Can BRET-based biosensors be used to characterize G-protein mediated signaling pathways of an insect GPCR, the \textit{Schistocerca gregaria} CRF-related diuretic hormone receptor?

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\textbf{ABSTRACT}

G protein-coupled receptors (GPCRs) are membrane-bound receptors that are considered prime candidates for the development of novel insect pest management strategies. However, the molecular signaling properties of insect GPCRs remain poorly understood. In fact, most studies on insect GPCR signaling are limited to analysis of fluctuations in the secondary messenger molecules calcium (Ca\textsuperscript{2+}) and/or cyclic adenosine monophosphate (cAMP). In the current study, we characterized a corticotropin-releasing factor-related diuretic hormone (CRF-DH) receptor of the desert locust, \textit{Schistocerca gregaria}. This \textit{Schgr-CRF-DHR} is mainly expressed in the nervous system and in brain-associated endocrine organs. The neuropeptide \textit{Schgr-CRF-DH} induced Ca\textsuperscript{2+}-dependent aequorin-based bioluminescent responses in CHO cells co-expressing this receptor with the promiscuous G\textalpha\textsubscript{16} protein. Furthermore, when co-expressed with the cAMP-dependent bioluminescence resonance energy transfer (BRET)-based CAMYEL biosensor in HEK293T cells, this receptor elicited dose-dependent agonist-induced responses with an EC\textsubscript{50} in the nanomolar range (4.02 nM). In addition, we tested if vertebrate BRET-based G protein biosensors, can also be used to detect direct G\textalpha protein subunit activation by an insect GPCR. Therefore, we analyzed ten different human BRET-based G protein biosensors, representing members of all four G\textalpha protein subfamilies; G\textalpha\textsubscript{s}, G\textalpha\textsubscript{i/o}, G\textalpha\textsubscript{q/11} and G\textalpha\textsubscript{12/13}. Our data demonstrate that stimulation of \textit{Schgr-CRF-DHR} by \textit{Schgr-CRF-DH} can dose-dependently activate G\textalpha\textsubscript{i/o} and G\textalpha\textsubscript{s} biosensors, while no significant effects were observed with the G\textalpha\textsubscript{q/11} and G\textalpha\textsubscript{12/13} biosensors. Our study paves the way for future biosensor-based studies to analyze the signaling properties of insect GPCRs in both fundamental science and applied research contexts.

1. Introduction

Neuropeptides regulate key biological processes, such as development, growth, metabolism, ecdysis, feeding, and reproduction, in a very precise and controlled manner. The majority of neuropeptides signal through G protein-coupled receptors (GPCRs), membrane-bound receptors which are often regarded as candidate targets for novel insect pest management strategies (Gäde and Goldsworthy, 2003; Verlinden et al., 2014). The GPCR superfamily has been studied extensively in vertebrates, since these receptors are pharmacological targets for many important therapeutic compounds (Pierce et al., 2002). In insects, however, the molecular signaling properties of GPCRs remain poorly understood. Fortunately, numerous in vitro assays have been developed to study the GPCR signaling cascades. Most of these assays focus on fluctuations in downstream intracellular secondary messenger levels, such as Ca\textsuperscript{2+} and cyclic AMP (cAMP). For instance, an aequorin bioluminescence assay is frequently utilized to monitor possible changes in intracellular Ca\textsuperscript{2+} levels. Similarly, a cAMP response element (CRE) dependent luciferase reporter assay, using a CRE\textsubscript{Luc} construct, enables the monitoring of variations in intracellular cAMP levels. Both
induced resulting in a larger distance between the donor is not stimulated by a ligand, the donor R

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et al., 2015). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.) A schematic representation of this assay (adapted from Galés et al., 2006) is depicted in Fig. 1B. While this assay has proven its value in the study of the G protein-mediated signaling pathways of several vertebrate GPCRs (Audet et al., 2008; Bellot et al., 2015; Bruzzone et al., 2014; Busnelli et al., 2012; Capra et al., 2013; Corbisier et al., 2015; Damian et al., 2015; De Henau et al., 2016; Galandrin et al., 2008, 2016; Galés et al., 2006; Garcia et al., 2018; Hansen et al., 2013; Leduc et al., 2016; Mollayeva et al., 2017; Saulière et al., 2012; Schmitz et al., 2014; Schrage et al., 2015), to our knowledge, it has never been employed to analyze G protein-mediated signaling pathways of insect GPCRs, despite the fact that insect GPCRs are typically characterized by heterologous expression in mammalian cell lines.

In the current study, we therefore verified the application of BRET-based assays for insect GPCR research. For this purpose, we identified and characterized a corticotropin-releasing factor (CRF)-related diuretic hormone (DH) receptor from the desert locust Schistocerca gregaria, an insect known for its extreme form of density dependent polyphenism (Cullen et al., 2017). The role of its ligand, CRF-DH, has been well established as a diuresis stimulator in the excretory system of various insects (Cannell et al., 2016; Coast and Kay, 1994; Furuya et al., 2000; Te Brugge et al., 2011). Besides its diuresis activating properties, CRF-DH is suggested to induce satiety in S. gregaria, Locusta migratoria, and Rhodnius prolixus (Van Wielendaal et al., 2012; Audsley et al., 1997; Goldsworthy et al., 2003; Lee et al., 2016; Mollayeva et al., 2018). In addition, CRF-DH initiates pre-ecysis in Manduca sexta (Kim et al., 2006), regulates sperm retention and storage in the spermatheca in Drosophila melanogaster (Lee et al., 2015) and retards oocyte growth in S. gregaria (Van Wielendaal et al., 2012).

While the physiological role of Schgr-DH was previously investigated in S. gregaria (Van Wielendaal et al., 2012), its receptor was never molecularly characterized. Moreover, this newly characterized receptor, Schgr-CFR-DHR, was also chosen as a proof-of-principle, since it is well-documented that CRF-DHs exert effects on their cellular targets through their interaction with receptors belonging to the secretin receptor-related GPCR family (family B), for which cAMP has been identified to act as an intracellular second messenger of CRF-DH in numerous insects, both in vivo and in vitro (Audsley et al., 1995; Baldwin et al., 2001; Clottens et al., 1994; Hector et al., 2009; Johnson

**Fig. 1. Schematic representation of both BRET1 and BRET2 systems.**

**A** Schematic representation of the cAMP reporter assay using the CAMYEL biosensor: the protein exchange factor directly activated by cAMP (EPAC) is flanked by yellow fluorescent protein (YFP) and Rluc. Upon binding of cAMP to EPAC, a conformational change is induced which results in a larger distance between YFP and Rluc. As a consequence, bioluminescence energy transfer (BRET1) decreases. Coelenterazine acts as a substrate for Rluc (Image adapted from Matthias and Nielsen, 2011). **B** Schematic representation of the BRET2-based G protein biosensors. The energy donor, Rluc8, is genetically coupled into the helical domain of Ga. GFP10 is N-terminally linked to Gγ. In resting state, when the G protein-coupled receptor (GPCR) is not stimulated by a ligand, the donor Rluc8 and the acceptor GFP10 are in proximity, inducing a high BRET2 signal. Upon binding of the ligand to the receptor, this GPCR is activated, inducing the activation of the Ga subunit. When the Ga subunit is activated, GDP is exchanged for GTP and a large interdomain movement in Ga is induced resulting in a larger distance between the donor Rluc8 and the acceptor GFP10, consequently leading to a lower BRET2 signal (Image adapted from Bellot et al., 2015). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
et al., 2004; Lee et al., 2016; Reagan, 1996; Tobe et al., 2005). In addition, the production of primary urine is dependent on cAMP, which increases cationic transport (such as K⁺ and Na⁺) into the Malpighian tubules (Beyenbach, 1995; O'Donnell et al., 1996). The first insect CRF-DHR receptor (CRF-DHR) was characterized in vitro in M. sexta (Reagan, 1995), followed by in vitro characterizations of additional homologues in following species; Acheta domesticus (Reagan, 1996), R. prolixus (Lee et al., 2016) and D. melanogaster (Hector et al., 2009; Johnson et al., 2004).

In the present study, Schgr-CRF-DHR was cloned from a desert locust brain cDNA library. Quantitative real-time PCR (qRT-PCR) revealed spatial expression patterns of Schgr-CRF-DHR in tissues derived from the locust central nervous system. We have further characterized this receptor in cell-based functional receptor assays: downstream signaling effects were studied by means of the aequorin bioluminescence assay and the BRET-based CAMYEL (ATCC MBA-277) biosensor. Furthermore, direct activation of G-proteins was measured using the BRET-based biosensors (Galés et al., 2006).

2. Materials and methods

2.1. Sequence analysis of Schgr-CRF-DHR

The nucleotide sequence of a putative CRF-DHR receptor (Schgr-CRF-DHR) was identified by means of a local BLAST scan of a brain-specific transcriptome database of gregarious S. gregaria created by illumina-sequence reads (unpublished data). As depicted below, amino acid sequences of the putative Schgr-CRF-DHR were compared with amino acid sequences of other insect CRF-DHRs that have been characterized in vitro: D. melanogaster (Drome-DHR4-R1; GenBank: NP_610960.1, and Drome-DHR4-R2; GenBank: NP_610789.3), R. prolixus (RhoGR-CRF-DHR2B; GenBank: KJ407397), M. sexta (Manse-CRF-DHR; GenBank: AAC64699.1) and A. domesticus (Achdo-CRF-DHR; GenBank: AAC47000.1).

A multiple sequence alignment and a percent identity matrix were created using the EMBO-EBI Clustal Omega Multiple Sequence alignment software (http://www.ebi.ac.uk/Tools/msa/clustalo/). Conservation of the amino acids was predicted using MEGA 7. Figures were created using T-COFFEE Multiple sequence alignment server (http://tcofee.crg.cat/apps/tcoffee/doc/tcoffee) and the BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html). Putative transmembrane regions (TM1-TM7) were predicted by the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). An additional percent identity matrix was created using receptor sequences from TM1 to TM7 using the EMB-EBI Clustal Omega Multiple Sequence alignment software.

2.2. Rearing of animals and tissue collection

S. gregaria was reared under crowded conditions (> 200 locusts/ cage) as described by Lismont et al. (2015). In the reported experiments, multiple tissues were collected from both immature and mature gregarious locusts of both sexes, as CRF-DHR plays a role in reproduction of this locust species (Van Wielendaele et al., 2012). The locusts were synchronized on the day of adult eclosion and placed in separate cages. Immature males and females were dissected on day three after their molt into adults. The locust tissues and organs of interest were dissected immediately snap frozen in liquid nitrogen to prevent RNA degradation. Until further processing, the tissues were stored at −80 °C.

2.3. RNA extraction and cDNA synthesis

Depending on the tissue, different RNA extraction methods were used. RNA extractions of the tissues collected in the MagNa Lyser green beads tubes were performed utilizing the RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD), according to the manufacturer's protocol. A DNase treatment (RNase-Free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. Because of their relatively small size, RNA extractions of the tissues collected in the RNase-free Screw Cap Microcentrifuge tubes were executed using the RNaseous-Micro Kit (Ambion) according to the manufacturer's instructions. The recommended DNase step was subsequently performed. RNA quantity and quality were verified using a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.). cDNA of all samples was synthesized using the PrimeScript RT reagent Kit (Perfect Real Time) from Takara, according to the manufacturer's instructions. The resulting cDNA was diluted tenfold.

2.4. Quantitative real-time PCR (qPCR)

All primers used for qPCR profiling are presented in Supplementary Table S1. All qPCR reactions were performed in duplicate in 96-well plates on a StepOne System (ABI Prism, Applied Biosystems), as described by Lexaerts et al. (2017). Suitable reference genes were selected, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ubiquitin conjugating enzyme 10 (Ubi) and CG13320, from a pool of candidate reference genes by means of the geNorm software (Van Hiel et al., 2009; Vandesompele et al., 2002). All qPCR results were calculated according to the comparative delta Ct method and a mix of all samples was used as a calibrator. qPCR was used to determine the tissue distribution of the Schgr-CRF-DHR transcript, in immature and mature adult locusts. GraphPad Prism 6 (GraphPad Software Inc.) was used to plot the data and to test the statistical significance, using ANOVA, of the observed differences between immature and mature transcript levels.

2.5. Molecular cloning of Schgr-CRF-DHR

The full-length sequence of Schgr-CRF-DHR was found in the S. gregaria transcriptome database, as described in 2.1. The complete ORF was PCR-amplified using cDNA of brains and Pwo DNA Polymerase (Roche). The nucleotide sequences of the specific forward primer, containing the CACC Kozak sequence at the 5’ side to facilitate translation in mammalian cells (Kozak, 1986), and reverse primer, are enlisted in Supplementary Table S2. Amplicons were cloned on a 1% agarose gel, purified using the GenElute Gel Extraction Kit (Sigma-Aldrich), cloned into a pcDNA3.1/V5-His-TOPO TA expression vector (Invitrogen) and transformed into One Shot TOP10 chemically competent E. coli cells (Invitrogen). The sequence of the insert was confirmed by Sanger sequencing. Bacteria harboring the correct receptor insert were grown overnight in LB medium supplemented with 100 ng/mL Ampicillin (Invitrogen), and plasmid DNA was isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich).
2.6. Cell culture and transfection

Pharmacological studies were carried out in a Chinese hamster ovary (CHO)-WTA11 cell line, genetically modified to stably express apao-αequorin, a zeocin resistance gene, and the promiscuous Ga116 subunit coupling to the phospholipase C and Ca2+ signaling cascade (Euroscreen, Belgium). CHO-PAM28 cells (stably expressing apao-αequorin and a puromycin resistance gene) and human embryonic kidney (HEK) 293T cells were used to assess effects on the Ca2+ and/or cAMP second messenger systems, respectively.

Cell culture and transfections of CHO-WTA11 and CHO-PAM28 cells were performed as described by Lismont et al. (2015). HEK293T cells were cultured in monolayers at 37 °C with a constant supply of 5% CO2. Further analysis was performed using Graphpad Prism 6 (GraphPad Software Inc.).

2.8. BRET1-based CAMYEL biosensor assay

The original BRET system described by Xu and co-workers (1999) is referred to as BRET1 and uses Renilla luciferase (RLuc) as a donor with benzyl-coelenterazine (coelenterazine h) as a substrate in combination with enhanced yellow fluorescent protein (EYFP). The BRET1-based CAMYEL biosensor can be used to measure cAMP levels in living cells upon GPCR activation. The core of this biosensor consists of the cAMP-binding protein EPAC, flanked by the BRET probes RLuc and EYFP. The sensor changes conformation in response to increasing levels of cAMP, resulting in a decrease of BRET intensity. In this study, this biosensor is used to measure bioluminescent changes resulting from increasing intracellular cAMP levels upon exposure of cells, transfected with the Schgr-CRF-DHR expression vector (experimental condition), to increasing concentrations of Schgr-CRF-DH. As a negative control condition, cells were transfected with the CAMYEL biosensor only.

One day post-transfection, the co-transfected HEK293T cells were detached using PBS supplemented with 0.2% EDTA (pH 8.0) and collected in a falcon tube. The cells were pelleted for 4 min at 800 rpm and resuspended at a density of 0.5 × 10⁶ cells/mL in DMEM/F-12 without phenolred (Gibco) supplemented with 1% fungigzone (Gibco), 10% fetal bovine serum (FBS; Gibco), 1% sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin (stock solution: 10 000 units/mL penicillin and 10 mg/mL streptomycin; Invitrogen). Cells were sub-cultured twice a week.

For the BRET1-based G protein biosensor analyses, HEK293T cells were co-transfected using the calcium phosphate method (Jordan et al., 1996). Thus, cells were co-transfected with 4 μg CAMYEL vector, combined with 10 μg Schgr-CRF-DHR pcDNA3.1 receptor expression construct and 6 μg pcDNA3.1 TOPO empty vector or with 16 μg of pcDNA3.1 TOPO empty vector only. The cell medium was replaced with 10 mL fresh HEK293T culture medium half an hour prior to transfection and the DNA mixture was prepared in MilliQ water to a final volume of 500 μL.

For the BRET2-based G protein biosensor analyses, HEK293T cells were transiently transfected using the calcium phosphate method (with 1) one of the ten Ga-Rluc constructs, G12-GFP10, Gβ1, pcDNA3.1: Schgr-CRF-DHR, constituting the experimental conditions, or (2) one of the ten Ga-Rluc constructs, G12-GFP10, Gβ1, and the pcDNA3.1 empty vector, constituting the control conditions. The ten Ga-Rluc constructs are: Ga11, Ga12, Ga13, Ga16, representing the Gα11 subfamily, Ga11 and Ga16 representing the Gα12/13 subfamily, Ga3, as a representative of the Gs subfamily, and Ga12 and Ga13 representing the Gα12/13 subfamily. In all cells, the G protein heterodimer αGβ1 is expressed with Ga3, representing one of the ten Ga constructs. The cell medium was replaced half an hour prior to transfection with 10 mL fresh culture medium and the mixtures of DNA constructs were prepared as described in Supplementary Table S3.1 for the experimental conditions, and in Supplementary Table S3.2 for the controls. All mixtures were brought to a concentration of ~20 μg DNA in 500 μL MilliQ water.

For transfections using the calcium phosphate method, 50 μL CaCl2 (2.5 M) was added to the DNA mixture. Next, 500 μL HEPES-buffered saline (HBS (2x); 280 mM NaCl, 50 mM HEPES and 1.5 mM Na2HPO4; pH 7.1) were added. The transfected cells were maintained in an incubator at 37 °C (5% CO2). An additional transfection was performed with 4 μg CAMYEL vector and 16 μg empty vector, without pcDNA3.1 receptor construct, as an extra control. The transfected cells were maintained in an incubator at 37 °C (5% CO2) and on the next day the medium was replaced by fresh culture medium.

2.7. Aequorin reporter assay in CHO cells

The aequorin reporter assay was performed as described by Lismont et al. (2015). This assay will be used to measure calcium mobilization after Schgr-CRF-DHR activation. Peptides (Table 1) were ordered from GL Biochem (Shanghai, China) at 95% purity and further purified by RP-HPLC fractions that were subsequently controlled by MALDI. The peptides (Table 1) were tested in a dilution series (0.01 fM – 10 μM). Further analysis was performed using Graphpad Prism 6 (GraphPad Software Inc.).
performed in two separate transfections [for both (1) the experimental condition with insect receptor and (2) the control without insect receptor] per Gα construct (Gαi1, Gαi2, Gαi3, Gαs, Gαo, Gαob, Gαs, Gαi1, Gαi3, Gαq, Gαg12, or Gαg13). When a significant reaction was obtained, a second screen was conducted in duplicate per Gα construct to examine whether the obtained reaction was dose-dependent by using several concentrations of peptide (0.10 nM–1 μM).

The experiment was conducted two days post-transfection. After removal of the culture medium, the HEK293T cells were detached using PBS supplemented with 0.2% EDTA (pH 8.0) and collected. The cells were pelleted by centrifugation (4 min, 1100 rpm) and resuspended to a concentration of 1 μg of proteins/mL. The concentration of proteins per sample was determined using the DC Protein Assay (BIO-RAD) according to the manufacturer’s instructions.

In the first experiment, cells were distributed (80 μg of proteins per well) to four wells in a 96-well microplate (Black/White Isoplate-96 Black Frame White Well; PerkinElmer Life Sciences). To the remaining two wells, 10 μL PBS (Gibco) was added as a blank. To the remaining two wells, 1 μM Schgr-CRF-DH peptide dissolved in PBS was added. BRET2 between luc8 (370–450 nm) was measured using an Infinite F200 reader (Tecan Group Ltd) 1 min after addition of the DeepBlueC. For each well, the BRET2 signal was calculated as the ratio of emission of GFP10 to 510–540 nm. The signal of the control cells should remain at the zero level. The magnitude of the BRET2 signal depends on the biosensor used. Therefore, the y-axis of the graphs is set in the same range as observed in earlier studies which use the same BRET2-based G protein biosensors to test receptor signaling of the Homo sapiens chemokine receptor (HomsaCCR2; Corbisier et al., 2015) for the Gαi/o subfamily, the H. sapiens β2-adrenergic receptor (Homsa-β2-AR; Saulière et al., 2012) for the Gαs subfamily, the H. sapiens angiotensin II type 1 receptor (Homsa-AT1R, Saulière et al., 2012) for the Gαq/11 subfamily and the H. sapiens thromboxane TPα receptor (TPα-R; Saulière et al., 2012) for the Gα12/13 subfamily.

The result of the blank was subtracted from the two other wells (resulting in a ΔBRET2 value). This experiment was performed in duplicate per transfection (two for each Gα construct) for both experimental and control cells.

### 2.10. G protein analysis in S. gregaria

All available D. melanogaster G protein subunit sequences were obtained from FlyBase (https://flybase.org/) and used as a query to scan a brain-specific transcriptome database of gregarius S. gregaria created by illumina-sequenced reads (unpublished data), to identify G protein subunit sequences in this species. All identified G protein subunit sequences are validated by using the online tool GprotPRED (http://aias.biol.uoa.gr/GprotPRED/) which is able to recognize Gα, Gβ and Gγ subunits and is able to assign Gα subunits to one of the four Gα protein subfamilies; namely Gαi1, Gαi2, Gαq11 or Gαq12 (Kostiou et al., 2016). In case that GprotPRED is unable to assign a Gα protein sequence to a Gα subfamily, the sequence is assigned to a Gα protein subfamily based on sequence identity with other insect Gα subunit sequences by using the BLAST tool on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A multiple sequence alignment was constructed for Gαi1, Gαi2, Gαq11, Gαq12, Gβ and Gγ using sequences of the H. sapiens G protein subunits used to construct the BRET2-based G protein biosensors, as well as all identified G protein subunit sequences of S. gregaria. Since there are three Gα subtypes and two Gα isoforms, sequences of the Gαi and Gαq are analyzed separately. Multiple sequence alignments and additional percent identity matrices are constructed as described in 2.1.

GenBank accession numbers of the G α protein G protein subunit sequences used to construct the BRET2-based G protein biosensors which are studied in the multiple sequence alignment are GenBank: P63096 for Homsa-Gαi1, GenBank: P04899 for Homsa-Gαi2, GenBank: P08754 for Homsa-Gαq1, GenBank: NP 066268.1 for Homsa-Gαq11, GenBank: NP 620073.2 for Homsa-Gαq12, GenBank: P63092 for Homsa-Gαq2, GenBank: P50148 for Homsa-Gαo, GenBank: B29992 for Homsa-Gα11, GenBank: Q03113 for Homsa-Gα12, GenBank: Q14344 for Homsa-Gα13, GenBank: P62873, for Homsa-Gβ1, and GenBank: P59768 for Homsa-Gγ1.


### 3. Results

#### 3.1. Cloning and sequence analysis of Schgr-CRF-DHR

The complete open reading frame (ORF) of the Schgr-CRF-DHR was found in a S. gregaria transcriptome database (unpublished data) and was verified by Sanger sequencing (LGC genomics) of the PCR ampli- cons obtained using the primers listed in Supplementary Table S1. The ORF of the Schgr-CRF-DHR cDNA consists of 1293 nucleotides encoding a 430 amino acids-long receptor (Fig. 1). Transmembrane topology prediction revealed the presence of seven hydrophobic regions forming the α-helical transmembrane segments (TM1–7) characteristic of GPCRs (Krogh et al., 2001; Sonnhammer et al., 1998). BLASTx searches revealed similarities of the cloned Schgr-CRF-DHR with other insect CRF-
Fig. 2. Multiple sequence alignment of CRF-DHRs from *D. melanogaster* (Drome-DH44-R1; GenBank acc. no. NP_610960.1, and Drome-DH44-R2; GenBank acc. no. NP_610789.3), *R. prolixus* (Rhopr-CRF-DHR2B; GenBank acc. no. KJ407397), *M. sexta* (Manse-CRF-DHR; GenBank acc. no. AAC46469.1), *A. domesticus* (Achdo-CRF-DHR; GenBank acc. no AAC47000.1) and *S. gregaria* (Schgr-CRF-DHR). Identical residues between the aligned sequences are highlighted in black, and conservatively substituted residues in grey. Amino acid position is indicated at the left and dashes indicate gaps that are introduced to maximize similarities in the alignment. Putative transmembrane regions of Schgr-CRF-DHR and Schgr-CRF-DHR (TM1-TM7) are indicated by grey bars. Conserved cysteine residues that are predicted to form disulfide bridges in the N-terminus, EL1 and EL2 are indicated (*). The intracellular loops (IL) and the C-terminus of the receptors are shown as well.
The Schgr-CRF-DHR sequence was submitted to GenBank at the National Center for Biotechnology Information (NCBI) and has received accession number GenBank: MN663112. A multiple sequence alignment with other in vitro functionally confirmed insect CRF-DH receptors is displayed in Fig. 2. Schgr-CRF-DHR displays the typical features of GPCRs belonging to the secretin receptor superfamily (Fig. 3). Insect CRF-DH receptors contain six conserved cysteine residues known to form disulfide bridges in the N-terminal region of all family B GPCRs, which is essential for ligand binding properties (Fredriksson et al., 2003). Furthermore, they contain two conserved cysteine residues in extracellular loop (EL)1 and EL2 known to form a disulfide bridge between these ELs, ensuring proper orientation of EL2 (Gether, 2000; Schiöth and Lagerström, 2008). The alignment (Fig. 2) shows that the Schgr-CRF-DHR sequence is highly conserved, especially in the transmembrane regions, the intracellular loops, and in some parts of the N- and C-terminal regions.

3.2. Transcript profiling

The tissue distribution of Schgr-CRF-DHR was studied in adult locusts using qRT-PCR. The results show that expression of Schgr-CRF-DHR is mainly restricted to the central nervous system (CNS) (Fig. 4). The relative transcript levels appear highest in the suboesophageal ganglion, the ventral nerve cord, the frontal ganglion and the brain, followed by a slightly lower expression in the optic lobes. The receptor transcript is also detected in the brain-associated endocrine organs, corpora cardiaca, corpora allata and prothoracic glands. However, no significant differences were found between the mature and immature female tissue samples that were compared in this study. No detectable relative expression levels were observed in the samples analyzed for peripheral tissues, such as fat body, flight muscle, intestine, epidermis, gonads and male accessory glands.

3.3. Pharmacological characterization of Schgr-CRF-DHR

3.3.1. The aequorin bioluminescence assay

Schgr-CRF-DHR elicits a sigmoidal dose-dependent response with an EC50 value in the high nanomolar range (30.65 nM, Table 1), when it is expressed in CHO-WTA11 cells (Fig. 5), indicating that Schgr-CRF-DH is an agonist of Schgr-CRF-DHR. However, no response is detected in CHO-PAM28 cells that do not contain the promiscuous Gα16 protein (results not shown), which suggests that coupling of this receptor to a detectable elevation of intracellular Ca2+ levels may not occur via the signaling components that are endogenously present in this cell line.

![Fig. 3. Schematic representation of Schgr-CRF-DHR. Schgr-CRF-DHR is a secretin-like GPCR with seven transmembrane regions. The residues highlighted in yellow are a typical feature of secretin-like GPCRs. The highly conserved disulfide bridges in the N-terminus and a typical disulfide bridge formed between a cystein (C) at the beginning of transmembrane 3 and in extracellular loop 2, are indicated in black. The conserved TNXFWMXVELXXLVV motif in transmembrane 3 is accentuated in white, according to the multiple sequence alignment in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image1)

![Fig. 4. Tissue distribution profile of Schgr-CRF-DHR in both immature and mature adult S. gregaria. The data represent mean values ± S.E.M. of three independent tissue samples run in duplicate, normalized relative to Ubi, CG13220 and GAPDH transcript levels. Significant differences between mature and immature samples were tested by ANOVA. Abbreviations used: Br = brain, OL = optic lobes, CC = corpora cardiaca, CA = corpora allata; PG = prothoracic glands, FrG = frontal ganglion, SOG = suboesophageal ganglion, VNC = ventral nerve cord, fGon = female gonads, FB = fat body, FM = flight muscle, FG = foregut, Cae = caecum, MT = Malpighian tubules, MG = midgut, HG = hindgut, Epi = epidermis, mGon = male gonads, ACCGL = male accessory glands.](image2)
in CHO-WTA11-Schgr-CRF-DHR cells. The aequorin bioluminescence assay is executed in two independent transfections. The bioluminescence is measured in triplicate per concentration of Schgr-CRF-DH and per transfection. Error bars represent the S.E.M and the 100% level refers to the maximal response level. The zero-response level corresponds to treatment with BSA blank. These results suggest an increase in cAMP levels upon stimulation of G protein biosensors.

In addition, CRF-DH-44 peptides derived from other insect species (Table 1), Trica-CRF-DH-I and Trica-CRF-DH-II from the red flour beetle Tribolium castaneum, and Acypi-CRF-DH from the pea aphid Acrhythosiphon pisum, were tested to investigate the ability of cross-species agonists to activate Schgr-CRF-DHR when it is expressed in CHO-WTA11 cells. We observed that Trica-CRF-DH-II (DH44-II) did not activate Schgr-CRF-DHR in the tested concentration range (Supplementary Fig. S1A). Furthermore, we observed partial sigmoidal activation of the locust receptor by Trica-CRF-DH-I and Acypi-CRF-DH (Supplementary Fig. S1A, with EC50 values in the (supra)micromolar range). There are some noticeable sequence differences between Schgr-CRF-DH and these other insect CRF-DH peptides (Supplementary Fig. S1B). For example, changes in the RLLL motif can be observed; in which Arg19 is replaced by Leu20 is replaced by Trp17 in Acypi-DH44. In addition, changes in the charged amino acid RRLRD motif can be observed in Acypi-DH44 and Trica-DH44-II whereas this motif is deleted in Trica-DH44-II.

3.3.2. The CAMYEL biosensor

A dose-dependent decrease in BRET signal is observed with an EC50 value in the nanomolar range (0.42 ± 8.8 μM, respectively; Table 1). There are some noticeable sequence differences between Schgr-CRF-DH and these other insect CRF-DH peptides (Supplementary Fig. S1B). For example, changes in the RLLL motif can be observed; in which Arg19 is replaced by Thr18 in Trica-DH44-II and by Lys27 in Acypi-DH44. Furthermore, Leu20 is replaced by Trp17 in Trica-DH44-II, and Leu26 is replaced by Lys15 in Trica-DH44-II and by Met29 in Acypi-DH44. In addition, changes in the charged amino acid RRLRD motif can be observed in Acypi-DH44 and Trica-DH44-II whereas this motif is deleted in Trica-DH44-II.

3.3.3. Measuring a direct activation of the Gα protein by using BRET-based G protein biosensors

The BRET signal measured in HEK293T-Schgr-CRF-DHR cells is reduced compared to the BRET signal measured in the control cells upon addition of 1 μM Schgr-CRF-DH for four out of five biosensors (Gα12, Gα13, Gαq, and Gα16) of the Gαq/o subfamily (Fig. 7B-E). Moreover, the BRET signals of Gα12, Gα13, Gαq, and Gα16 are dose-dependent with an EC50 value in the high nanomolar range (Fig. 8B-E), indicating that these biosensors are truly activated. Although the BRET signal is decreased for the Gα13 biosensor (Fig. 7A) and a trend towards a reduced signal seems present when increasing concentrations of Schgr-CRF-DH are applied, no sigmoidal dose-dependent relationship is observed (Fig. 8A). In addition, a lower BRET signal is measured for the Gαq biosensor in the HEK293T-Schgr-CRF-DHR cells compared to the control cells (Fig. 7F). Moreover, this biosensor induces a dose-dependent decrease in BRET signal with an EC50 value in the high nanomolar range (Fig. 8F), indicating that this biosensor is truly activated. Furthermore, neither the Gα13 biosensor, nor the Gαq biosensor (Fig. 7G-H, Fig. 8G) appear to be activated.

When testing a single peptide dose, the Gα12 biosensor generates a reduced BRET signal in the HEK293T-Schgr-CRF-DHR cells compared to the control cells (Fig. 7I). However, when multiple concentrations of Schgr-CRF-DH are applied, no clear dose-dependence is detected (Fig. 8I). Finally, Gα13 does not induce a significantly lower BRET signal in the HEK293T-Schgr-CRF-DHR cells compared to the control cells (Fig. 7J).

3.3.4. Identification of G proteins in S. gregaria

To interpret the data of the BRET-based G protein biosensors, we also performed a BLAST analysis to identify G protein subunit sequences in the genome database of D. melanogaster and the transcriptome database of S. gregaria (unpublished data). The Schgr-Gα protein subunit sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI) and have received accession number GenBank: MN663113 for Schgr-Gα12, GenBank: MN663114 for Schgr-Gα13, GenBank: MN663115 for Schgr-Gαo, GenBank: MN663116 for Schgr-Gαq, and GenBank: MN663117 for Schgr-Gα12/13. By means of a multiple sequence alignment and a percent identity matrix, we compared these putative insect G protein subunit sequences with the human G protein subunits that were used to construct the BRET-based G protein biosensors. We compared the G protein subunits per G protein subfamily, with the exception of the Gαq/o subfamily for which we analyzed the Gαq and Gαo sequences separately since this subfamily comprises five biosensors. Overall, the insect and human sequences of Gαq, Gαo, Gαq, and Gαq/o subunits are very similar. The insect Gα protein subunits show a higher sequence identity with each other than with the human G protein subunits (Supplementary Figs. S2-S5 and Supplementary Tables S4-S7). They reported a sequence identity of 87% with the human Gαq subunit and a sequence identity of 80% with the human and mouse Gαo subunits.

However, insect and human members of the Gα12/13 subfamily show less sequence identity than the other Gα protein subfamilies. Overall, the insect Gα12/13 subunits only show a sequence identity of 51-59% with the human Gα12 and Gα13 subunits and of 62% with each
other (Supplementary Fig. S6 and Supplementary Table S8).

In addition, we also identified two putative Gβ and two putative Gγ subunit sequences in the transcriptome database of *S. gregaria*. These *Schgr*-Gβ and *Schgr*-Gγ protein subunit sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI) and have received accession number GenBank: MN663118 for *Schgr*-Gβ1, GenBank: MN663119 for *Schgr*-Gβ2, GenBank: MN663120 for *Schgr*-Gγ1 and GenBank: MN663121 for *Schgr*-Gγ2. By looking at the multiple sequence alignment and the percent identity matrix, we concluded that *Schgr*-Gβ1 and *Drome*-Gβ1, with a sequence identity of 76–79% are more related to the *Homsa*-Gβ subunit that was used to construct the BRET²-based G protein biosensor. The same range of sequence identity is seen when different human Gβ subunit types are compared, e.g. when comparing four *Homsa*-Gβ1,4 sequences 78–88% identity is observed. However, other insect β subunits, *Drome*-Gβ2, *Drome*-Gβ3 and *Schgr*-Gβ2, only share a sequence identity of 40–55% of with this human subunit (Supplementary Fig. S7 and Supplementary Table S9).

We identified two putative Gγ sequences in *S. gregaria*, none of these is showing a high sequence identity with the human Gγ2 sequence that was used to construct the Gγ2-GFP10 biosensor (Supplementary Fig. S8 and Supplementary Table S10). The sequence identity of this human subunit merely ranges from 30% with *Drome*-Gγ3A to 43% with *Drome*-Gγ1. Nevertheless, *Schgr*-Gγ1 and *Drome*-Gγ1 on one hand and *Schgr*-Gγ2 and *Drome*-Gγ3A on the other seem to be orthologues showing a sequence identity of 66% and 87.5%, respectively.

4. Discussion

4.1. Molecular cloning and receptor sequence analysis

In this study, *Schgr*-CRF-DHR was successfully cloned and characterized with cell-based functional receptor assays. As shown in the multiple sequence alignment (Fig. 2), the amino acid sequence of *Schgr*-CRF-DHR is well-conserved when compared with other insect CRF-DHRs that have been characterized *in vitro*: *Drome*-DH44-R1 and *Drome*-DH44-R2 (*D. melanogaster*), *Rhopr*-CRF-DHR2B (*R. prolixus*), *Manse*-CRF-DHR (*M. sexta*) and *Achdo*-CRF-DHR (*A. domesticus*), especially in the 7TM regions, parts of the N- and C-terminal regions, and the intracellular loops. The latter may be indicative for a conserved coupling of these receptors to downstream effectors, such as G proteins.

4.2. Tissue distribution analysis and functions of *Schgr*-CRF-DHR

The distribution of the *Schgr*-CRF-DHR transcript (Fig. 4) is mainly restricted to the nervous system (Fig. 4). This is similar to *Drome*-DH44-R1 distribution patterns that were mainly observed in the brain and VNC (Hector et al., 2009; Lee et al., 2015), and probably to *Aedia*-DH44-R2 transcript abundance as well, which was solely detected in *A. aegypti* heads (Jagge and Pietrantonio, 2008). In addition, in line with the spatial expression profile of *Rhopr*-CRF/DH-R2B (Lee et al., 2016), gene expression of *Schgr*-CRF-DHR was also detected in different brain-associated endocrine organs. *Schgr*-CRF-DHR expression levels were also observed in the frontal ganglion, which belongs to the stomatogastric nervous system. However, no transcript levels were observed in Malpighian tubules or reproductive organs, which is in contrast to the spatial expression profile of *Rhopr*-CRF/DH-R2B (Lee et al., 2016).
Transcripts for Drome-DH44-R2 (Hector et al., 2009) and Aedae-DH44-R1 (Jagge and Pietrantonio, 2008) were also observed in Malpighian tubules.

Although CRF-DH plays a pronounced role in the regulation of diuresis, feeding, and reproduction in S. gregaria (Van Wielendaele et al., 2012) and other insects (Audsley et al., 1995, 1997; Te Brugge and Orchard, 2002; Goldsworthy et al., 2003; Tobe et al., 2005), this is not entirely reflected in the observed tissue distribution, since relative Schgr-CRF-DHR transcript levels are low or undetectable in several tissues that are involved in these processes. This infers that this is probably not the only Schgr-CRF-DH receptor, and/or that the peptide regulates some of these processes in an indirect manner. The former hypothesis is supported by the fact that in several insect species two distinct CRF-DHR receptors have been identified (Caers et al., 2012; Hector et al., 2009; Jagge and Pietrantonio, 2008; Johnson et al., 2004; Lee et al., 2016), which can be an indication for a functional differentiation between both receptors. Moreover, differences in in vitro receptor signaling between both CRF-DHRs were observed in D. melanogaster (Hector et al., 2009) and R. prolixus (Lee et al., 2016). For instance, Drome-DH44-R2 is much more sensitive to Drome-DH44 than Drome-DH44-R1 and sensitivity of the latter to evoke a translocation of arrestin to the transmembrane is rather low. Furthermore, down-regulation of Drome-DH44-R2 by RNAi, results in a higher sensitivity to osmotic challenges in D. melanogaster (Hector et al., 2009). Alternatively, if Schgr-CRF-DHR would regulate physiological processes in an indirect manner, this could be obtained via neuronal control and/or via the regulation of the activity of endocrine organs.

High expression of Schgr-CRF-DHR in the brain, the optic lobes, the suboesophageal ganglion and the ventral nerve cord, suggests a prominent role for this receptor within the central nervous system. Moreover, expression levels are also high in the frontal ganglion, which is part of the stomatogastric nervous system. Both parts of the nervous system interact to regulate and coordinate the processes of feeding and digestion. The suboesophageal ganglion, for instance, innervates the mouthparts and the salivary glands and thus controls food intake (Audsley and Weaver, 2009; Nation, 2015). The frontal ganglion, on the other hand, innervates the foregut, enhancing gut motility and progression of the food bolus towards the midgut, where digestion and nutrient absorption are taking place (Holtf et al., 2019). Van Wielendaele et al. (2012) also point out that the decrease in food intake, as observed after injecting locusts with Schgr-CRF-DH, also may result from a decreased peripheral sensitivity to food stimuli, such as olfactory or gustatory stimuli, that affect central functions like appetite and feeding behavior.

4.3. Pharmacological receptor characterization and G protein mediated signaling pathways

The data obtained with the aequorin bioluminescence assay indicate that Schgr-CRF-DH is an agonist of Schgr-CRF-DHR and that activation of this receptor in absence of Go16 has no detectable effect on the PLC/Ca2+ pathway. This is in accordance with the available literature on other characterized CRF-DHRs in R. prolixus (Lee et al., 2016) and D. melanogaster (i.e. Drome-DH44-R2; Hector et al., 2009). In contrast, a stimulatory effect on the PLC/Ca2+ pathway has been reported for Drome-DH44-R1 (Hector et al., 2009).
In addition, we have analyzed the activity of three other CRF-DHs derived from the pest species, *T. castaneum* (Trica-CRF-DH-I and Trica-CRF-DH-II) and *A. pisum* (Acypi-CRF-DH). While CRF-DH peptides from these two insect species also display some agonistic activity to Schgr-CRF-DHR, our results point towards a much higher affinity of the desert locust CRF-DH peptide to Schgr-CRF-DHR, which is indicative for a long-term co-evolution between natural (locust) ligand-receptor couples (Markov et al., 2008). The observed higher cross-species activity between Schgr-CRF-DHR and Trica-CRF-DH-I (DH44-I) can probably be attributed to a higher structural similarity between this peptide and Schgr-CRF-DH in comparison to Acypi-CRF-DH (DH44) and Trica-CRF-DH-II (DH44-II). This is evidenced by amino acid sequence alignments, as well as by the sequence logos provided for CRF-DH in DINeR, a Database for Insect Neuropeptide Research (Yeoh et al., 2017). Clearly, when compared to Schgr-CRF-DH, the sequence of the least active (or even inactive) peptide, Trica-CRF-DH-II (DH44-II), is also the most divergent one (Supplementary Fig. S1B). The most noticeable difference in the sequence of this peptide is the substitution of various amino acids in the RLLL motif; Arg19, a positively charged amino acid, is replaced by a positively charged Lys20 residue. In addition, hydrophobic Leu21 is replaced by a bigger hydrophobic amino acid Trp18. Moreover, changes in the charged amino acid RLRLD motif can be observed in Acypi-DH44 and Trica-DH44-I. Most notably, the positively charged Arg20 residue is replaced by a negatively charged Asp residue in Acypi-DH44 and this motif is deleted in Trica-DH44-II. These amino acid substitutions might induce conformational changes within the analyzed peptides and/or might be intrinsic for binding capability to the Schgr-CRF-DHR. However, we cannot exclude that other structural motifs might also be of importance for ligand-receptor interaction, but this would require more detailed structural analyses.

The data obtained with the cAMP dependent CAMYEL biosensor assay indicate that cellular CAMP levels increase upon activation of Schgr-CRF-DHR by Schgr-CRF-DH. A stimulatory effect on cAMP levels is in agreement with the data previously reported for other in vitro characterized CRF-DHs: activation of Achoo-CRF-DHR, Manse-CRF-DHR, Rhopr-CRF-DHR2B and both Drome-DH44-R1 and Drome-DH44-R2 by their respective peptide agonists induces the cAMP/protein kinase A pathway (Hector et al., 2009; Johnson et al., 2004; Lee et al., 2016; Reagan, 1995, 1996).

In addition, the downstream G protein mediated signaling pathways are studied in more detail using BRET2-based G protein biosensors, which can measure direct activation of the G protein itself. The Gaq biosensor is activated in a dose-dependent manner upon exposure to Schgr-CRF-DHR, in line with the results obtained for the CAMYEL biosensor-based assay. Interestingly, the magnitude of the ABRET2 signal is comparable with the magnitude of the ABRET2 signal detected with the *H. sapiens* β2-adrenergic receptor (Saulière et al., 2012), a receptor known to activate the Gaq protein. In addition, four out of five biosensors (Ga12q, Ga13, Gaq, and Gaiq) of the Gaq/o subfamily are activated in a dose-dependent manner when Schgr-CRF-DHR is stimulated by Schgr-CRF-DH. Notably, the magnitudes of the ABRET2 signals of the Gaq/o biosensors are also comparable to those previously detected with the *H. sapiens* chemokine receptors known to activate Gaq/o proteins (Corbisier et al., 2015). The fact that both Gaq/o and Gai/o subfamilies are activated may appear contradictory. However, activation of the Gai/o subfamily does not necessarily result in a decrease in intracellular cAMP levels. Together with the high conservation of metazoan Gai/o subunits, the fact that this insect receptor can activate four mammalian members of this Ga subfamily suggests that it may also be capable of signaling to endogenous insect Gaq/o proteins. Clearly, this hypothesis remains to be confirmed for in vivo situations in the insect.

Furthermore, neither the Gaq, nor the Gai/o biosensor, both belonging to the Gai/o11 subfamily, are activated by agonist-induced Schgr-CRF-DHR. This conclusion is further reinforced when the magnitude of the obtained ABRET2 signal (Fig. 7G and H) is compared to the magnitude of the ABRET2 signal detected with the *H. sapiens* angiotensin II type 1 receptor (Saulière et al., 2012), which is a receptor known to activate Gaq/o11 proteins. Moreover, this is in agreement with the results obtained with the aequorin bioluminescence assay in CHO-PAM28 cells.

In addition, no clear dose-dependent ABRET2 is observed with the Ga12q biosensor when increasing concentrations of Schgr-CRF-DH are applied (Fig. 8H), particularly when the measured ABRET2 signal (Fig. 8H) is compared to the magnitude of the signal detected with the *H. sapiens* thromboxane TPa receptor (Saulière et al., 2012), which is a receptor known to activate Ga12q subunit proteins. Moreover, Ga13, which belongs to the same subfamily, does not induce a significantly lower BRET2 signal in the HEK293T-Schgr-CRF-DHR cells compared to the control cells. Whether this results from a lack of activation of members of the Ga12q/o11 subfamily by this receptor type or from ineffective coupling of an insect receptor to mammalian Ga12q/o subunits that display only limited structural conservation with their insect homologues, remains an open question.

These BRET2-based G protein biosensors are also used to verify which specific Ga subunit isoform(s) can be activated within a particular Ga subunit family. However, only one Gaq and one Gai/o subunit sequence are identified in *S. gregaria*. Therefore, knowledge of mammalian Gaq or Gai/o isoform activation may not be very relevant for the natural situations encountered in insect cells and organisms. Overall, we conclude that the G protein subunit sequences of *D. melanogaster* and *S. gregaria* are more similar to each other than to their human counterparts. With exception of the Ga12q/o11 subfamily, the Gaq subunits show a high sequence similarity. The Gj/o subunits seem to be less conserved, although it should be noted that Homsa-Gj/o also shows a sequence identity of 51 – 53% with the four other Homsa-Gj/o subunit isoforms (Dupré et al., 2009). The Gf subunit sequences, on the other hand, display a very limited degree of conservation, especially between insects and humans. Nevertheless, since many insect receptors are being molecularly characterized in frequently cultured mammalian cell lines, this knowledge remains of high importance to allow for correct interpretations of *in vitro* receptor studies.

5. Conclusion

Taking all this together, we can conclude that a Schgr-CRF-DHR sequence was determined and validated in *vivo*. We have demonstrated that this locust neuropeptide receptor responds to its predicted agonist, Schgr-CRF-DH. Agonist-induced activation of the receptor has a stimulatory effect on the AC/cAMP pathway, but in absence of the proriscuous Ga16 subunit it has no detectable effect on the PLC/Ca2+ pathway. These findings are in agreement with data obtained with the BRET1-based CAMYEL biosensor (ATCC MBA-277), which appears to be very well suited for pharmacological characterization of this insect member of GPCR family B.

In addition, the cross-species agonist study might be indicative for the intrinsic potential of the Schgr-CRF-DHR to be selectively activated by an agonist. In the future, essential amino acids for receptor binding could be confirmed using peptide analogues (or similar techniques), as the identification of a target ligand binding site is an important step for structure-based rational agonist design for GPCRs. These essential characteristics might avoid or reduce non-target effects in insecticides targeting this receptor would be developed in the future.

We have also shown that the BRET2-based biosensor assays, consisting of the human Gaq, Gai/q, Ga12q/o combinations, can accurately monitor the activation of an insect GPCR, the Schgr-CRF-DHR receptor. Nevertheless, although the different Ga subfamilies are highly conserved, with the exception of Ga12q/o11, insect genomes tend to encode a lower number of isoforms within each subfamily. Moreover, Gq/o subunits appear to differ more profoundly from their mammalian counterparts, especially in comparison to the more conserved Ga subfamilies. Therefore, it might also prove to be useful to design BRET2-based signal transduction assays.
based biosensors with insect G proteins. By performing these assays in insect cells, the native structure and mode of action of both the GPCR and the G proteins would be respected.

Our study paves the way for further biosensor research, as well as for the future development of novel biosensors consisting of specific insect-derived components for studying insect GPCRs, thereby evoking the desired cellular responses in a signaling context that would more closely mimic the natural situation. Such assays might be of great value, for instance for analyzing the signaling characteristics and the application potential of novel insect pest-management compounds targeting this physiologically very important receptor category.

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