidence from *B. mori* indicates that expression of the receptor is strongly upregulated after a sudden decline of the 20-hydroxyecdysone titer. Considering the observation of sudden PTSP release coinciding with a drop of the 20E titer, it has been proposed that PTSP/PTSPR signaling is involved in the fine-tuning of ecdysteroid titers by suppressing prothoracic gland activity.³²

Little is known about the physiological effects of the B-type allatostatins in Crustacea. For *C. borealis*, three out

of the ten Cb-AST-B peptides identified by mass spectrometric analysis affect the pyloric rhythm in a state-dependent manner.²⁷

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