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Neuropeptides affecting the transfer of juvenile hormones from males to females during mating in *Spodoptera frugiperda*

Intisar T.E. Hassanien, Manuela Grötzner, Martina Meyering-Vos, Klaus H. Hoffmann *

Animal Ecology I, University of Bayreuth, 95448 Bayreuth, Germany

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ABSTRACT

In the polyandric moth, *Spodoptera frugiperda*, juvenile hormone (JH) is transferred from the male accessory reproductive glands (AG) to the female bursa copulatrix (BC) during copulation (see Hassanien et al., 2014). Here we used the RNA interference technique to study the role of allatoregulating neuropeptides in controlling the synthesis and transfer of JH during mating.

Knockdown of *S. frugiperda allatostatin C* (*Spofr-AS type C*) in freshly emerged males leads to an accumulation of JH in the AG beyond that in the control and mating results in a higher transport of JH I and JH II into the female BC. Knockdown of *S. frugiperda allatotropin 2* (*Spofr-AT2*) significantly reduces the amount of JH in the AG as well as its transfer into the female BC during copulation. Knockdown of *S. frugiperda allatostatin A* (*Spofr-AS type A*) and *S. frugiperda allatotropin* (*Spofr-AT*; Hassanien et al., 2014) only slightly affects the accumulation of JH in the AG and its transfer from the male to the female.

We conclude that Spofr-AS type C and Spofr-AT2 act as true allatostatin and true allatotropin, respectively, on the synthesis of JH I and JH II in the male AG. Moreover, both peptides seem to control the synthesis of JH III in the corpora allata of adult males and its release into the hemolymph.

1. Introduction

Juvenile hormones (JH) play a crucial role in the regulation of insect larval development, metamorphosis, and adult reproduction (Riddiford, 2012). The concentration of JH in the hemolymph is ascertained mainly by its synthesis in the corpora allata (CA) (Gilbert et al., 2000). More recent research showed that the male accessory reproductive glands (AG) of some Lepidoptera (Park et al., 1998), Diptera (Borovsky et al., 1994), and Coleoptera (Tian et al., 2010) synthesize and store JH and that the JH can be transferred to the female during copulation.

In the noctuid moth, *Spodoptera frugiperda*, mating results in elevated JH titers in the hemolymph of the females, as well as in a higher number of deposited eggs, compared to virgins (Hassanien et al., 2014). However, it remained unclear how elevation of JH titers and increase in egg laying is achieved in response to copulation. Recently, we could show a transfer mainly of JH I and JH II from the male AG to the bursa copulatrix (BC) of the female during mating (Hassanien et al., 2014), but it remained unresolved whether JH biosynthesis in the male and JH transport from the male to the female are affected by allatoregulating neuropeptides.

Biosynthesis of JH in the corpora allata (CA) of insects can be either stimulated or inhibited by neuropeptides, which are termed allatotropin (AT) and allatostatin (AS) (Stay, 2000). An allatotropin was first characterized from *Manduca sexta* (Kataoka et al., 1989) and a structurally related AT was found in dipterans (Li et al., 2003). Three families of structurally unrelated AS have been isolated and characterized from various insect orders: the type A AS (FGLamides), type B AS [W(X_6)Wamides], and type C AS (*M. sexta* type; lepidopteran AS) (Stay, 2000). Allatostatins and AT are pleiotropic in function. In addition to their allatoregulatory activities, they modulate the contraction of visceral muscles (Hoffmann et al., 1999; Weaver and Audsley, 2009), affect midgut enzyme activities (Fúse et al., 1999; McNeil and Tobe, 2001), or control the synthesis of ovarian ecdysteroids (Lorenz et al., 1997) and vitellogenins (Martin et al., 1996). Whether the allatoregulatory neuropeptides act on sites of JH biosynthesis beyond the CA has not yet been studied (De Loof et al., 2010).

The presence of Manse-AS and Manse-AT in *S. frugiperda* was verified by cloning the cDNA, which encode the precursors of Manse-AS and Manse-AT, respectively (Abdel-latief et al., 2003). The two genes were termed *Sopfr-AS* and *Spofr-AT* and are expressed in brains of larvae, pupae and adults with variable intensity (Abdel-latief et al., 2004a). A gene encoding *Spofr-AS type A* was isolated from brain cDNA containing nine FGLamides (Abdel-latief et al., 2004b). The gene is expressed in the brain and midgut of

* Corresponding author. Tel.: +49 921 552469; fax: +49 921 552784.
E-mail address: Klaus.hoffmann@uni-bayreuth.de (K.H. Hoffmann).

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larvae and adults, but also in the reproductive tissues of adult moths. A cDNA encoding a novel *S. frugiperda* preprohormone was isolated and cloned by Abdel-latief et al. (2004c) and contained a decapeptide sequence RVRGNPISCF-OH. It strongly stimulates the synthesis and release of JH *in vitro* by the CA and was termed *Spofr-AT2*.

In the present paper, we use the RNA interference (RNAi) technique for a specific knockdown of the preprohormones of allatoregulating neuropeptides (Spofr-AS type A, Spofr-AS type C, Spofr-AT and Spofr-AT2) in freshly ecdysed males of S. *frugiperda* and then measure the amounts of the JH homologs JH I to JH III in the male AG and in the female BC before and after mating by liquid chromatography–mass spectrometry (LC–MS). In a previous experiment, knockdown of the *Spofr-AT* gene in newly ecdysed males by RNAi hardly affected the amount of JH transferred and had no effect on the oviposition rate of the females mated with such treated males (Hassanien et al., 2014). The present study provides evidence that Spofr-AS type C and Spofr-AT2 control the male to female JH transfer during copulation.

2. Materials and methods

2.1. Insect rearing

Eggs and pupae of *S. frugiperda* were provided by Bayer CropScience (Monheim, Germany) and reared at 27 °C and 70% rh under a L16: D8 photoperiod as described by Oeh et al. (2000). Males were used in the RNAi experiments within 4 h after adult emergence. For mating, males were added to the females on day 1 of adult life in a ratio of 1:1, if not otherwise indicated. In the polyandric moth, mating occurs every 24 h. Eggs laid by the females were collected on each day of adult life.

2.2. dsRNA synthesis

A PCR method was used to generate the templates for the dsRNA syntheses corresponding to 298–621 nucleotides (nt) of the Spofr-AT isoform A sequence (Abdel-latief et al., 2004b; Hassanien et al., 2014), 293–570 nt of the Spofr-AS type C sequence (Abdel-latief et al., 2003; Griebler et al., 2008), 23–218 nt of the Spofr-AT2 sequence (Abdel-latief et al., 2004c; Griebler et al., 2008), and 289–758 nt of the Spofr-AS type A sequence (Abdel-latief et al., 2004b; Meyering-Vos et al., 2006). Amplification of the transcripts and generation of the dsRNA were carried out as previously described by Hassanien et al. (2014).

2.3. Injections

1.0–1.5 μg dsRNA in 2 μl noctuid Ringer (Davenport and Wright, 1985) were injected once with a 10 μl Hamilton syringe (Hamilton AG, Bonaduz, CH) into the third segment of the ventral abdomen of adult males within the first 4 h after adult emergence. Specificity and efficiency of the gene knockdown in *S. frugiperda* larvae and adults were measured continuously during the experiments and had been shown also in previous reports (Meyering-Vos et al., 2006; Griebler et al., 2008; Terenius et al., 2011; Hassanien et al., 2014).

2.4. Hemolymph and tissue collection

Hemolymph was collected from adult moths through the intersegmental membranes with a 20 μl micropipette (Blaubrand® intra Mark Brand, Wertheim, Germany). Since adult moths have a low hemolymph volume, hemolymph from 4 to 5 animals had to be combined to get a final volume of 20 μl. The JH was extracted as previously described by Westerlund and Hoffmann (2004).

Moths were dissected under a binocular microscope, and covered with cricket Ringer saline (Lorenz et al., 1997). Male accessory reproductive glands (AG) and the bursa copulatrix (BC) of the females were isolated and removed from adhering tissue. Organs were transferred into a mixture of 200 μl methanol/isooctane (1:1; v/v) and carefully grinded with a glass homogenizer (0.5 ml; Motor cordless Kontes, Vineland, USA) for 90 s, vortexed and incubated at room temperature for 20 min. The extracts were stored at −70 °C until use.

2.5. Juvenile hormone titers

Concentrations of the juvenile hormone homologs (JH I, JH II, and JH III) in hemolymph and tissues were quantified by the LC–MS method as described previously (Westerlund and Hoffmann, 2004).

2.6. Statistics

Statistical analysis was performed using the Student's *t*-test or the Mann–Whitney *U*-test. A level of $P < 0.05$ was considered as significant.

3. Results

3.1. Efficiency of RNA interference

Single injection of 1.0–1.5 μg dsRNA into freshly ecdysed male moths resulted in 75% and 97% reduction of gene expression for Spofr-AT, Spofr-AT2 and Spofr-AS type C and 40–80% for Spofr-AS type A. A dsRNA fragment of *Spofr-sulfakinin* served as a control for effects caused by dsRNA. Injection of *Spofr-SK* dsRNA never affected the expression of the allatoregulatory genes. Further, we included a Ringer-injected control group in each of our experiments. Because of pecuniary aspects we could not recheck the transcript reduction in each individual experiment.

3.2. JH titer in the hemolymph of female and male adults

We measured the JH titers in the hemolymph of virgin and mated females and of adult males during the first 6 days of adult life (not shown in figures). Adult females and males contained measurable amounts of the three JH homologs JH I, JH II and JH III. In males (<10 pg/μl total JH), JH concentrations were much lower than in females and JH I remained below detection limit. In mated females (up to 70 pg/μl total JH), JH titers were generally higher than in virgins (<40 pg/μl total JH) (Hassanien et al., 2014) and mating seemed to induce a shift towards JH I and JH II, with less JH III.

3.3. Effects of Spofr-AS type A gene knockdown

The AG of freshly emerged males contained significant amounts of JH I and JH II, but only traces of JH III (Fig. 1A). In the glands of unmated males, the amounts of JH I and JH II increased during the next 48 h of adult life, whereas JH III remained low. Mating on day 1 after emergence led to a drastic decrease of JH I and JH II in the AG (Fig. 1B), but JH I and JH II recovered again after 24 h without mating. A second mating on day 2 resulted in another depletion of JH I and JH II from the AG. Mating thus resulted in a reduction of JH I and JH II in the AG (see also Fig. 2A). The BC of newly ecdysed females contained only traces of JH (Fig. 2B) and the JH content remained low also on day 2 of adult life, if no mating had occurred (not shown). However, shortly after mating, a drastic increase in