

# Myoinhibiting peptides are the ancestral ligands of the promiscuous *Drosophila* sex peptide receptor

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**Abstract** Male insects change behaviors of female partners by co-transferring accessory gland proteins (Acps) like sex peptide (SP), with their sperm. The *Drosophila* sex peptide receptor (SPR) is a G protein-coupled receptor expressed in the female's nervous system and genital tract. While most Acps show a fast rate of evolution, SPRs are highly conserved in insects. We report activation of SPRs by evolutionary conserved myoinhibiting peptides (MIPs). Structural determinants in SP and MIPs responsible for this dual receptor activation are characterized. *Drosophila* SPR is also expressed in embryonic and larval stages and in the adult male nervous system, whereas SP expression is

restricted to the male reproductive system. MIP transcripts occur in male and female central nervous system, possibly acting as endogenous SPR ligands. Evolutionary consequences of the promiscuous nature of SPRs are discussed. MIPs likely function as ancestral ligands of SPRs and could place evolutionary constraints on the MIP/SPR class.

**Keywords** Allatostatin · GPCR · Insect · Myoinhibiting peptide · Post-mating response · Sex peptide receptor

## Abbreviations

Acps	Accessory gland proteins
CNS	Central nervous system
GPCR	G protein-coupled receptor
MIP	Myoinhibiting peptide
SP	Sex peptide
SPR	Sex peptide receptor

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## Introduction

Myoinhibiting peptides (MIPs) take their name from the ability to inhibit spontaneous muscle contractions of insect gut and oviduct preparations [1, 2]. MIPs belong to the W(X)<sub>6</sub>Wamide peptide family and are also termed B-type allatostatins or prothoracicostatic peptides, according to their biological activity in crickets (inhibition of juvenile hormone production in the corpora allata) and the silkworm (suppression of ecdysteroid biosynthesis in the prothoracic gland), respectively [3, 4]. In *Drosophila*, five different isoforms are encoded by a single MIP peptide precursor gene [5, 6] and MIPs have been detected by mass spectrometry in different *Drosophila* tissues [7, 8]. The biological functions of *Drosophila* MIPs, as well as the

identity of the corresponding receptor(s) are unknown. In order to elucidate the roles that these pleiotropic peptides play in insects, we searched for a *Drosophila* MIP receptor and found that the G protein-coupled receptor (GPCR) encoded by *CG16752* is activated by MIPs in the nanomolar concentration range.

Interestingly, this GPCR was recently described as a functional receptor for sex peptide (SP) [9]. SP is a 36-amino acid peptide that is one of the many seminal fluid proteins that male flies transfer to females during copulation. SP is responsible for various post-mating changes in female behavior: it decreases the willingness to re-mate, induces egg production and egg laying, stimulates food intake, enhances antimicrobial peptide synthesis and reduces female longevity [10–15]. The SP receptor (SPR) was found to be expressed in the female's genital tract and nervous system and downregulation by RNAi abolished female post-copulatory behaviors [9, 16, 17]. Interestingly, SPR is also expressed in male central nervous system (CNS). This represents a confusing observation since its ligand, SP, is produced in male accessory glands only and transferred to females during copulation [18, 19].

Another intriguing observation is the structural conservation of insect SPRs. This is unexpected, since SP itself is not well conserved. Apart from a few *Drosophila* species, SP-like peptides have only been identified in the moth *Helicoverpa armigera* [20–22].

We now show that insect SPRs can also be activated by MIPs and that MIP transcripts are present in the CNS of *Drosophila* females and males and thus could serve as SPR ligands in tissues where SP itself is absent. The fact that a single GPCR can respond to two functionally unrelated ligand systems with almost equal potency and efficiency is quite rare and we identified structural determinants in the ligands and in the receptor that are important for this dual activation. Finally, we position the evolutionary well-conserved family of MIP-like peptides as the ancestral ligands of SPRs.

## Materials and methods

### Molecular cloning and expression constructs

cDNA-fragments encoding SPRs from *Drosophila melanogaster*, *Tribolium castaneum*, and *Bombyx mori* were generated by means of PCR reactions using species-specific cDNA mixes of different stages/tissues as templates and including the following primers: 5'-caccatggacaactacggacgtactg-3' and 5'-ctagaggaccgtctcgttggtg-3' (for *Drosophila*), 5'-caccatggcgagatggcgtcgaactcga-3' and 5'-tctagattacaaaacagtttcattggtacaa-3' (for *Tribolium*) and 5'-caccatggcggtcaccatagacaattcaacgaacga-3' and 5'-ctcgag

ttaaagcacagtttcgtgtgtacag-3' (for *Bombyx*). Pwo (*Pyrococcus woesei*) DNA polymerase was used according to the manufacturer's protocol (Roche). The obtained PCR products were directionally cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen) and sequenced.

### Site-directed mutagenesis of *Drosophila* SPR cDNA

The wild-type *D. melanogaster* SPR cDNA fragment cloned in pcDNA3.1D/V5-His-TOPO (Invitrogen) was used as the template to mutate the naturally occurring "QRY" motif into "DRY" using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol and using the following primers: Fwd 5'-ctaactctggccctcgccgttgatagatacatctacgttg-3' and Rev 5'-caaacgtagatgtatctatcaacggcgagggccagagtag-3' (mutated nucleotides are underlined). The correct insert of the plasmid was checked by sequencing before further use in cell-based assays.

### Cell culture and transfections

CHO-WTA11 cells (Euroscreen, Belgium) stably expressing apo-aequorin and  $G\alpha_{16}$  were cultured at 37°C, 5% CO<sub>2</sub> in Ham's F-12 medium, supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml zeocin and 10% heat-inactivated fetal calf serum (Invitrogen). HEK cells were cultivated in D-MEM medium (Invitrogen) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Transfections were performed with FuGENE6 according to the manufacturer's protocol (Roche).

### Peptides

A library of 76 *Drosophila* peptides was synthesized by means of Fmoc polyamide chemistry (Sigma-Aldrich and GLS). The correct mass of these peptides was determined by MALDI-TOF-MS. MIPs and SP were further purified with HPLC and checked for correct mass and identity by either MALDI-TOF-MS or sequencing. Peptides were stored at -20°C as freeze-dried stocks that were dissolved in appropriate buffer directly prior to use.

### Aequorin assay

Transfected and control CHO-WTA11 cells were grown until 90% confluency, detached using 1× PBS with 100 µM EDTA, spun (6 min, 150 g) and resuspended at 5 × 10<sup>6</sup> cells/ml in D-MEM/F-12 without phenol red supplemented with 0.1% BSA. 'Coelenterazine h' (Invitrogen) was added to a concentration of 5 µM and the cells were incubated in the dark at room temperature for 3–4 h.

After coelenterazine loading, cells were diluted ten times and incubated for another 30 min.

Test substances were dissolved in 50  $\mu$ l D-MEM/F-12, 0.1% BSA and dispensed in triplicate into the wells of a white 96-well plate. Light emission was recorded (EG&G Microplate Luminometer LB96V, Berthold) for 30 s immediately after injection of 50  $\mu$ l cell suspension into each well. Cells were then lysed by a second injection of 50  $\mu$ l 0.3% Triton X-100, followed by an 8-s monitoring period. Results were calculated by means of Winglow software (PerkinElmer) as the fractional luminescence, i.e., the ratio of the agonist generated signal and the total luminescence (agonist + lysed cells), thereby correcting for potential well-to-well variation in the number of injected cells [23]. The resulting data were then transferred to, and processed by SigmaPlot (SSI) software.

#### cAMP assay

HEK cells (mock- or receptor-transfected) were dispensed in 24-well plates at a density of 250,000 cells/well. After 24 h, cells were washed with PBS and incubated for 30 min in 200  $\mu$ l PBS containing 200  $\mu$ M 3-isobutyl-1-methylxanthine, 10  $\mu$ M forskolin and the experimental concentration of peptide agonist (six wells per condition). Forskolin was added to stimulate cAMP production, since this treatment accentuates the SPR-mediated cAMP reduction. The incubation was stopped by adding two volumes of ice-cold 100% ethanol. The extracts were collected in polypropylene tubes, centrifuged to remove cell debris, and the supernatants dried. Each sample was dissolved in 250  $\mu$ l of assay buffer (0.05 M Tris, 4 mM EDTA; pH 7.5) and cyclic AMP concentrations were determined according to the manufacturer's protocol (cAMP  $^3$ H Assay System, GE Healthcare).

#### Fly stocks and behavioral assay

*Drosophila melanogaster* (Canton S strain) flies were kept at 25°C on a 12:12 light:dark cycle and maintained on a diet of standard corn meal-yeast-agar medium. Three to four-day-old virgins were injected in the abdomen (FemtoJet, Eppendorf) with 50 nl peptide (6 pmol/female) dissolved in *Drosophila* Ringer (46 mM NaCl, 182 mM KCl, 3 mM CaCl<sub>2</sub>, 10 mM Tris; pH 7.2). Injections with Ringer alone were used as controls. Injected flies were placed in food vials (24-well plates; 1–2 flies/well) and the number of eggs laid was counted 24 and 48 h later. MIP RNAi flies were obtained from the Vienna *Drosophila* RNAi Center (stock 5294).

#### Real-time PCR analyses

Total RNA from different stages and tissues from *D. melanogaster* was extracted according to the manufacturer's

protocol (RNeasy Mini kit, Qiagen). In combination with this extraction procedure, a DNase treatment ('RNase-free DNase set', Qiagen) was performed to eliminate potential genomic DNA contamination. After quantification and verification of the RNA quality (Agilent Bioanalyzer, Agilent Technologies), the resulting total RNA was reverse transcribed (Superscript III, Invitrogen). To minimize variations during the cDNA synthesis step, all RNA samples were reverse transcribed simultaneously. Furthermore, several negative control reactions, i.e., without the reverse transcription enzyme were prepared and analyzed in parallel with the unknown samples during the quantitative PCR assay. PCR reactions were performed in a 35- $\mu$ l reaction volume following the manufacturer's instructions for the Sybr greenI-assay (Applied Biosystems): the final concentration of the primers was 300 nM and 200 ng of transcribed RNA was used per well. Primer sequences were: 5'-agcagccgaatgtcaggaat-3' and 5'-gaatttcgactctggcagtttc-3' (*CG16752*), 5'-gtaagcggaacccacatg-3' and 5'-cctgtgtacggcgattctc-3' (*MIP*), 5'-gcctacaaagtccaattccaa-3' and 5'-gcgggcccaattaaga-3' (*SP*) and 5'-gaagtcctggtgcacaa cgt-3' and 5'-aaacgcggttctgcatgag-3' (*rp49*). Relative standard curves were generated by serial (5 $\times$ ) dilutions of a mix of *Drosophila* cDNA from different stages and tissues. Reactions were run in triplicate (ABI Prism 7000 SDS, Applied Biosystems) using the following thermal cycling profile: 50°C (2 min), 95°C (10 min), followed by 40 steps of 95°C for 15 s and 60°C for 60 s. After 40 cycles, samples were run for the dissociation protocol, showing a single melting peak. Results were analyzed using the ABI Prism 7000 SDS software and, in order to compensate for differences in loading and RT efficiency, values were normalized relative to *rp49*.

## Results

*Drosophila* sex peptide receptor is activated by SP and MIPs

*CG16752*, initially an orphan *Drosophila* GPCR, was co-expressed with G $\alpha_{16}$  (a promiscuous G protein that couples most GPCRs to phospholipase C $\beta$  activation and consequently to the mobilization of intracellular Ca<sup>2+</sup>) and aequorin in CHO-cells, allowing for a rapid detection of receptor mediated Ca<sup>2+</sup>-responses [24]. Using 10  $\mu$ M of each peptide from an extensive library of 76 *Drosophila* peptides, it was shown that both SP and MIPs were able to generate intracellular Ca<sup>2+</sup>-increases in receptor—but not in mock-transfected cells. Recently, Yapici et al. [9] reported that *CG16752* is indeed a functional receptor for SP (henceforth referred to as SPR) and that SPR is both necessary and sufficient to induce different post-mating

**Table 1** *Drosophila* MIP and SP amino acid sequences and corresponding EC<sub>50</sub> values (nM) on different insect SP receptors

Peptide	Sequence	Drm-SPR	Bom-SPR	Trc-SPR
MIP1	AWQSLQSSW <sub>a</sub>	15.1	6.9	210
MIP2	AWKSMNVAV <sub>a</sub>	23.4	20.8	>1,000
MIP3	EPTWNNLKGMW <sub>a</sub>	24.9	4.1	16.7
MIP4	DQWQKLHGGW <sub>a</sub>	6.8	6.1	22.2
MIP5	pQAQGWNKFRGAW <sub>a</sub>	10.8	4.6	8.9
SP	WEWPWNRKPTKFP <sub>IP</sub> SPNPRDKWCRLNLGPAWGGR <sub>C</sub>	14.8	54.6	10.7
DUP99B	pQDRNDTEWISQKDREKWCRLNLGPYLGGR <sub>C</sub>	ND	ND	ND

Conserved tryptophan residues are indicated in **bold**, a: amide. Hydroxylated prolines are underlined, as well as cysteine residues involved in disulfide bonding. DUP99B (Ductus ejaculatorius peptide 99B, a less potent SPR ligand) is given for comparison. *Drm* *Drosophila melanogaster*, *Bom* *Bombyx mori*, *Trc* *Tribolium castaneum*, ND not determined

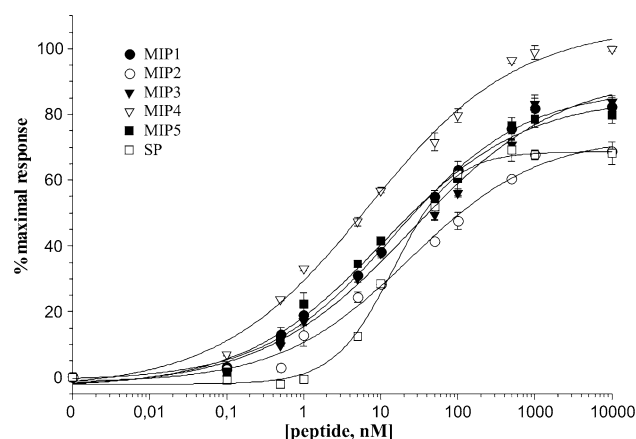
responses in female fruit flies (decreased receptivity, increased oviposition). The *Drosophila* MIP peptide precursor encodes five different MIP isoforms (Table 1). Both SP and all five MIPs activate SPR with comparable efficiencies and potencies (EC<sub>50</sub> values range from 6.8 to 24.9 nM, Fig. 1; Table 1), with MIP4 being the most potent and efficacious ligand. When MIPs and SP were applied together, no additional or synergistic receptor mediated activation was detected as compared to the same total concentration of MIP or SP only (data not shown).

#### MIPs activate orthologous insect SP receptors

Since SPR orthologs, as well as MIP precursor orthologs, appear in most sequenced insect genomes, we determined whether these receptors could also be activated by MIPs. Both the silk moth (*B. mori*) and the red flour beetle (*T. castaneum*) SPRs dose-dependently responded to *Drosophila* MIPs. Except for MIP2, EC<sub>50</sub> values in the nanomolar range were obtained (Fig. 2; Table 1). However, efficiencies of *Drosophila* MIPs 1, 4, and 5 and SP were substantially lower for the *Tribolium* receptor when compared to the *Drosophila* and *Bombyx* SPR. It should be noted that functional responses on the *Tribolium* SPR were only acquired when a (partial) Kozak sequence (CACC) preceded the receptor open reading frame, which might provide an explanation of why Yapici et al. [9] did not record SP activation on the *Tribolium* SPR.

#### Identification of common structural determinants necessary for SP and MIP induced SPR activation

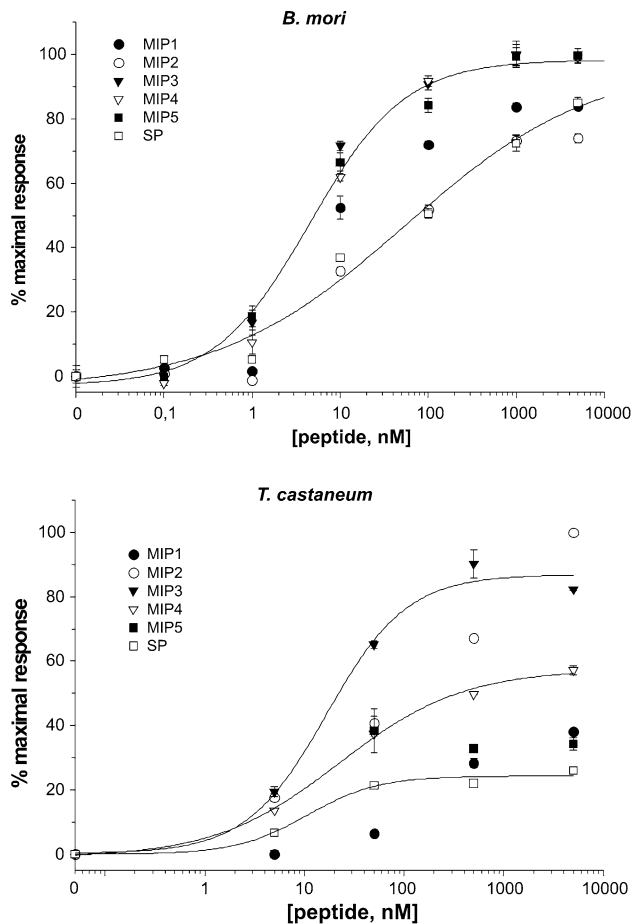
Except for two partially conserved C-terminal tryptophan residues, no obvious primary sequence similarity occurs between *Drosophila* SP and MIPs. Therefore, one explanation for the agonistic properties of both SP and MIPs may reside in shared two-dimensional structural determinants. We hypothesized that the conserved tryptophan residues in MIPs (i.e., the C-terminal W(X)<sub>6</sub>W motif) are



**Fig. 1** SPR is a receptor for both SP and MIPs. CHO-cells co-expressing aequorin, Gα<sub>16</sub>, and *Drosophila* SPR dose-dependently increase intracellular Ca<sup>2+</sup>-concentration when challenged with *Drosophila* SP and MIPs 1–5. Data points represent mean ± SD of three independent measurements done in triplicate and are given in percentage of the maximal response. The zero response level corresponds to treatment with buffer only

likely to play a pivotal role in biological activity. Similarly, SP also harbors a Trp-rich region (i.e., W(X)<sub>8</sub>W) that may represent a major structural feature. To test this hypothesis, WTA11 cells were transiently transfected with *Drosophila* SPR and different synthetic SP and MIP analogs were tested for their ability to stimulate the receptor. In each experiment SP or MIP4 (i.e., the most potent SPR ligand) were used as controls.

In these assays we included an N-terminally truncated form of SP, designated as SP<sub>8–36</sub> (K-Hyp-TKF-Hyp-I-Hyp-S-Hyp-N-Hyp-RDKW<sub>1</sub>CRLNLGPAW<sub>2</sub>GGRC; with Hyp being hydroxylated P), and showed that this form is equally efficient in eliciting a receptor-mediated response than SP itself. Substitution of W<sub>1</sub> with Ala (SP<sub>8–36</sub> AW) reduces the efficiency of SP<sub>8–36</sub> significantly and an additional Ala substitution of W<sub>2</sub> (SP<sub>8–36</sub> AA) almost totally abolished its activity, clearly demonstrating the major importance of the two tryptophan residues for signaling via SPR (Fig. 3a).



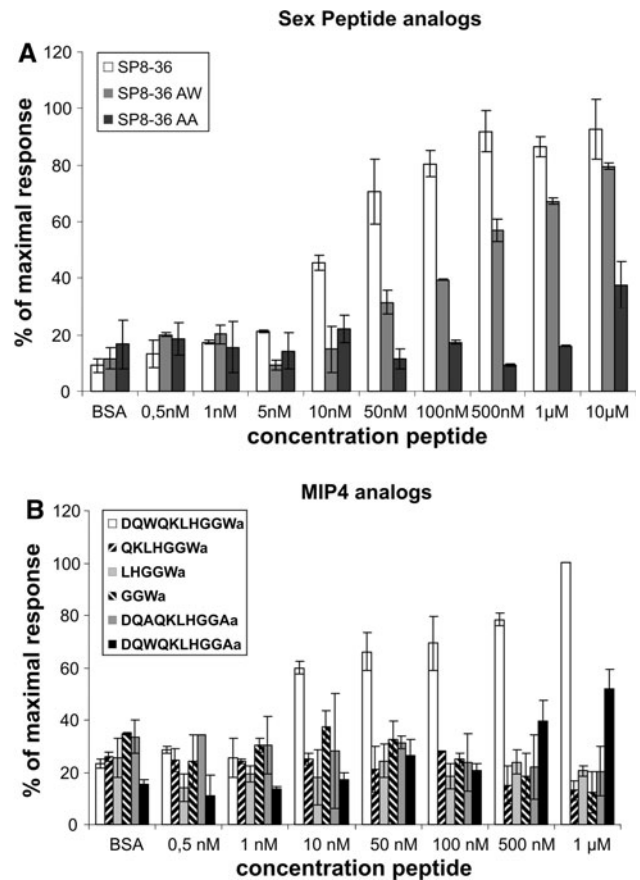
**Fig. 2** Orthologous insect SPRs are activated by SP and MIPs. Dose-response curves in CHO-cells co-expressing aequorin,  $G\alpha_{16}$  and the *Bombyx* (upper panel) or *Tribolium* SPR (lower panel), when challenged with *Drosophila* SP and MIPs 1–5. Data are represented as mean  $\pm$  SD of three independent measurements done in triplicate and are given in percentage of the maximal response. For clarity, only dose-response curves for MIPs 3 and 4 and SP were drawn

MIP4 analogs were synthesized and functionally tested in the aequorin assay. Whereas MIP4 itself generates a clear response on SPR expressing cells, a MIP4 analog lacking the two conserved tryptophan residues (DQAQKLHGGAA) is inactive. Other analogs that lack the N-terminal DQW residues (QLHGGWa) or with the C-terminal Trp substituted by Ala (DQWQKLHGGAA), fail to activate SPR as well (Fig. 3b).

Together, these data support the idea that the C-terminal tryptophan residues are crucial for the activity of MIP peptides and SP on SPR.

*Drosophila* SPR is a constitutively active  $G\alpha_{i/o}$  coupled-receptor

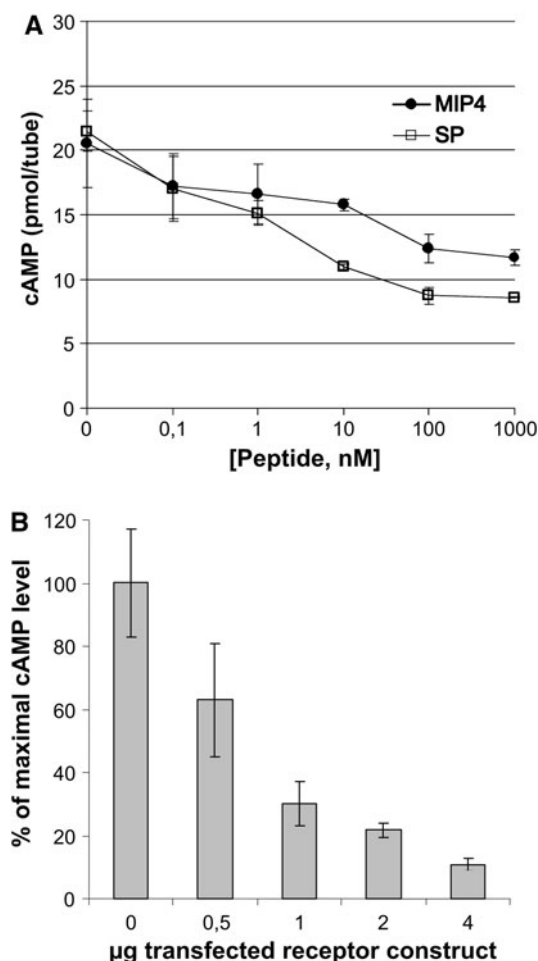
It was previously suggested that SPR couples to the cAMP pathway, as mutant flies with abnormal cAMP levels did



**Fig. 3** C-terminal Trp residues in SP and MIPs are important for activity on SPR. **a** The C-terminal Trp residues in SP ( $W_1(X)_8$   $W_2$ GGRC) are crucial for its activity. Three different SP analogs were tested in the aequorin assay to monitor their agonistic properties on SPR. An N-terminally truncated form of SP lacking the first seven amino acids ( $SP_{8-36}$ ) was shown to be an equally potent and efficient agonist as SP itself. Replacement of Trp residue  $W_1$  by an Ala residue ( $SP_{8-36}$  AW) results in a severe drop of potency and efficiency. When both  $W_1$  and  $W_2$  are substituted by Ala ( $SP_{8-36}$  AA), peptide activity is almost totally abolished. Data are represented as mean  $\pm$  SD of an experiment done in duplicate and are given in percentage of the maximal response. The same experiment was repeated twice with similar results. **b** Different analogs of MIP4 were tested in the same assay. N-terminally truncated forms of MIP4 that lack the first Trp residue lose their agonistic activity. Also, when the fully conserved Trp residues are replaced with Ala (DQAQKLHGGAA or DQWQKLHGGAAa), the activity of the peptide is abolished. All data are represented as mean  $\pm$  SD of an experiment done in duplicate and are given in percentage of the maximal response. Parallel experiments were performed twice with similar results

not properly react to SP [25]. Additionally, when *Drosophila* SPR was not co-expressed with  $G\alpha_{16}$ ,  $Ca^{2+}$ -responses induced by SP and MIP were very weak, probably indicating the coupling of the receptor to  $G\alpha_{i/o}$  or  $G\alpha_s$  G proteins (data not shown). Therefore, we performed cAMP measurements on SPR-transfected HEK cells. All MIPs and SP dose-dependently decreased intracellular cAMP concentrations (Fig. 4a for dose-response curves for



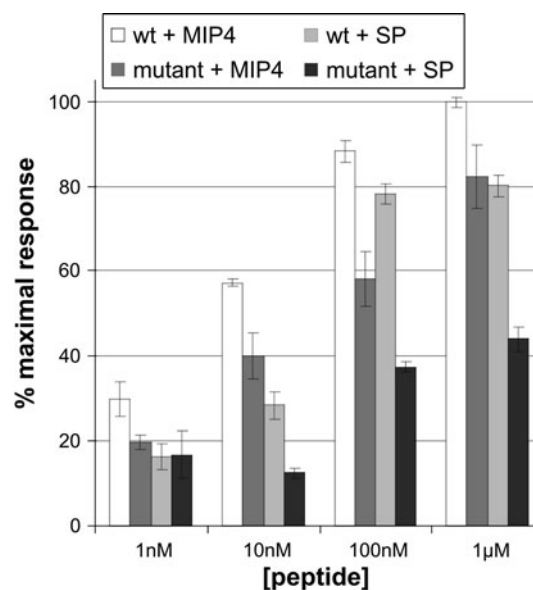


**Fig. 4** *Drosophila* SPR is coupled to a decrease in cAMP levels. **a** Dose–response curves of HEK cells transfected with *Drosophila* SPR when challenged with MIP4 or SP. Data points correspond to mean cAMP concentration (pmol/tube)  $\pm$  SD ( $n = 8$ ). **b** *Drosophila* SPR is a constitutively active GPCR. Basal cAMP levels of HEK cells decrease when transfected with increasing amounts of *Drosophila* SPR expression construct per 25 cm<sup>2</sup> culture flask. The total amount of DNA was kept constant using empty expression vector. Data are represented as mean  $\pm$  SD ( $n = 15$ ).

MIP4 and SP). Interestingly, by comparing basal cAMP levels between SPR-transfected and mock-transfected cells, a ligand-independent cAMP-decreasing activity of the receptor was apparent. The level of this cAMP decrease depended on the amount of transfected receptor expression construct (Fig. 4b). Moreover, *Bombyx* SPR also constitutively induced a decrease of cAMP levels when expressed in HEK cells (data not shown).

A conserved QRY motif in invertebrate SPRs is important for SP-mediated receptor activation

Most class A (rhodopsin family) GPCRs contain a well-conserved Glu/Asp-Arg-Tyr (E/DRY) sequence that is present at the boundary between transmembrane domain 3



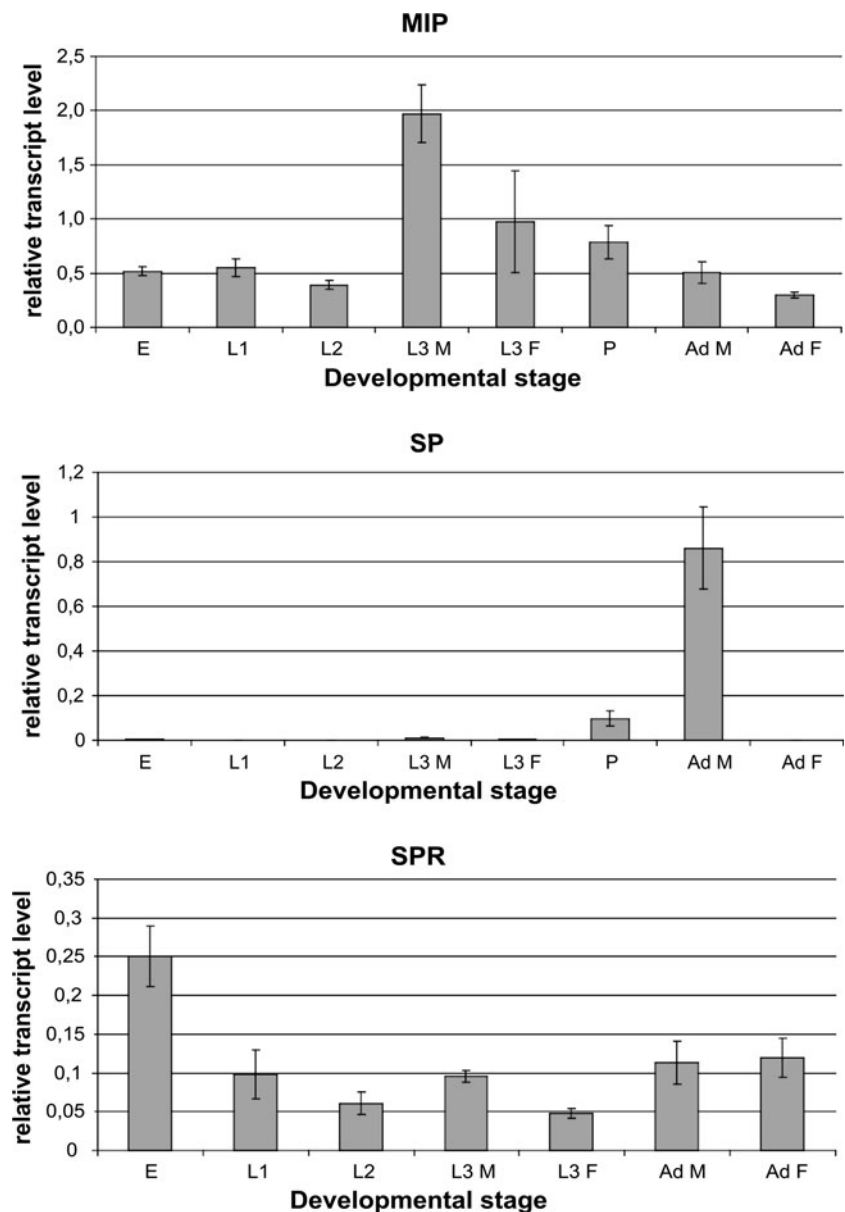
**Fig. 5** The conserved QRY motif of SPR differentially influences SP- and MIP-mediated responses. *Drosophila* wild-type SPR (containing a QRY motif) and mutated SPR (containing a DRY motif) respond differently to *Drosophila* SP and MIPs (MIP4 displayed as a representative example). Data are represented as mean  $\pm$  SD ( $n = 6$ ).

and intracellular loop 2. The E/DRY motif has been shown to be important for keeping unbound class A GPCRs in an inactive conformation [26]. Sequence comparison of 24 orthologous invertebrate SPRs indicated that this E/DRY motif was naturally replaced by a QRY sequence. Reintroducing the DRY sequence in *Drosophila* SPR by mutagenesis did not abolish the constitutive activity, but rendered the receptor less responsive to SP (i.e., a decrease in SP efficiency). Interestingly, the ligand inducible function for MIPs was much less affected, indicating that this effect is not simply due to a change in receptor stability and expression (Fig. 5).

MIP, SP, and SPR developmental and spatial expression patterns

During development, MIP transcript levels are boosted in male third instar larvae, as measured by real-time PCR. Transcripts are also more abundant in adult males than in females. As anticipated, SP transcripts are mainly found in adult males (with some expression in pupae). SPR expression was detected in all developmental stages in males and females (Fig. 6). More specifically, we detected SPR expression in female, but also in male heads, an observation that was also made by Yapici et al. [9]. While SP transcripts are clearly absent, MIP transcripts are highly expressed in male and female heads. As expected, SP transcripts are vastly abundant in male reproductive organs (Fig. 7).

**Fig. 6** Relative quantification of MIP, SP, and SPR transcript levels in different stages of *Drosophila melanogaster*. Samples were analyzed in triplicate and the assays were repeated twice with a biological replicate. Data represent mean values  $\pm$  SD, normalized relative to the endogenous control. *Ad* adult, *E* embryo, *F* female, *L1–3* larval stages 1–3, *M* male, *P* pupae



MIP injection does not induce egg-laying in virgin females

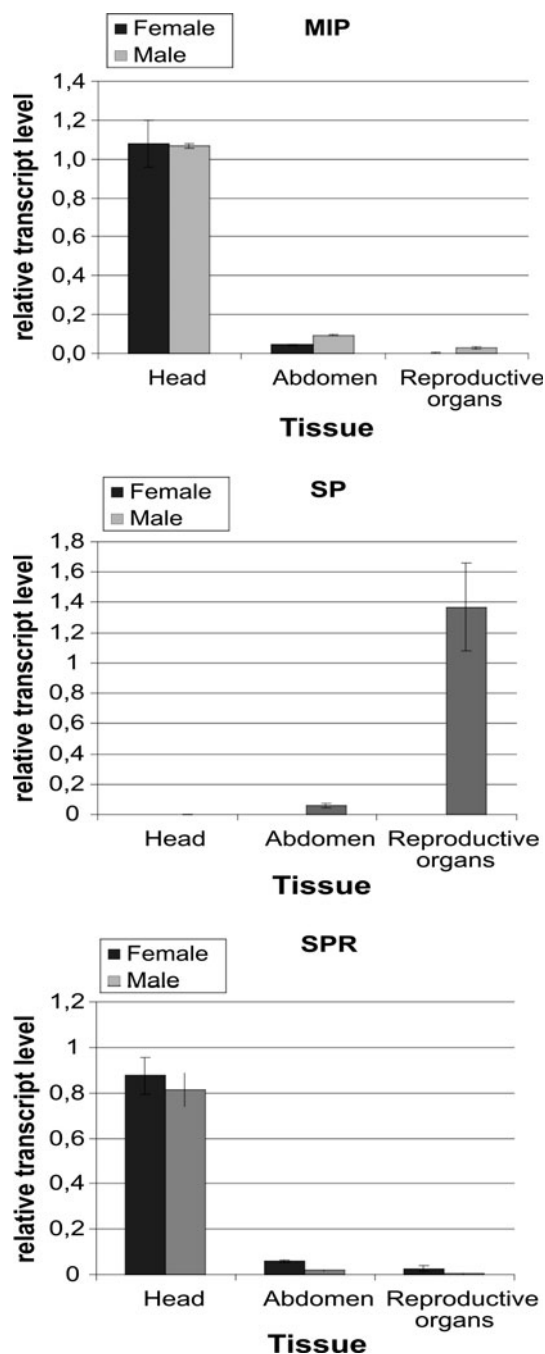
Knockdown of MIP expression using a UAS-RNAi transgene targeting MIP, combined with a general *Gal4* driver (*actin5C-Gal4*) or a pan-neuronal driver (*elav-Gal4*), did not result in a modification of oviposition rate (data not shown). Additionally, when MIPs were injected in the abdomen of virgin females (50 nl containing 6 pmol peptide), no increase in egg-laying rate could be observed 1 or 2 days post-injection. This was in contrast to virgins that were injected with 6 pmol SP and that clearly showed an increase in oviposition. When MIPs and SP were injected together, no differences in egg laying rate were monitored when compared to SP injection alone (Fig. 8). Thus, in

virgin females, MIPs are not essential for oviposition and do not induce egg laying at the injected dose.

## Discussion

MIPs and SP activate insect sex peptide receptors

While MIPs contain 9–12 amino acids with a C-terminal W(X)<sub>6</sub>Wamide consensus sequence, SP is a 36-amino acid peptide that includes different functional domains [10, 15, 27]. We demonstrate that the two C-terminal Trp residues of MIPs as well as of SP are necessary for total receptor activation. Moreover, DUP99B (Ductus ejaculatorius peptide 99B), another male reproductive peptide that is



**Fig. 7** Relative quantification of MIP, SP, and SPR transcript levels in different tissues of *Drosophila melanogaster*. Samples were analyzed in triplicate and the assays were repeated twice with a biological replicate. Data represent mean values  $\pm$  SD, normalized relative to the endogenous control. Abdomen: all abdominal tissues except reproductive tissues. Male reproductive organs: testes, seminal vesicles, accessory glands, ejaculatory duct and ejaculatory bulb. Female reproductive organs: ovaria, oviducts, spermatheca, and uterus

homologous to SP but that lacks the most C-terminal Trp residue, is less potent in activating SPR when compared to SP [9, 28]. While the spacing, position, and amidation of

these Trp residues differ between MIPs and SP (Table 1), SP contains a C-terminal disulfide bridge that is required for activity and the dual activation of SPR could thus be explained by conservation of the Trp residues at the secondary structure level. The C-terminal part of SP (SP<sub>8-36</sub>) is indispensable for the induction of post-mating responses [27, 29] and is gradually released from sperm tails by cleavage at a trypsin-like proteolytic cleavage site [30]. We further substantiate this finding by showing that SP<sub>8-36</sub> activates SPR with equal potency and efficiency as the complete SP.

The comparable efficiencies and potencies displayed by both ligands on *Drosophila* SPR are remarkable. Whether they bind to the same binding site or different locations on the receptor is currently unknown, but no reciprocal influence (i.e., no antagonism or extra stimulation) on SPR activation could be detected when both peptides were applied together. Few other *Drosophila* GPCRs have been reported to functionally respond to peptides that originate from different precursors, but in these cases there is either extensive homology between the agonists, or their corresponding EC<sub>50</sub>-values differ considerably [31–33].

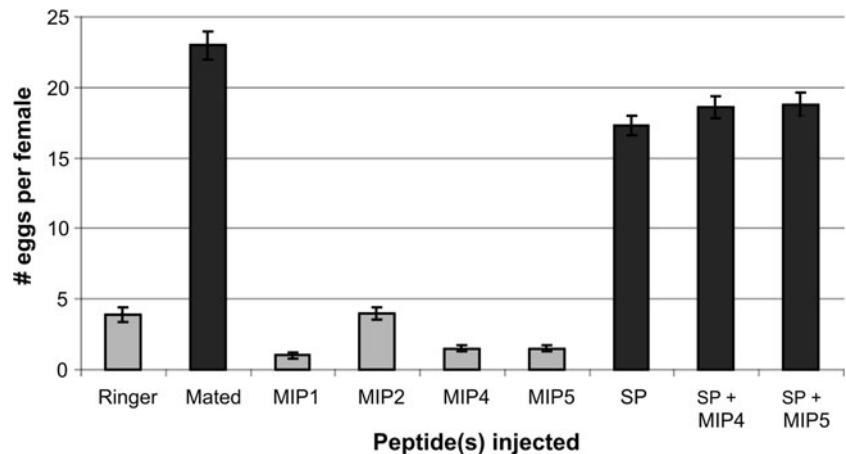
Are there other MIP receptors present in *Drosophila*? The existence of more than one GPCR for a single peptide family has been demonstrated multiple times in fruit flies, e.g., for tachykinins [32, 34]. Johnson et al. [31] reported GFP-labeled  $\beta$ -arrestin translocation to the cell membrane in *CG14484*-expressing HEK cells when applying 1  $\mu$ M MIP1 for 20 min. It would be interesting to determine whether a second MIP receptor could be stimulated by SP. However, we did not detect any MIP- or SP-induced responses when *CG14484* was expressed in the CHO- $G\alpha_{16}$ -aequorin cell line. It should be noted that the orphan GPCR *CG14593* is highly related to *CG14484* and thus represents another putative MIP receptor in *Drosophila*. Moreover, it cannot be ruled out that as yet undiscovered additional MIP receptors exist or that different conformations of SPR are present in vivo that confer differential MIP or SP specificity.

#### MIPs and SPR are expressed in the CNS

How can a single receptor be activated by two different ligands and still retain specific responses in certain physiological situations? A combination of factors can be of influence: the two ligands could be expressed at different time points or in diverse tissues or the accessibility of the receptor for the agonists could differ. Indeed, SP shows a very restricted male-specific expression pattern [10]. Yapici et al. [9] already reported the expression of SPR in the male CNS, but could not attribute a function to this since SP expression is absent from the male CNS. This is in contrast to MIPs that are produced in the brain and ventral



**Fig. 8** Induction of oviposition upon injection of SP and/or MIPs in virgin females. Six picomoles of the indicated peptides dissolved in Ringer's solution were injected into the abdomen of virgin females and the number of eggs laid/female per 24 h was counted. Mated flies: no peptide injected. Data represented as mean  $\pm$  SEM ( $n = 30$ )



nerve cord in *Drosophila* as well as in many other insects and thus form a likely ligand for SPR in these tissues [2, 35–37].

#### Functional interactions of MIPs and SP

Injecting MIPs into the abdomen of virgin flies does not enhance oviposition (in contrast to SP injections) indicating the possibility of MIPs not reaching the SPR target. This could be caused by an inability to access the genital tract, as has been shown for ectopically expressed SP (in the fat body) which could not induce an epithelial immune response in the oviduct (another SP effect in females) [15, 38]. Proteolytic breakdown of injected MIPs might be involved also, as reported for SP, which specifically binds to sperm tails to protect it from degradation by proteases [30, 39, 40]. In vivo, male transfer of MIPs during mating is very unlikely, since males deficient in SP only, do not provoke post-mating responses in females [39]. In addition, we could not detect MIP immunoreactivity in male accessory glands or in the female reproductive system of the fruit fly (data not shown).

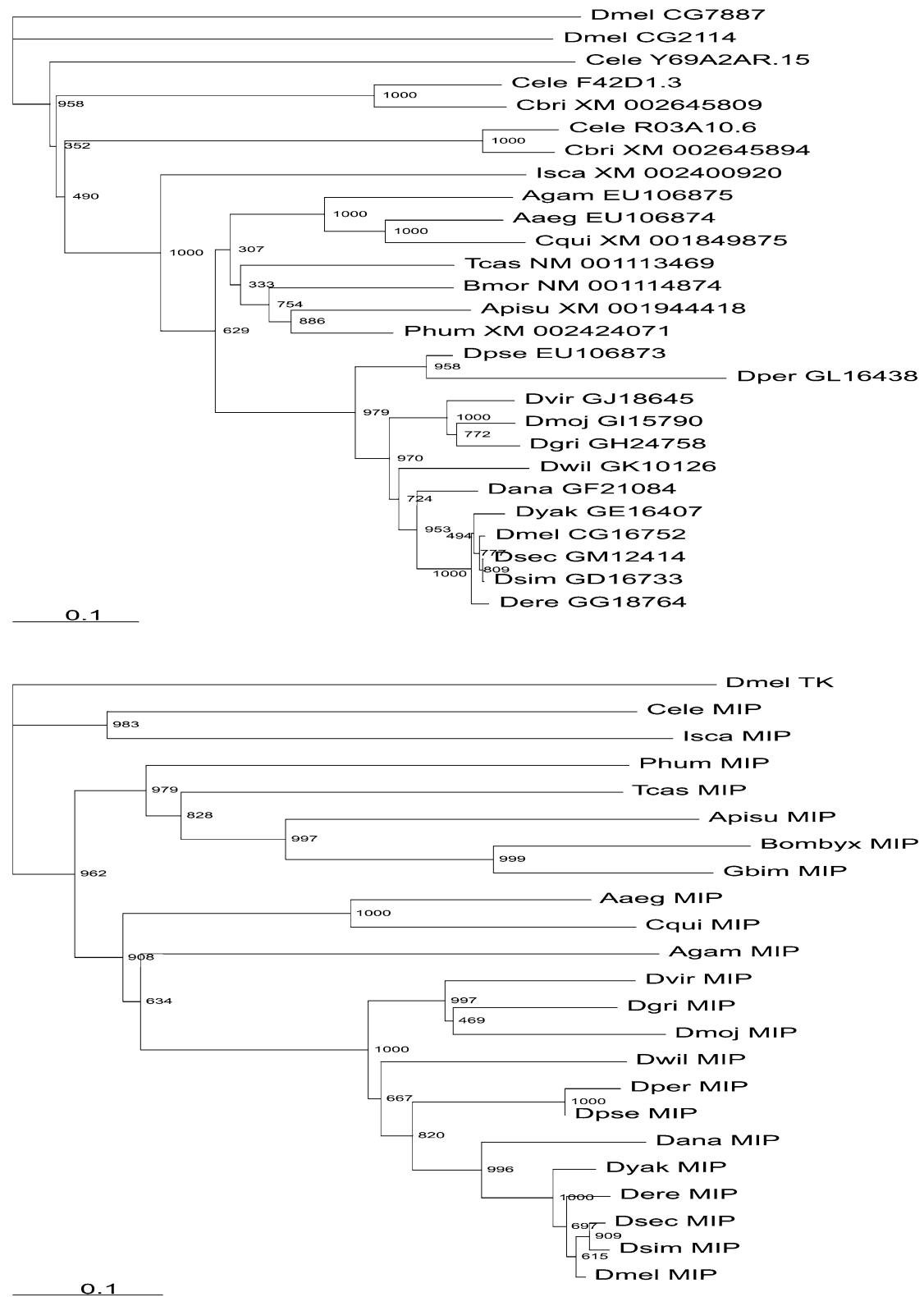
No definite functions have been ascribed to *Drosophila* MIPs, although an eliciting role in ecdysis has been postulated [37]. Yamanaka et al. [41] recently showed that *B. mori* prothoracicostatic peptides (MIP orthologs) reduce ecdysteroid synthesis via SPR. Although this was not further investigated in the silk moth, ecdysteroid levels play an important role in the control of oocyte maturation and are influenced by mating in *Drosophila* [42].

#### Evolutionary considerations

Reproductive proteins, such as the accessory gland proteins (Acps) from insects, show a rapid adaptive evolution [43–47], which can be driven by sexual conflict [48, 49]. The general conceptual context explaining the rapid evolution of male sex-related genes has been discussed in a review by

Singh and Kulathinal [50]. Since *Drosophila* seminal proteins reduce female lifespan (depending on the food conditions) [11, 13, 51, 52], this could theoretically lead to sexually antagonistic coevolution, where females would counter-adapt to the male-derived peptide effects [53, 54], e.g., by modulating receptors for Acps. Unambiguous SP orthologs are difficult to discover in insect genomes other than *Drosophila* [9, 55, 56]. This is in sharp contrast to the corresponding SPRs and to MIPs which form an evolutionary well-conserved ligand-receptor couple in insects (for phylogenetic trees of MIPs and SPRs, see Fig. 9). Since MIPs too bind SPRs, they could place strong evolutionary constraints, forming an explanation for the discrepancy in conservation of the SP–SPR pair. It is thus tempting to speculate that SP hijacked the female insect MIP receptor in some invertebrate species during evolution while MIPs retained their endogenous function as SPR ligands in males and in tissues or stages where SP is absent.

Interestingly, the fruit fly as well as the silkworm SPR both showed considerable constitutive (ligand-independent) activity in cellular expression systems. Class A GPCRs, to which SPR belongs, possess a conserved Asp/Glu-Arg-Tyr (E/DRY) motif that is important in keeping the receptor in an inactive conformation. Nonconservative mutations of the Asp residue often result in constitutive activity [26]. All invertebrate SPR orthologs contain a charge-neutralizing substitution in the DRY pattern (i.e., Q instead of D) that is not present in the closest *Drosophila* SPR relatives. Surprisingly, changing the QRY motif back to a DRY sequence did not abolish constitutive activity, but decreased the efficiency of SP for the receptor. It thus seems that SPRs evolved a naturally occurring substitution of Asp in the DRY motif, which has affected the functional properties of the receptor protein. MIPs and SP further decrease cAMP levels when binding to SPR and thus do not function as inverse agonists. Also, Q-PCR data do not indicate that the receptor is transcriptionally regulated by mating (that is, transcript levels in virgins and mated



**Fig. 9** Phylogenetic trees for *Drosophila* SPR (upper panel) and MIP (lower panel) orthologs. The protein set was aligned using ClustalX [57] and a tree was calculated using the neighbor-joining method. Bootstrap support values (1,000 replicas) are indicated. *Drosophila* species: *ananassae*, *erecta*, *grimshawi*, *melanogaster*, *mojavensis*, *persimilis*, *pseudoobscura*, *sechellia*,

*simulans*, *virilis*, *willistoni*, *yakuba*. Aaeg, *Aedes aegypti*; Agam, *Anopheles gambiae*; Apisu, *Acyrtosiphon pisum*; Bmor, *Bombyx mori*; Cbri, *Caenorhabditis briggsae*; Cele, *Caenorhabditis elegans*; Cqui, *Culex quinquefasciatus*; Gbim, *Gryllus bimaculatus*; Isca, *Ixodes scapularis*; Phum, *Pediculus humanus corporis*; Tcas, *Tribolium castaneum*

females are similar). Noteworthy, females with elevated cAMP levels due to the *dunce* mutation show increased remating rates, even after SP injection [25], indicating that tightly regulated cAMP levels are crucial for proper SPR responses.

## Conclusions

While the *Drosophila* SPR has been clearly shown to be responsible for initiating SP-mediated post-mating responses in the adult female fly, we now refine and expand these findings by proving that this GPCR can be very effectively activated by a seemingly distinct class of peptide ligands; i.e., the family of MIPs/B-type allatostatins. Furthermore, this remarkable feature is preserved with orthologous receptors from other insects. While SP genes, as most reproductive genes, evolve rapidly, its binding partner, SPR, is unexpectedly well conserved. This could now be explained by the fact that SPRs bind to a second, well-conserved peptide family that causes evolutionary constraints. It is therefore very likely that, at some point in evolution, male-derived SP hijacked the MIP receptor and co-evolved as a natural peptide mimetic of the more ancestral MIP agonists. In addition, the MIP/SPRs evolved a nonconservative amino acid substitution in a domain that is involved in generating constitutive activity and SP-mediated activation. As this is the first report on the complete functional characterization of a *Drosophila* MIP receptor, these findings will undoubtedly stimulate future research on this pleiotropic family of brain-gut peptides. Additionally, it will facilitate characterization of receptor orthologs from various medical, veterinary, and agricultural pest species, such as the malaria mosquito, which may constitute promising targets for the development of novel control agents or strategies.

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