



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

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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^{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, *Schistocerca gregaria*. This *Schgr*-CRF-DHR is mainly expressed in the nervous system and in brain-associated endocrine organs. The neuropeptide *Schgr*-CRF-DH induced Ca^{2+} -dependent aequorin-based bioluminescent responses in CHO cells co-expressing this receptor with the promiscuous $\text{G}\alpha_{16}$ 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_{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 $\text{G}\alpha$ protein subunit activation by an insect GPCR. Therefore, we analyzed ten different human BRET-based G protein biosensors, representing members of all four $\text{G}\alpha$ protein subfamilies; $\text{G}\alpha_s$, $\text{G}\alpha_{i/o}$, $\text{G}\alpha_{q/11}$ and $\text{G}\alpha_{12/13}$. Our data demonstrate that stimulation of *Schgr*-CRF-DH by *Schgr*-CRF-DH can dose-dependently activate $\text{G}\alpha_{i/o}$ and $\text{G}\alpha_s$ biosensors, while no significant effects were observed with the $\text{G}\alpha_{q/11}$ and $\text{G}\alpha_{12/13}$ 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^{2+} and cyclic AMP (cAMP). For instance, an aequorin bioluminescence assay is frequently utilized to monitor possible changes in intracellular Ca^{2+} levels. Similarly, a cAMP response element (CRE) dependent luciferase reporter assay, using a $\text{CRE}_{(6x)}$ -Luc construct, enables the monitoring of variations in intracellular cAMP levels. Both

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assays and the cell lines used in these assays have proven their value in insect GPCR characterization studies (Bil et al., 2016; Caers et al., 2016a, 2016b; Dillen et al., 2013; Horodyski et al., 2011; Huang et al., 2014; Lenaerts et al., 2017; Lismont et al., 2015, 2018; Marchal et al., 2018; Meeusen et al., 2002; Mertens et al., 2002; Poels et al., 2007, 2009, 2010; Vandersmissen et al., 2013; Verlinden et al., 2013, 2015; Vleugels et al., 2013; Vuerinckx et al., 2011; Zels et al., 2014). However, the bioluminescence measured using the CRE_(6x)-Luc construct is not directly induced by cAMP but is dependent on the phosphorylation of the CRE binding protein (CREB). As there is some speculation that CREB can also be phosphorylated by calcium/calmodulin-dependent protein kinase, bioluminescence measured in this way may also be caused by increased Ca²⁺ levels (Johannessen et al., 2004). Therefore, several alternative methods have been developed to monitor changes in intracellular cAMP levels in cell based GPCR assays. The bioluminescence resonance energy transfer (BRET¹)-based assay, using YFP-Epac-Rluc (CAMYEL) as a biosensor for cAMP (ATCC MBA-277), is such an alternative method. Using this biosensor, decreasing or increasing levels of intracellular cAMP upon GPCR activation are monitored by an increase or a decrease in BRET¹ signal, respectively (Jiang et al., 2007L). A schematic representation of this CAMYEL biosensor-based cAMP reporter assay (adapted from Matthiesen and Nielsen, 2011) is presented in Fig. 1A. This assay was developed to study the biology, pharmacology and signaling of GPCRs that regulate the adenylyl cyclase (AC)/cAMP pathway (Jiang et al., 2007). However, by only monitoring fluctuations in second messenger levels, we cannot precisely infer which specific Gα protein(s) is (are) activated upon receptor activation. Therefore, to further study vertebrate G protein mediated signaling pathways, BRET²-based G protein biosensors were developed to detect direct activation of the Gα subunit after GPCR activation. To study their coupling with mammalian GPCRs, ten different human G protein-based biosensors representing all four Gα protein subfamilies (Gα_{i/o}, Gα_s, Gα_{q/11} and Gα_{12/13}) have been validated. Each individual biosensor consists of three constructs, Gα-Rluc8, Gβ₁ and Gγ₂-GFP10, which form a α_xβ₁γ₂ G protein heterotrimer, with α_x representing one of ten different Gα subunit constructs. These biosensors enable the real-time detection of Gα activation upon receptor stimulation (Denis et al., 2012; Galés et al., 2006) while simultaneously verifying which particular member of this family is activated (Galés et al., 2006). 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., 2009; Maurice et al., 2010; Peverelli et al., 2013; Rives et al., 2012; Onfroy 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 Wielendaele 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-ecdysis 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 Wielendaele et al., 2012).

While the physiological role of *Schgr*-DH was previously investigated in *S. gregaria* (Van Wielendaele et al., 2012), its receptor was never molecularly characterized. Moreover, this newly characterized receptor, *Schgr*-CRF-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

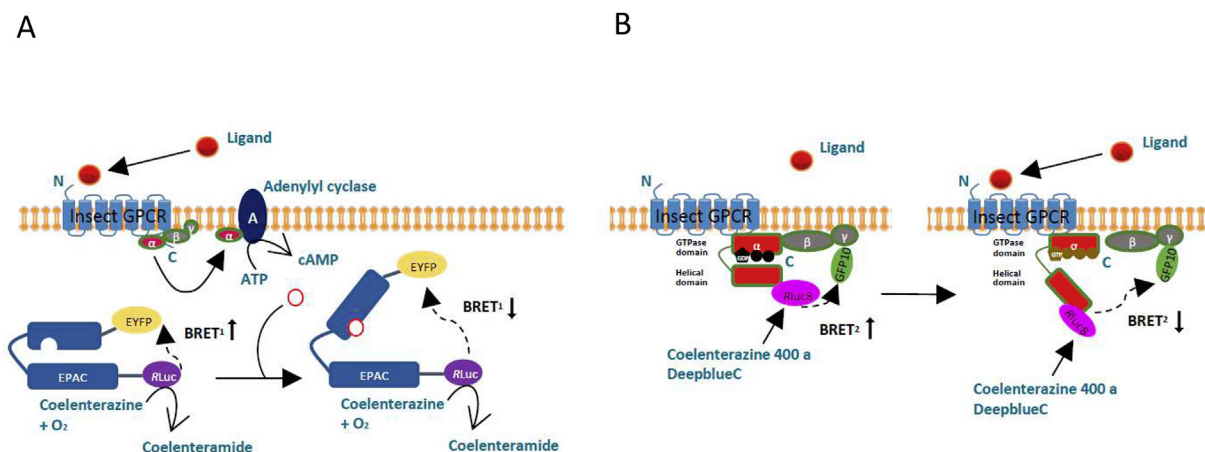


Fig. 1. Schematic representation of both BRET¹ and BRET² systems. A) Schematic representation of the cAMP reporter assay using the CAMYEL biosensor: the protein exchange factor directly activated by cAMP (EPAC) is flanked by yellow fluorescent protein (YFP) and Rluc. Upon binding of cAMP to EPAC, a conformational change is induced which results in a larger distance between YFP and Rluc. As a consequence, bioluminescence energy transfer (BRET)¹ decreases. Coelenterazine acts as a substrate for Rluc (Image adapted from Matthiesen 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 Gα. 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 Gα subunit. When the Gα subunit is activated, GDP is exchanged for GTP and a large interdomain movement in Gα 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-DH 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 BRET¹-based CAMYEL (ATCC MBA-277) biosensor. Furthermore, direct activation of G-proteins was measured using the BRET²-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-DH 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-sequenced 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-DH44-R1; GenBank: [NP_610960.1](#), and Drome-DH44-R2; GenBank: [NP_610789.3](#)), *R. prolixus* (Rhopr-CRF-DHR2B; GenBank: [KJ407397](#)), *M. sexta* (Manse-CRF-DHR; GenBank: [AAC46469.1](#)) and *A. domesticus* (Achdo-CRF-DHR; GenBank: [AAC47000.1](#)).

A multiple sequence alignment and a percent identity matrix were constructed by using the EMB-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://tcoffee.crg.cat/apps/tcoffee/do:mcoffee>) 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-DH plays a role in reproduction of this locust species (Van Wielendaele et al., 2012). The locusts were synchronized on the day of adult ecdysis 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 under a binocular microscope and rinsed in locust Ringer solution (1L: 8.766 g NaCl; 0.188 g CaCl₂; 0.746 g KCl; 0.407 g MgCl₂; 0.336 g NaHCO₃; 30.807 g sucrose; 1.892 g trehalose). In this way, brain, optic lobes, ventral nerve cord, gonads, fat body, flight muscle, foregut, caeca, Malpighian tubules, midgut, hindgut, male accessory glands, as well as a piece of the epidermis with its cuticle, were removed and collected in 2.0 mL tubes containing MagNa Lyser green beads (Roche). Additionally, the corpora cardiaca, corpora allata, the prothoracic gland, the frontal ganglion, and the suboesophageal ganglion were

collected in RNase-free Screw Cap Micro centrifuge tubes. The same tissues were collected for the samples taken from mature insects. Mature males were dissected 12–13 days after molting, when they had a yellow cuticle coloration, an indication of gregarious male maturity. Mature females were similarly dissected 10–13 days after molting, but only if they were in the vitellogenic stage of ovarian maturation. During this stage, the oocyte size strongly increases from 1.7 to 7 mm and oocytes start showing a yellow color (Tobe and Pratt, 1975). For this study, tissues were only collected from females with oocyte sizes ranging from 3 to 6 mm. For every tissue or organ in each condition, four pools were dissected consisting of eight animals each. All samples were immediately snap frozen in liquid nitrogen to prevent RNA degradation. Until further processing, the samples 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 RNAqueous-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 Lenaerts 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 analyzed 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 apo-aequorin, a zeocin resistance gene, and the promiscuous $G\alpha_{16}$ subunit coupling to the phospholipase C and Ca^{2+} signaling cascade (Euroscreen, Belgium). CHO-PAM28 cells (stably expressing apo-aequorin and a puromycin resistance gene) and human embryonic kidney (HEK) 293T cells were used to assess effects on the Ca^{2+} 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% CO_2 in 10 cm Nunc Cell Culture/Petri Dishes (Thermo Scientific) in DMEM (Gibco) supplemented with 1% fungizone (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 CAMYEL biosensor assay, 5×10^6 HEK293T cells were transiently transfected using the calcium phosphate method (Jordan et al., 1996). Thus, cells were co-transfected with 4 μ g CAMYEL vector, combined with 10 μ g *Schgr*-CRF-DHR pcDNA3.1 receptor expression construct and 6 μ g pcDNA3.1 TOPO empty vector or with 16 μ g 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 μ L.

For the BRET²-based G protein biosensor analyses, HEK293T cells were co-transfected using the calcium phosphate method with (1) one of the ten $G\alpha$ -Rluc8 constructs, $G_{\gamma 2}$ -GFP10, $G\beta_1$, pcDNA3.1: *Schgr*-CRF-DHR, constituting the experimental conditions, or (2) one of the ten $G\alpha$ -Rluc8 constructs, $G_{\gamma 2}$ -GFP10, $G\beta_1$ and the pcDNA3.1 empty vector, constituting the control conditions. The ten $G\alpha$ -Rluc8 constructs are: $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oa}$, $G\alpha_{ob}$ representing the $G_{ai/o}$ subfamily, $G\alpha_{11}$ and $G\alpha_q$ representing the $G\alpha_{q/11}$ subfamily, $G\alpha_s$ as a representative of the G_s subfamily, and $G\alpha_{12}$ and $G\alpha_{13}$ representing the $G\alpha_{12/13}$ subfamily. In all cells, the G protein heterodimer $\alpha_x \beta_1 \gamma_2$ is expressed with $G\alpha_x$ representing one of the ten $G\alpha$ 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 μ g DNA in 500 μ L MilliQ water.

For transfections using the calcium phosphate method, 50 μ L $CaCl_2$ (2.5 M) was added to the DNA mixture. Next, 500 μ L HEPES-buffered saline [HBS (2x); 280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4 ; pH 7.1] were added. The transfected cells were maintained in an incubator at 37 °C (5% CO_2). An additional transfection was performed with 4 μ g CAMYEL vector and 16 μ g empty vector, without pcDNA3.1 receptor construct, as an extra control. The transfected cells were maintained in an incubator at 37 °C (5% CO_2) 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 μ M). 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 0.5×10^6 cells/mL in DMEM/F-12 without phenolred (Gibco) supplemented with 1% fungizone (Gibco), 10% fetal bovine serum (FBS; Gibco), 1% sodium pyruvate (Invitrogen), and 1% penicillin/streptomycin. Cells were seeded in white MicroWell 96-Well Optical-Bottom Plates with Polymer Base (catalog no. 165306; Thermo Scientific) by adding 100 μ L of the cell solution (~50 000 cells) to each well. Subsequently, the cells were incubated overnight at 37 °C (5% CO_2).

The actual experiment was conducted two days post-transfection. Experiments were performed in the presence of IBMX (end concentration 40 μ M), to prevent cAMP breakdown, and coelenterazine *h* (end concentration 5 μ M; Promega), which is used as a substrate for Rluc.

The HEK293T cells were rinsed with PBS, and subsequently resuspended in PBS. Next, cells were incubated for 5 min with the IBMX/coelenterazine *h* mix prior to the addition of *Schgr*-CRF-DH peptide at different concentrations (0.10 nM - 1 μ M). BRET¹ readings were collected 5 min after stimulation using the Mithras LB940 (Berthold Technologies) and the ratio of emission of YFP (520–570 nm) to Rluc (370–480 nm) was calculated. The results are expressed as BRET fold-change, i.e. the ratio of the BRET signal evoked in response to addition of the neuropeptide, over the BRET signal induced in response to addition of the buffer only. Measurements were taken in duplicate per concentration of peptide in two independent transfections for both the experimental condition (with insect receptor), and the negative control (without insect receptor). Calculations were made using the output files from the Microwin2000 (Mikrotek) in Excel (Microsoft) and further analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.).

2.9. BRET²-based G protein biosensor assay

An advanced BRET system (BRET)² uses different GFP variants in combination with the coelenterazine variant DeepBlueC, also referred to as coelenterazine 400a, which permits the measurement of the acceptor emission spectrum without any overlap of the donor emission spectrum. The BRET²-based G protein biosensors can be used to measure activation of G proteins in living cells by monitoring conformational changes of G proteins. This technology relies on the interdomain movement that occurs within heterotrimeric G proteins upon GDP/GTP exchange, resulting in a decrease of the BRET signal between probes inserted within $G\alpha$ and $G\gamma$ subunits (Galés et al., 2006). This technology detects the early signaling event that occurs after receptor activation and permits discrimination between different G protein subtypes activated by a receptor. To test whether the BRET²-based G protein biosensors react to insect GPCRs, enabling the detection of the activation of specific G proteins, a first screen was conducted whereby only one high dose of peptide (1 μ M) was applied. This pilot experiment was

Table 1Primary structure of the peptides used in the current study to activate of *Schgr*-CRF-DHR with respective EC₅₀ values.

Neuropeptide Name	Amino acid sequence	EC50 value
<i>Schgr</i> -CRF-DH	MGMGPSLSIVNPMQDLRQLLLEIARRRLDAEEQIKANKDFLQQLamide	30,65 nM
<i>Trica</i> -DH44-I	AGALGESGASLSIVNSLDVLRNRLLEIARKKAKEGANRRNRQILLSLamide	0,42 mM
<i>Trica</i> -DH44-II	SPTISITAPIDVLRKTWAKENMRKQMQINREYLNKQLamide	/
<i>Acypi</i> -DH44	NGAMQGESPRSRPSLSIVNSLDVLRQKLMYEVARRHVDENQKVLSQLNHLKSLamide	8,8 mM

Abbreviations: *Schgr* = *Schistocerca gregaria*, *Trica* = *Tribolium castaneum* and *Acypi* = *Acyrtosiphon pisum*.

performed in two separate transfections [for both (1) the experimental condition with insect receptor and (2) the control without insect receptor] per Gα construct (Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_{oa}, Gα_{ob}, Gα_s, Gα₁₁, Gα_q, Gα₁₂ or Gα₁₃). When a significant reaction was obtained, a second screen was conducted in three separate transfections 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 first 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 supplemented. Exactly 1 min later, 5 μM coelenterazine 400a/DeepBlueC (Gentaur) was added. BRET² between Rluc₈ (370–450 nm) and GFP₁₀ (510–540 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 Rluc₈. 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.

A significant reduction in ΔBRET² signal in the experimental cells (with insect receptor) compared to the control cells (without receptor) may indicate that there is an activation of the Gα protein. The ΔBRET² 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 (*Homsa*-CCR2; Corbisier et al., 2015) for the Gα_{i/o} subfamily, the *H. sapiens* β₂-adrenergic receptor (*Homsa*-β₂-AR; Saulière et al., 2012) for the Gα_s subfamily, the *H. sapiens* angiotensin II type 1 receptor (*Homsa*-AT1R, Saulière et al., 2012) for the Gα_{q/11} subfamily and the *H. sapiens* thromboxane TPα receptor (TPα-R; Saulière et al., 2012) for the Gα_{12/13} subfamily.

If the ΔBRET² signal is significantly reduced in comparison to the control, we can conclude that the biosensor is truly activated. Therefore, HEK293T cells were co-transfected again in triplicate with insect receptor and the G protein biosensor constructs. The protocol of this assay is similar to the procedure described above but instead of adding cells to four wells, cells were added to six wells to test multiple concentrations of peptide (0.10 nM - 1 μM) including the PBS that serves as a blank. The result of the blank (first well) was subtracted from the other wells to calculate ΔBRET². This procedure was performed in duplicate per transfection (in three transfections for each Gα construct) and the data were analyzed using the Excel output file (Microsoft) and GraphPad Prism 6 (GraphPad Software Inc.).

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 gregarious *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 Gα_{i/o}, Gα_s, Gα_{q/11} or Gα_{12/13} (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 Gα_i, Gα_o, Gα_s, Gα_{q/11}, Gα_{12/13}, 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_o isoforms, sequences of the Gα_i and Gα_o are analyzed separately. Multiple sequence alignments and additional percent identity matrices are constructed as described in 2.1.

GenBank accession numbers of the *H. sapiens* 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*-Gα_{i1}, GenBank: P04899 for *Homsa*-Gα_{i2}, GenBank: P08754 for *Homsa*-Gα_{i3}, GenBank: NP_066268.1 for *Homsa*-Gα_{oa}, GenBank: NP_620073.2 for *Homsa*-Gα_{ob}, GenBank: P63092 for *Homsa*-Gα_s, GenBank: P50148 for *Homsa*-Gα_q, GenBank: P29992 for *Homsa*-Gα₁₁, GenBank: Q03113 for *Homsa*-Gα₁₂, GenBank: Q14344 for *Homsa*-Gα₁₃, GenBank: P62873, for *Homsa*-Gβ₁, and GenBank: P59768 for *Homsa*-Gγ₁.

GenBank accession numbers of the *D. melanogaster* G protein subunit sequences used to identify *S. gregaria* G protein subunit sequences are GenBank: CG10060 for *Drome*-Gα_i, GenBank: CG2204 for *Drome*-Gα_o, GenBank: CG2835 for *Drome*-Gα_{s60A}, GenBank: CG12232 for *Drome*-Gα_{73B}, GenBank: CG17759 for *Drome*-Gα_q, GenBank: CG17760 for *Drome*-Gα_{SD21019p}, GenBank: CG17678 for *Drome*-Gα_{12/13}, GenBank: CG10545 for *Drome*-Gβ₁, GenBank: CG8770 for *Drome*-Gβ₂, GenBank: CG10763 for *Drome*-Gβ₅, GenBank: CG8261 for *Drome*-Gγ₁ and GenBank: CG3694 for *Drome*-Gγ_{30A}.

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 amplicons 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-

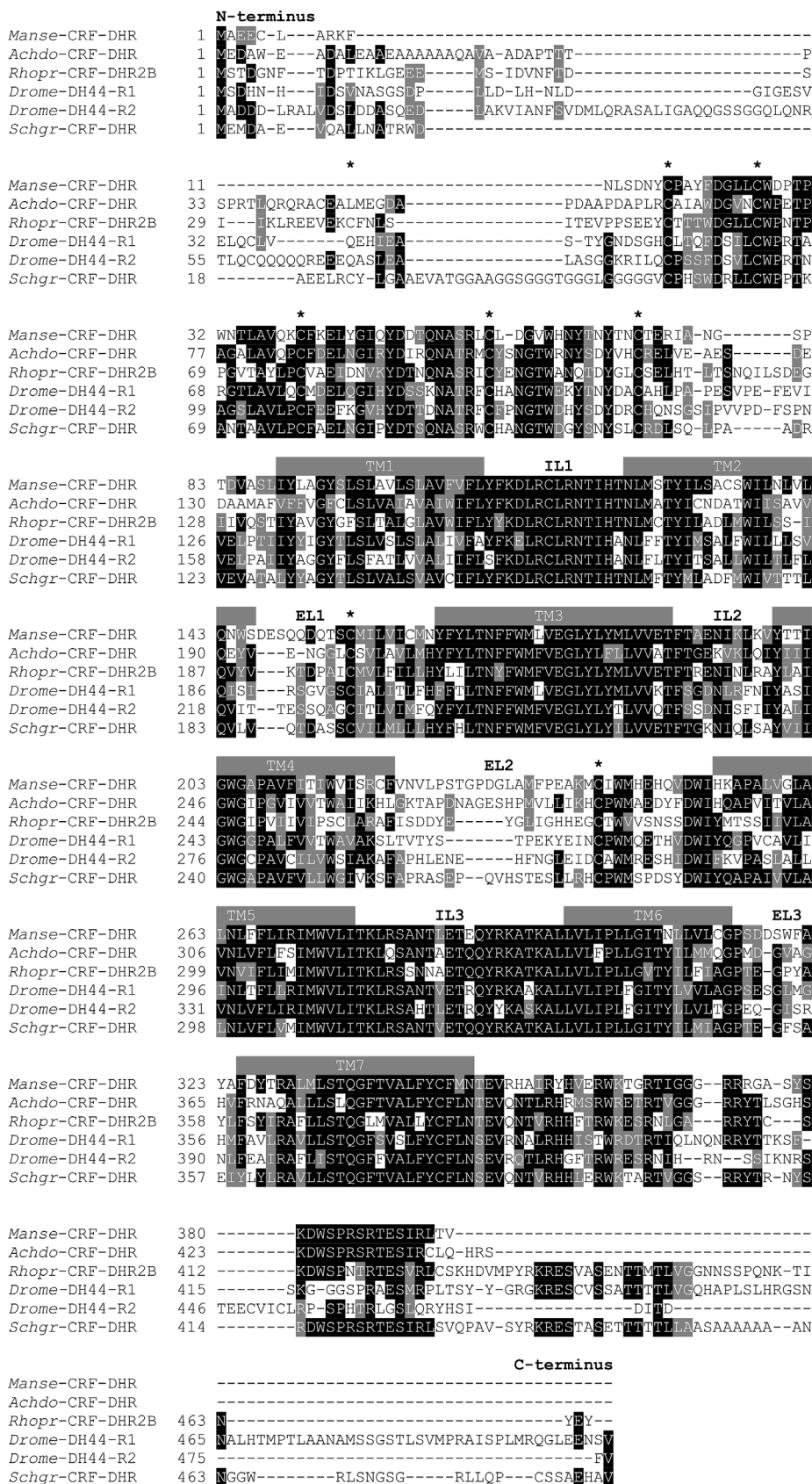


Fig. 2. Multiple sequence alignment of CRF-DHRs from *D. melanogaster* (Drome-DH44-R1; GenBank acc. no. NP_610960.1, and Drome-DH44-R2; GenBank acc. no. NP_610789.3), *R. prolixus* (Rhopr-CRF-DHR2B; GenBank acc. no. KJ407397), *M. sexta* (Manse-CRF-DHR; GenBank acc. no. AAC46469.1), *A. domesticus* (Achdo-CRF-DHR; 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.

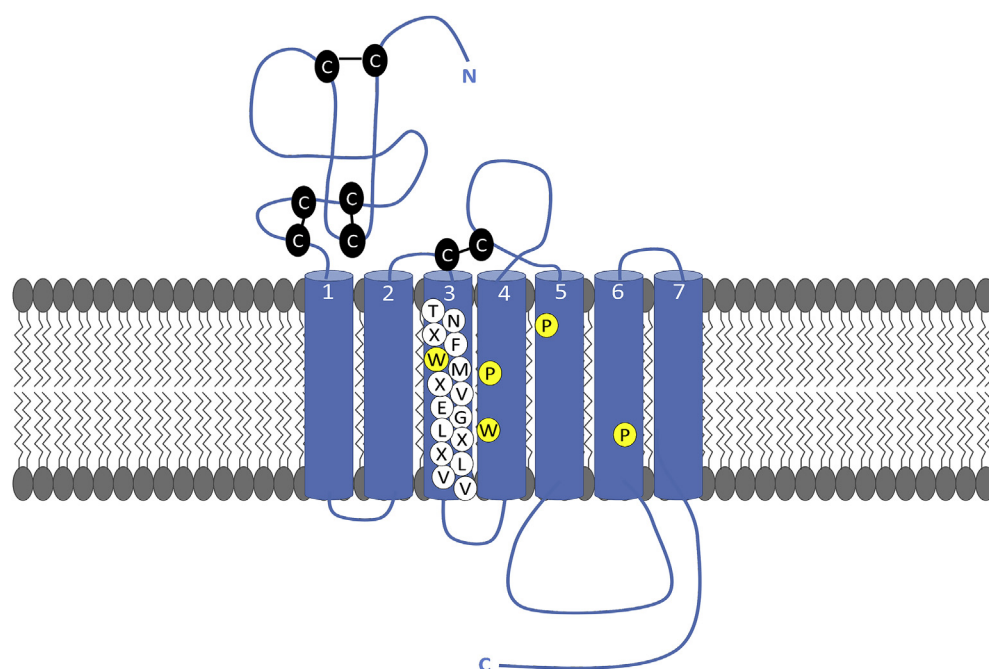


Fig. 3. Schematic representation of *Schgr*-CRF-DHR. *Schgr*-CRF-DHR is a secretin-like GPCR with seven transmembrane regions. The residues highlighted in yellow are a typical feature of secretin-like GPCRs. The highly conserved disulphide bridges in the N-terminus and a typical disulphide bridge formed between a cysteine (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.)

DHRs. 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.

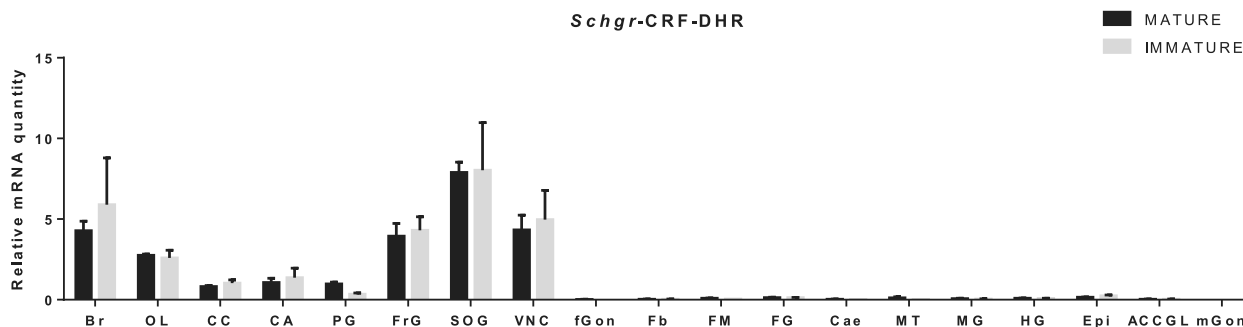


Fig. 4. Tissue distribution profile of *Schgr*-CRF-DHR in both immature and mature adult *S. gregaria*. The data represent mean values $\pm$ S.E.M. of three independent tissue samples run in duplicate, normalized relative to Ubi, CG13220 and GAPDH transcript levels. Significant differences between mature 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.

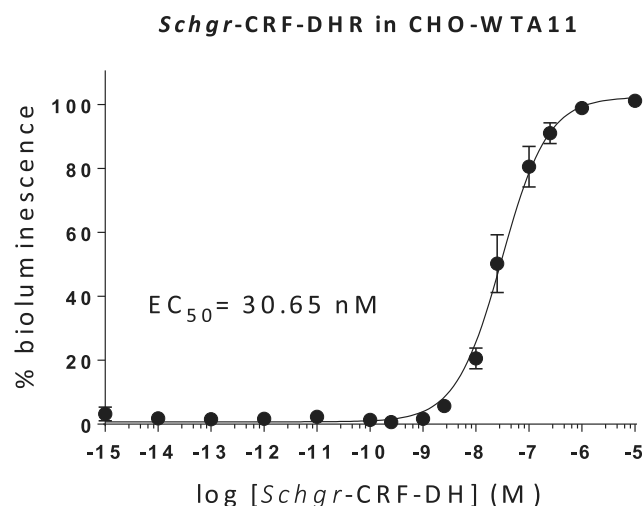


Fig. 5. Dose-response curve for aequorin-based bioluminescent responses induced in CHO-WTA11-Schgr-CRF-DHR cells. The aequorin bioluminescence assay is executed in two independent transfections. The bioluminescence is measured in triplicate per concentration of Schgr-CRF-DH and per transfection. Error 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 medium only. In addition, the EC_{50} value is indicated.

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 *Acyrtosiphon 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 EC_{50} values in the (supra)micromolar range (0.42 μ M and 8.8 μ M, 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 Thr¹⁶ in *Trica*-DH44-II and by Lys²⁷ in *Acypi*-DH44. Furthermore, Leu²⁰ is replaced by Trp¹⁷ in *Trica*-DH44-II, and Leu²² is replaced by Lys¹⁹ 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-I whereas this motif is deleted in *Trica*-DH44-II.

3.3.2. The CAMYEL biosensor

A dose-dependent decrease in BRET¹ signal is observed with an EC_{50} value in the nanomolar range (4.02 nM) in cells transfected with Schgr-CRF-DHR (Fig. 6). The control remains stable in the same range as the blank. These results suggest an increase in cAMP levels upon stimulation of Schgr-CRF-DHR by Schgr-CRF-DH.

3.3.3. Measuring a direct activation of the $G\alpha$ protein by using BRET²-based G protein biosensors

The Δ BRET² signal measured in HEK293T-Schgr-CRF-DHR cells is reduced compared to the Δ BRET² signal measured in the control cells upon addition of 1 μ M Schgr-CRF-DH for four out of five biosensors ($G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oa}$ and $G\alpha_{ob}$) of the $G\alpha_{i/o}$ subfamily (Fig. 7B-E). Moreover, the Δ BRET² signals of $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{oa}$ and $G\alpha_{ob}$ are dose-dependent with an EC_{50} value in the high nanomolar range (Fig. 8B-E), indicating that these biosensors are truly activated. Although the Δ BRET² signal is decreased for the $G\alpha_{i1}$ 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

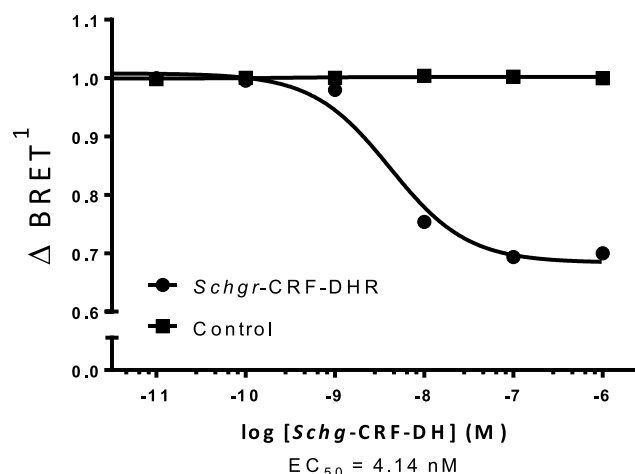


Fig. 6. Results of CAMYEL biosensor: BRET1 measured in HEK293T cells transfected with Schgr-CRF-DHR1 (●) or without Schgr-CRF-DHR1 [control (■)] in two independent transfections. Measurements are taken in duplicate for each concentration of Schgr-CRF-DH (10 pM - 1 μ M). Data ($\pm$ S.E.M) are presented relative to the baseline (buffer only).

is observed (Fig. 8A). In addition, a lower Δ BRET² signal is measured for the $G\alpha_s$ biosensor in the HEK293T-Schgr-CRF-DHR cells when compared to the control cells (Fig. 7F). Moreover, this biosensor induces a dose-dependent decrease in Δ BRET² signal with an EC_{50} value in the high nanomolar range (Fig. 8F), indicating that this biosensor is truly activated. Furthermore, neither the $G\alpha_{i1}$ biosensor, nor the $G\alpha_q$ biosensor (Fig. 7G-H, Fig. 8G) appear to be activated.

When testing a single peptide dose, the $G\alpha_{i2}$ biosensor generates a reduced Δ BRET² 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. 8H). Finally, $G\alpha_{i3}$ does not induce a significantly lower Δ BRET² 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 BRET²-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- $G\alpha$ protein subunit sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI) and have received accession number GenBank: MN663113 for Schgr- $G\alpha_i$, GenBank: MN663114 for Schgr- $G\alpha_o$, GenBank: MN663115 for Schgr- $G\alpha_s$, GenBank: MN663116 for Schgr- $G\alpha_q$ and GenBank: MN663117 for Schgr- $G\alpha_{i2/13}$. 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 subunit sequences that were used to construct the BRET²-based G protein biosensors. We compared the G protein subunits per G protein subfamily, with the exception of the $G\alpha_{i/o}$ subfamily for which we analyzed the $G\alpha_i$ and $G\alpha_o$ sequences separately since this subfamily comprises five biosensors. Overall, the insect and human sequences of $G\alpha_i$, $G\alpha_o$, $G\alpha_s$ and $G\alpha_{q/11}$ subunits are very similar. The insect $G\alpha$ 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 *D. melanogaster* $G\alpha_q$ subunit and a sequence identity of 80% with the human and mouse $G\alpha_q$ subunits.

However, insect and human members of the $G\alpha_{i2/13}$ subfamily show less sequence identity than the other $G\alpha$ protein subfamilies. Overall, the insect $G\alpha_{i2/13}$ subunits only show a sequence identity of 51–59% with the human $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits and of 62% with each

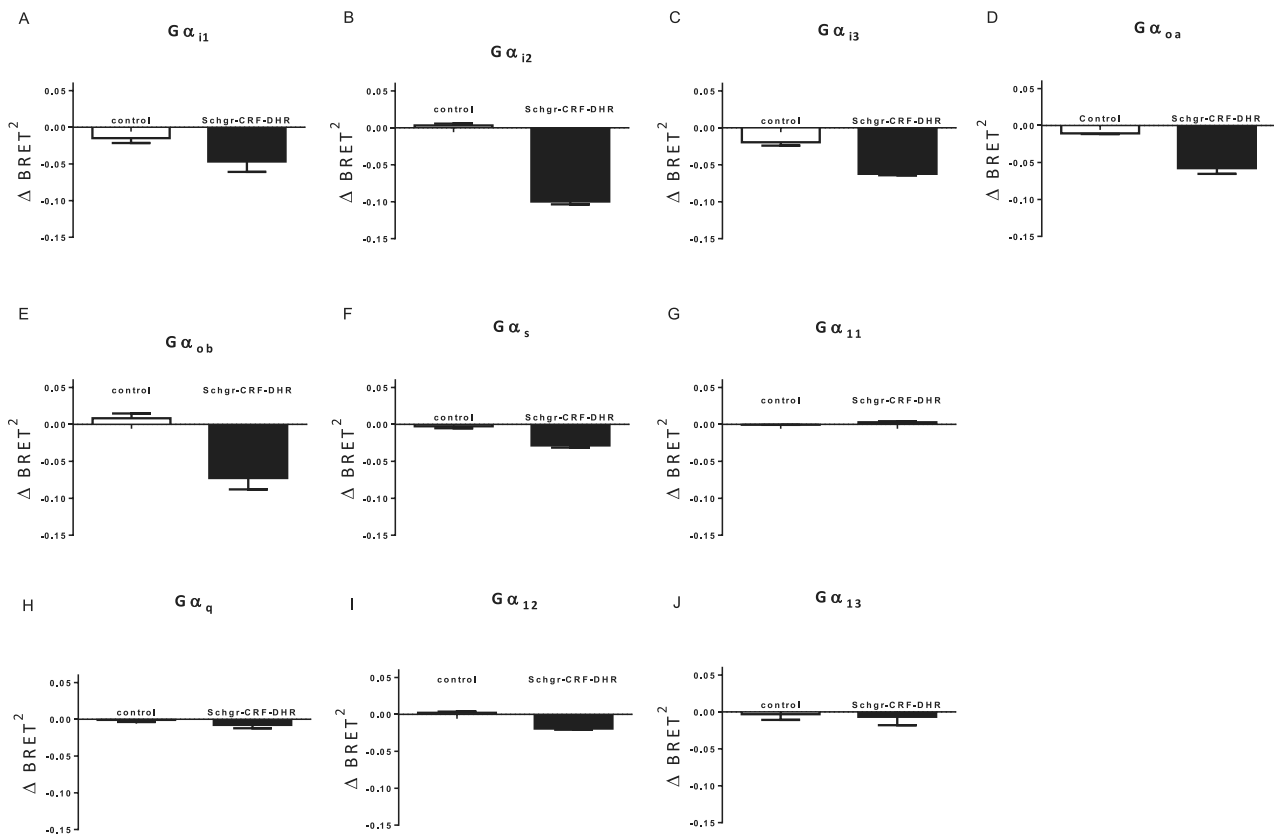


Fig. 7. Results of the HEK293T-Schgr-CRF-DHR cells (Schgr-CRF-DHR) and HEK293T cells (Control) co-transfected with BRET²-based G protein biosensors upon stimulation of 1 μ M Schgr-CRF-DH. A) Gα_{i1} biosensor, B) Gα_{i2} biosensor, C) Gα_{i3} biosensor, D) Gα_{oa} biosensor, E) Gα_{ob} biosensor, F) Gα_s biosensor, G) Gα_q biosensor, H) Gα₁₁ biosensor, I) Gα₁₂ biosensor, J) Gα₁₃ biosensor. The data represent the means $\pm$ S.E.M of Δ BRET² measured in triplicate in two independent transfections.

other (Supplementary Fig. S6 and Supplementary Table S8).

In addition, we also identified two putative G β and two putative G γ subunit sequences in the transcriptome database of *S. gregaria*. These Schgr-G β and Schgr-G γ protein subunit sequences were submitted to GenBank at the National Center for Biotechnology Information (NCBI) and have received accession number GenBank: MN663118 for Schgr-G β ₁, GenBank: MN663119 for Schgr-G β ₂, GenBank: MN663120 for Schgr-G γ ₁ and GenBank: MN663121 for Schgr-G γ ₂. By looking at the multiple sequence alignment and the percent identity matrix, we concluded that Schgr-G β ₁ and Drome-G β ₁, with a sequence identity of 76–79% are more related to the *Homsa*-G β ₁ subunit that was used to construct the BRET²-based G protein biosensor. The same range of sequence identity is seen when different human G β subunit types are compared, e.g. when comparing four *Homsa*-G β _{1–4} sequences 78–88% identity is observed. However, other insect β subunits, *Drome*-G β ₂, *Drome*-G β ₅ and Schgr-G β ₂, only share a sequence identity of 40–55% of with this human subunit (Supplementary Fig. S7 and Supplementary Table S9).

We identified two putative G γ sequences in *S. gregaria*, none of these is showing a high sequence identity with the human G γ ₂ sequence that was used to construct the G γ ₂-GFP10 biosensor (Supplementary Fig. S8 and Supplementary Table S10). The sequence identity of this human subunit merely ranges from 30% with *Drome*-G γ _{30A} to 43% with *Drome*-G γ ₁. Nevertheless, Schgr-G γ ₁ and *Drome*-G γ ₁ on one hand and Schgr-G γ ₂ and *Drome*-G γ _{30A} 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-DH

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 *Aedae*-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).

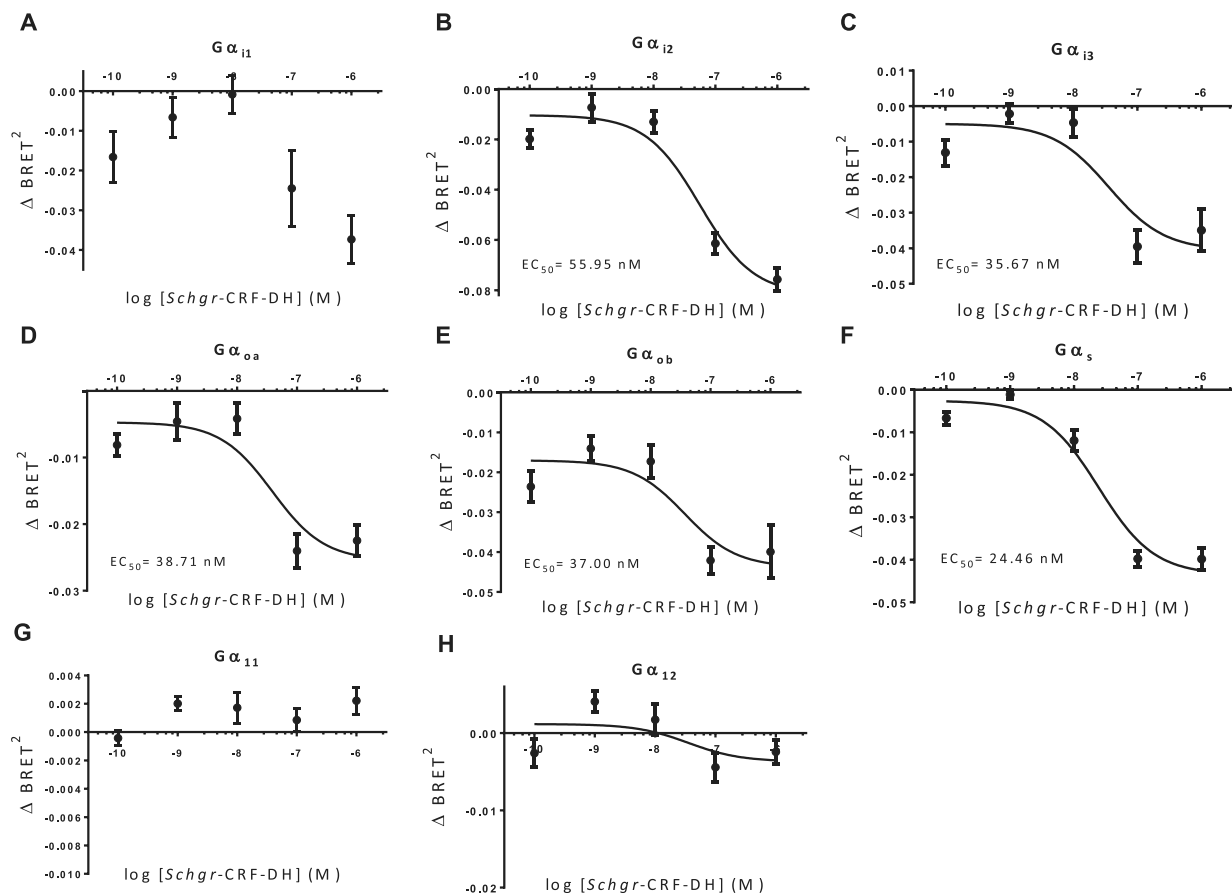


Fig. 8. Dose-dependent responses in HEK293T-Schgr-CRF-DHR1 cells co-transfected with BRET²-based G protein biosensors consisting of Gβ₁, Gγ₂-GFP10 and several Gα subunits upon stimulation of 0.01 nM–1 μM Schgr-CRF-DH. The data represent the means ± S.E.M of three independent transfections measured in duplicate per concentration. The EC₅₀ value is indicated in case a dose-dependent reaction is observed.

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; Goldworthy 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-DH 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*. This agrees with the fact that expression of *Drome-DH44-R2* is 200-fold higher in the Malpighian tubules than *Drome-DH44-R1* (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 subesophageal 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 subesophageal 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 (Holtof 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 Gα₁₆ 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 RLLI motif; Arg¹⁹, a positively charged amino acid, is replaced by a polar uncharged Thr¹⁶ and the hydrophobic Leu²³ residue is replaced by the positively charged Lys²⁰ residue. In addition, hydrophobic Leu²¹ is replaced by a bigger hydrophobic amino acid Trp¹⁸. Moreover, changes in the charged amino acid RRLRD motif can be observed in *Acypi*-DH44 and *Trica*-DH44-I. Most notably, the positively charged Arg³⁰ 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-DHRs: activation of *Achdo*-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 Gα_s biosensor is activated in a dose-dependent manner upon exposure to *Schgr*-CRF-DH, in line with the results obtained for the CAMYEL biosensor-based assay. Interestingly, the magnitude of the ΔBRET² signal is comparable with the magnitude of the ΔBRET² signal detected with the *H. sapiens* β2-adrenergic receptor (Saulière et al., 2012), a receptor known to activate the Gα_s protein. In addition, four out of five biosensors (Gα_{i2}, Gα_{i3}, Gα_{oa} and Gα_{ob}) of the Gα_{i/o} subfamily are activated in a dose-dependent manner when *Schgr*-CRF-DHR is stimulated by *Schgr*-CRF-DH. Notably, the magnitudes of the ΔBRET² signals of the Gα_{i/o} biosensors are also comparable to those previously detected with the *H. sapiens* chemokine receptors known to activate Gα_{i/o} proteins (Corbisier et al., 2015). The fact that both Gα_{i/o} and Gα_s subfamilies are activated may appear contradictory. However, activation of the Gα_{i/o} subfamily does not necessarily result in a decrease in intracellular cAMP levels. Together with the high conservation of metazoan Gα_{i/o} subunits, the fact that this insect receptor can activate four mammalian members of this Gα subfamily suggests that it may also be capable of signaling to endogenous insect Gα_{i/o} proteins. Clearly, this hypothesis remains to be confirmed for *in vivo* situations in the insect.

Furthermore, neither the Gα_q, nor the Gα₁₁ biosensor, both belonging to the Gα_{q/11} subfamily, are activated by agonist-induced *Schgr*-CRF-DHR. This conclusion is further reinforced when the magnitude of the obtained ΔBRET² signal (Fig. 7G and H) is compared to the

magnitude of the ΔBRET² signal detected with the *H. sapiens* angiotensin II type 1 receptor (Saulière et al., 2012), which is a receptor known to activate Gα_{q/11} 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 ΔBRET² is observed with the Gα₁₂ biosensor when increasing concentrations of *Schgr*-CRF-DH are applied (Fig. 8H), particularly when the measured ΔBRET² signal (Fig. 8H) is compared to the magnitude of the signal detected with the *H. sapiens* thromboxane TPα receptor (Saulière et al., 2012), which is a receptor known to activate Gα_{12/13} proteins. Moreover, Gα₁₃, 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 Gα_{12/13} subfamily by this receptor type or from ineffective coupling of an insect receptor to mammalian Gα_{12/13} 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 Gα subunit isoform(s) can be activated within a particular Gα subunit family. However, only one Gα_i and one Gα_o subunit sequence are identified in *S. gregaria*. Therefore, knowledge of mammalian Gα_i or Gα_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 Gα_{12/13} subfamily, the Gα subunits show a high sequence similarity. The Gβ subunits seem to be less conserved, although it should be noted that *Homsa*-Gβ₅ also only shares a sequence identity of 51 – 53% with the four other *Homsa*-Gβ subunit isoforms (Dupré et al., 2009). The Gγ 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 vitro*. 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 promiscuous Gα₁₆ 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 case insecticides targeting this receptor would be developed in the future.

We have also shown that the BRET²-based biosensor assays, consisting of the human Gα_x-Gβ_{1γ2} combinations, can accurately monitor the activation of an insect GPCR, the *Schgr*-CRF-DHR receptor. Nevertheless, although the different Gα subfamilies are highly conserved, with the exception of Gα_{12/13}, insect genomes tend to encode a lower number of isoforms within each subfamily. Moreover, Gβγ subunits appear to differ more profoundly from their mammalian counterparts, especially in comparison to the more conserved Gα subfamilies. Therefore, it might also prove to be useful to design BRET²-

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ibmb.2020.103392>.

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