Cellular Signalling 28 (2016) 1152–1162

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Isolation and characterization of the corticotropin-releasing factor-related diuretic hormone receptor in *Rhodnius prolixus*

Hae-Ri Lee, Meet Zandawala [1], Angela B. Lange, Ian Orchard *

Department of Biology, University of Toronto Mississauga, Mississauga, ON, L5L1C6, Canada

ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form 18 May 2016
Accepted 24 May 2016
Available online 27 May 2016

Keywords:
G protein-coupled receptor
Corticotropin-releasing factor
Cyclic AMP
Diuretic hormone
Disease vector
Diuresis

ABSTRACT

Rhodnius prolixus, the vector of human Chagas disease, is a hemipteran insect that undergoes rapid post-feeding diuresis following ingestion of a blood meal that can be up to 10 times its initial body weight. Corticotropin-releasing factor-related diuretic hormone (Rhopr-CRF/DH) and serotonin are neurohormones that are synergistic in increasing rates of fluid secretion by Malpighian tubules during this rapid post-feeding diuresis. A Rhopr-CRF/DH receptor transcript has now been isolated and characterized from fifth instar *R. prolixus*. The receptor is a family B1 (secretin) G protein-coupled receptor (GPCR) and was deorphaned in a heterologous cellular system using Chinese hamster ovary (CHO) cells stably expressing a promiscuous G-protein (Gα16). This assay was also used to demonstrate the presence of Rhopr-CRF/DH in the haemolymph of *R. prolixus* in response to blood-gorging. Two additional cell lines were used in this heterologous assay to verify that the cyclic adenosine monophosphate (cAMP) pathway and not the inositol triphosphate (IP_3) pathway was stimulated upon activation of the receptor. Lastly, quantitative PCR demonstrated strong receptor expression in digestive tissues, upper Malpighian tubules and reproductive tissues. Identification of the Rhopr-CRF/DH receptor now provides tools for a more detailed understanding into the precise coordination of diuresis and other physiological processes in *R. prolixus*.

1. Introduction

When the blood-feeding bugs, *Rhodnius prolixus*, gorge on a blood meal, they initiate rapid and prolonged diuresis to reduce the bulk of their mass and to concentrate the nutrients of the blood meal, while maintaining volume, osmotic and ionic balance of the hemolymph [1–5]. During this diuresis, *R. prolixus* acts as the vector of human Chagas disease by transmitting the parasite *Trypanosoma cruzi* in the urine, close to the feeding site. Rapid diuresis begins within 2–3 min of feeding and lasts for the next 3 h at a high rate of 400–700 nL/min, during which time over 50% of the mass of the blood meal is excreted as urine [3–5]. Water, sodium ions and chloride ions from the ingested blood are absorbed into the hemolymph across the anterior midgut (AMG). This is countered by the upper Malpighian tubules (MTs), which secrete sodium, potassium, and chloride ions and water, resulting in an isoosmotic fluid that is rich in potassium ions. As the fluid moves down to the lower MTs, potassium and chloride ions are reabsorbed with minimal water into the hemolymph and hypo-osmotic primary urine that is low in potassium and high in sodium empties into the hindgut for elimination [6–8]. This entire process in *R. prolixus* is largely controlled by diuretic hormones released from neurosecretory cells (NSCs) found within the central nervous system (CNS) [2–4,8].

Insect diuretic hormones include the biogenic amines, tyramine and serotonin, and multiple families of neuropeptides, such as corticotropin-releasing factor (CRF)-related diuretic hormone (CRF/DH), calcitonin (CT)-like diuretic hormone (CT/DH), kinins and cardioacceleratory peptide 2b (CAP_{2b}) [9]. Only two insect diuretic hormones have been shown to be released into the hemolymph in response to feeding. These are serotonin in *R. prolixus* [10] and the CRF/DH in *Locusta migratoria* [11]; however, many of the other neuropeptides possess diuretic activity.

Corticotropin-releasing factor/hormone is commonly known as the stress hormone in vertebrates but CRF orthologs act as diuretic peptides in insects [12]. The first insect CRF/DH was sequenced from *Manduca sexta* where diuretic effects were shown *in vivo* [13]. Since then, other CRF/DHs have been sequenced from *Periplaneta americana* [14],

Abbreviations: AMG, anterior midgut; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; CT, calcitonin; CAP2b, cardioacceleratory peptide 2b; CNS, central nervous system; CHO, Chinese hamster ovary; CRF, corticotropin-releasing factor; cAMP, cyclic adenosine monophosphate; CNG, cyclic nucleotide-gated; DH, diuretic hormone; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; G□16, G protein alpha 16; IP3, inositol trisphosphate; MT, Malpighian tubules; MTGM, mesothoracic ganglionic mass; NSCs, neurosecretory cells; PDF, pigment-dispersing factor; PKA, protein kinase A; PLC, phospholipase C; qPCR, Quantitative Polymerase Chair Reaction; TEP, transepithelial potential; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride.

* Corresponding author.
E-mail address: ian.orchard@utoronto.ca (I. Orchard).
[1] Present address: Department of Zoology, Stockholm University, S-10691 Stockholm, Sweden.

http://dx.doi.org/10.1016/j.cellsig.2016.05.020
0898-6568/

L. migratoria [15], *Drosophila melanogaster* [16], *R. prolixus* [17]and a second CRF/DH from *M. sexta* [18]. CRF/DHs have been shown to increase fluid excretion *in vivo* and *in vitro* and to increase cAMP content in MTs [14,15,19,20]. Besides diuretic effects, CRF/DH has been shown to induce satiety in *M. sexta* and *Schistocerca gregaria* [21,22], initiate pre-ecdysis behavior in *M. sextra* [23] retard reproduction in *Schistocerca gregaria* [22], modulate normal rest: activity rhythms, as well as control sperm ejection and storage in *D. melanogaster* [24,25].

The CRF/DH in *R. prolixus* (Rhopr-CRF/DH) is 49 amino acids long and is found in NSCs in the brain and in the mesothoracic ganglionic mass (MTGM) as well as their associated neurohemal sites [17,26]. Physiologically, Rhopr-CRF/DH increases secretion rates in MTs and increases the rate of absorption across the AMG [17]. Rhopr-CRF/DH acts synergistically with serotonin to increase secretion rates *via* the cAMP second messenger pathway [27–29]. Unlike serotonin, Rhopr-CRF/DH has no effect on the reuptake of potassium ions in the lower MTs [30]. Rhopr-CRF/DH also stimulates contractions of *R. prolixus* hindgut [31].

CRF/DH receptors belong to the family of secretin-like GPCRs and are categorized under subfamily B1 that primarily use cAMP as their secondary messenger. Other members of this family include CT/DH and pigment-dispersing factor (PDF) receptors in insects [32]. Parallel to its ligand, CRF/DH receptors have been isolated and cloned in a number of insect species. Like the CRF/DH peptide, the *M. sexta* CRF/DH receptor was the first to be cloned, closely followed by the sequencing of the CRF/DH receptor in *Acheta domesticus* [33,34]. Other insect CRF/DH receptor orthologs have been found including two *Drosophila* CRF/DH (also known as DH_{44}) receptors: DH_{44}-R1 (encoded by CG8422) and DH_{44} – R2 (encoded by CG12370) [35,36]. The first *Drosophila* CRF/DH receptor (DH_{44}-R1) stimulates increases in both cAMP and calcium ions upon receptor activation, while the second (DH_{44}-R2) only increases levels of cAMP [35,36].

Here we have isolated and characterized the Rhopr-CRF/DH receptor transcript from fifth instar *R. prolixus*. The receptor was deorphaned using a heterologous cellular assay. This assay was also used to demonstrate the presence of Rhopr-CRF/DH in the hemolymph of *R. prolixus* in response to blood-gorging. Quantitative PCR (qPCR) demonstrates strong receptor expression in digestive and reproductive tissues. We have also verified that this receptor couples to the cAMP second messenger pathway.

2. Materials and methods

2.1. Animals

Fifth instar and adult *R. prolixus* were maintained in incubators at 25 °C and 60% humidity. Insects were routinely fed through a membrane on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA; supplied by Cedarlane Laboratories Inc., Burlington, ON, Canada) once during each instar. Tissues were dissected from insects 4–6 weeks post-feeding as the previous instar, under nuclease-free phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada).

2.2. Molecular cloning

D. melanogaster CRF/DH receptor protein sequences (CG12370, isoform A accession no. NP_725175.3 and isoform B accession no. NP_610789.3) were used as the query to search the *R. prolixus* preliminary genome assembly (June 2009 release). A forward gene-specific primer (CRFR2 FOR1: 5′ GGTCTACTCTGTTGGCCGAATAC 3′) and a reverse gene-specific primer (CRFR2 REV 1: 5′ AGATAAGCGAG CAAGGTTTGTACTAC 3′) were designed based on the resultant hits and used to amplify the partial cDNA sequences encoding Rhopr-CRF/DH-R2A and B using a fifth instar *R. prolixus* CNS cDNA library [37] as the template. PCR was performed using an S1000 thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada) with the following temperature cycling profile: initial denaturation (94 °C for 3 min) then 40 cycles of denaturation (94 °C for 30 s), annealing (57 °C for 30 s) and extension (72 °C for 2 min) and a final extension for 10 min at 72 °C. Positive amplicons were cloned and sent for sequencing at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, ON, Canada) as previously described [38]. Since Rhopr-CRF/DH-R2A encoded a protein that comprised only six transmembrane domains (data not shown; partial sequence submitted to NCBI under the accession number: KU942308) and was thus atypical, we focused on Rhopr-CRF/DH-R2B from hereon. The 5′ and 3′ untranslated regions of Rhopr-CRF/DH-R2B cDNA were obtained using a modified 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR technique as described [38]. Rhopr-CRF/DH-R2B cDNA specific 5′ and 3′ RACE primers are listed in Tables 1 and 2 respectively. The full sequence was amplified using primers listed in Table 3, cloned and sent for sequencing as described above. The sequence has been submitted to NCBI under the accession number: KJ407397.

Table 1
5′ RACE primers.

Primer	Sequence(5′-3′)
CRFR2raceRO	TTTGGTTTGAAGTAAGAGTGTGTAG
CRFR2raceR1	GCCCAAGTACCATTTTCATAGC
CRFR2raceR2	ACACAGGGTAGATAAGCCGTG
CRFR2raceR3	GGTGTATTCGGCCAACAGAG

Table 2
3′ RACE primers.

Primer	Sequence (5′-3′)
CRFR2raceF1	CTGATGGTAGCGTTGTTATACTGC
CRFR2raceF2	GTAACCTGGGAGCAAGACG
CRFR2raceF3	GTCTCCAAATACCAGAACTGAAAGTG
CRFR2raceF4	CAGTGGCCAGCGAGAATAC

2.3. Sequence analysis

Rhopr-CRF/DH-R2 gene structure was determined using WebScipio [39]. Membrane topology of Rhopr-CRF/DH-R2A and B protein sequences was predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The potential phosphorylation sites were predicted using NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) and potential N-linked glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align Rhopr-CRF/DH-R2A and B protein sequences with the following CRF and CRF/DH receptors: M. sexta (AAC46469.1), *A. domesticus* (AAC47000.1), *D. melanogaster* (NP_610960.1 and NP_610789.3) and *Homo sapiens* (NP_001138618.1 and NP_001874.2). The alignment figure was obtained from BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html). The secondary structure was predicted using Phyre V. 2. 0 (www.sbg.bio.ic.ac.uk/phyre2/) and analyzed using the UCSF Chimera software (http://www.cgl.ucsf.edu/chimera).

Phylogenetic analysis of deuterostomian CRF and protostomian CRF/DH receptors was performed using maximum-likelihood. Briefly, sequences were aligned using a MAFFT v7.017 plugin in Geneious 8.0.5 [40] and the alignment was trimmed using BMGE [41]. Maximum-

Table 3
Primers to amplify the complete sequence.

Primer	Sequence (5′-3′)
FOR-CRFR2B	CATCTTGAATACATTCGTGACG
REV-CRFR2B	CAAAAATTCTACTTCACCACTTAGAC

likelihood analysis (LG substitution model) was conducted using PhyML 3.0 and the consensus tree of 1000 bootstrap iterations was generated using Geneious 8.0.5.

2.4. Expression vectors

Primers (CRFR2B FOR: 5′ CATCTTGAATACATTCGTGACG 3′, CRFR2B FOR- KOZAK: 5′ GCCACCATGAGTACTGATG 3′ and CRFR2B REV: 5′ CAAAAATTCTACTTCACCACTTAGAC 3′) were used to amplify the Rhopr-CRF/DH-R2B ORF and introduce a Kozak translation initiation sequence at the 5′ end. The resulting product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sub-cloned into pIRES2-ZsGreen1 (Clontech, Mountain View, CA, USA) for expression in mammalian cells.

2.5. Cell culture and transfection

Chinese hamster ovary (CHO) cells stably expressing the human G-protein Gα16 (CHO/G16) [42] were used to deorphan the receptor. CHO/G16 cells were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F12-Ham (DMEM/F12) supplemented with 5% fetal bovine serum (FBS), 1% penicillin and streptomycin (pen/strep) and 50 mg/ml Hygromycin B.

To determine if Rhopr-CRF/DH-R2B couples with Gq alpha subunits (and subsequently increases intracellular calcium concentration), CHO cells stably expressing the cytoplasmic luminescent reporter aequorin were used (CHOK1-aeq) [43]. Human embryonic kidney (HEK)-293 cells stably expressing a modified cyclic-nucleotide-gated (CNG) channel (HEK293/CNG) (previously available through BD Biosciences, Mississauga, ON, Canada) were used to test if Rhopr-CRF/DH-R2B naturally works through the phospholipase C/inositol triphosphate (PLC/IP_3) pathway or the adenylate cyclase (cAMP) pathway. In this cell line, the receptor links to its natural second messenger. If the receptor activates PLC/IP_3, then intracellular calcium is released and detected by luminescence *via* aequorin reporter. If the receptor activates adenylate cyclase, then the increase in cAMP opens a cyclic nucleotide-gated (CNG) ion channel, calcium enters from the extracellular medium, and is again detected by luminescence *via* the aequorin reporter. Each pathway can be blocked; the PLC/IP_3 pathway by U73122, a specific PLC inhibitor, or the cAMP pathway by using calcium-free medium. HEK293/CNG cells were grown in DMEM/F12 supplemented with 10% FBS, 1% pen/strep and 100 mg/mL Geneticin.

All cell lines were maintained in incubators at 37 °C in 5% CO_2. X-tremeGENE 9 DNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA) was used to transiently co-transfect CHO/G16 and HEK293/CNG cells with the expression vectors containing the receptor and aequorin using the manufacturer recommended protocol. CHOK1-aeq cells were transiently transfected with only the expression vector containing the receptor. Transfection using the empty expression vector was also performed as a negative control.

2.6. Bioluminescence functional receptor assay

The assay using CHO/G16 cells was done as previously described [32]. Forty-eight hours post-transfection, cells were dislodged using PBS with EDTA and resuspended in bovine serum albumin (BSA) media: DMEM with 10% BSA in water (1%) and pen/strep (1%). Before the functional assay, coelenterazine h (Promega, Madison, WI, USA) was added to the cells at a 5 μM final concentration and incubated for 3 h with stirring in the dark at room temperature. After incubation, the mixture was diluted 10-fold using BSA media. Peptides were prepared in BSA media and plated in triplicates on a 96-well plate. Cells were injected automatically and the luminescence was recorded over 15 s using a Wallac Victor2 plate reader (Perkin Elmer, Sand Diego, CA, USA). The following peptides were used in this functional assay:

Rhopr-CRF/DH (MQRPQGPSLSVANPIEVLRSRLLLEIARRRMKEQDASR VSK NRQYLQQIamide), and Rhopr-CT/DH (GLDLGLSRGFSGSQAAKHL MGLAAANYAGGPamide). Both peptides were custom synthesized by GenScript (Piscataway, NJ, USA) at >95% purity. *D. melanogaster* Pigment Dispersing Factor (PDF; NSELINSLLSLPKNMNDAamide) was synthesized by GeneMed Synthesis (San Antonion, TX, USA) at >95% purity (a kind gift from Dr. Cornelis Grimmelikhuijzen, University of Copenhagen). The same protocol was used for the CHOK1-aeq cells testing only Rhopr-CRF/DH and using ATP as a positive control.

The HEK293/CNG cells were used with the same protocol as the CHO/G16 cell lines; however only the Rhopr-CRF/DH was used as the ligand and bioluminescence was measured over 30 s. In addition, HEK293/CNG cells were used in two different conditions post-incubation with coelenterazine h. One subset of cells was incubated in 10 μM of U73122 for 10 min before recording luminescence in response to Rhopr-CRF/DH. The second subset of cells were washed and suspended in calcium-free DMEM supplemented with 10 μM ethylene glycol tetraacetic acid (EGTA) to chelate any free calcium in the media immediately before recording luminescence in response to Rhopr-CRF/DH. The peptide for the second subset of cells was also prepared in calcium-free DMEM. The subset of cells suspended in calcium-free DMEM and EGTA were also tested in response to Rhopr-CRF/DH following a wash off and incubation in control DMEM containing calcium.

All results were analyzed using Prism5 software (http://www.graphpad.com/).

2.7. Measurement of Rhopr-CRF/DH in the haemolymph

This heterologous cellular receptor assay was also used to measure Rhopr-CRF/DH in the hemolymph of unfed and blood-fed *R. prolixus*. Hemolymph samples were taken from unfed and fed insects at various times after the start of feeding. Hemolymph (20 μl) was pooled and collected at the specific time points and placed in 60 μl of methanol: acetic acid: H_20 (90:9:1) on ice to precipitate large proteins and to acidify the peptides. The samples were then mixed using a vortex and centrifuged for 10 min at 8500*g*. The supernatant was removed and then allowed to reduce in volume in a Savant Speed-Vac Concentrator (Farmingdale, NY, USA) until approximately 8–10 μl of solution remained. The samples were then made up to 20 μl with BSA media (buffer). The receptor assay was set up as described above and a range of Rhopr-CRF/DH (10^{-13} M to 10^{-5} M) was tested and the bioluminescent response quantified to produce a standard curve. The bioluminescence response from the hemolymph samples were then placed on the standard curve to determine the concentration of Rhopr-CRF/DH in the blood. Haemolymph from starved *R. prolixus* which does not contain sufficient biological activity to stimulate MT secretion *in vitro* [44] acted as control and was used to set the detection limit of the assay since there is some interference from haemolymph.

2.8. Quantitative PCR

The following tissues were dissected from both sexes of fifth instar *R. prolixus*: CNS, dorsal vessel, salivary glands, prothoracic gland and accompanying fat body, outer lobe of thoracic gland fat body, foregut, anterior midgut, posterior midgut, hindgut, upper and 2/3 lower Malpighian tubules, the lower 1/3 of the lower MT, and reproductive tissues. In adults, only the reproductive tissues were dissected: testes, male accessory reproductive tissues, ovaries and female accessory reproductive tissues. RNA was extracted using PureLink® RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, USA) and cDNA was synthesized using iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The resulting cDNA was diluted 10-fold and used as template for qPCR. A Rhopr-CRF/DH-2BR fragment was amplified using the forward primer, qPCR-CRFR2B-FOR (5′ CTGATGACTATGAATATGGATTAATTGG 3′) and the reverse primer, qPCR-CRFR2B-REV (5′ CGGTATTTAAAAAGCAGTATAACAACG 3′). Three

housekeeping genes (alpha-tubulin, beta-actin, and ribosomal protein 49) were also amplified using the primers described earlier [32]. All qPCR reactions were done on the Mx4000 Quantitative PCR System (Stratagene, Mississauga, On, Canada) using SsoFastTM EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Delta-delta Ct method was used to calculate relative expression of the receptor transcript and geometric averaging of transcript levels of the housekeeping genes was used to the normalize expression levels. Three independent biological replicates were done with two technical replicates each including a no template negative control. Products for each target were run on a gel to confirm amplicon size and verified by sequencing.

3. Results

3.1. R. Prolixus CRF/DH receptor

Two genes for the Rhopr-CRF/DH receptor were found, Rhopr-CRF/DH-R1 and Rhopr-CRF/DH-R2. Rhopr-CRF/DH-R2 was pursued in more detail since it is the *Drosophila* DH_{44}-R2 that is found in MTs and

A

									5′	CATCTTGAATACATTCGTGACGATTTATGTAGGGAAACATGAGTACTG									-48
ATG	AGT	ACT	GAT	GGG	AAT	TTT	ACG	GAT	CCT	ACC	ATT	AAA	CTA	GGG	GAG	GAA	GAA	ATG	AGT	60
MET	Ser	Thr	Asp	Gly	Asn	Phe	Thr	Asp	Pro	Thr	Ile	Lys	Leu	Gly	Glu	Glu	Glu	Met	Ser	20
ATT	GAT	GTT	AAT	TTC	ACG	GAT	TCC	ATC	ATC	AAA	CTT	AGG	GAG	GAA	GTA	GAA	AAA	TGT	TTT	120
Ile	Asp	Val	Asn	Phe	Thr	Asp	Ser	Ile	Ile	Lys	Leu	Arg	Glu	Glu	Val	Glu	Lys	Cys	Phe	40
AAT	CTT	TCA	ATA	ACC	GAA	GTG	CCA	CCA	TCG	GAG	GAA	TAT	TGC	ACG	ACC	ACG	TGG	GAT	GGT	180
Asn	Leu	Ser	Ile	Thr	Glu	Val	Pro	Pro	Ser	Glu	Glu	Tyr	Cys	Thr	Thr	Thr	Trp	Asp	Gly	60
CTA	CTC	TGT	TGG	CCG	AAT	ACA	CCG	CCT	GGG	GTC	ACG	GCT	TAT	CTA	CCC	TGT	GTA	GCT	GAG	240
Leu	Leu	Cys	Trp	Pro	Asn	Thr	Pro	Pro	Gly	Val	Thr	Ala	Tyr	Leu	Pro	Cys	Val	Ala	Glu	80
ATT	GAC	AAT	GTC	AAA	TAC	GAT	ACT	AAT	CAA	AAT	GCA	AGT	CGT	ATT	TGC	TAT	GAA	AAT	GGT	300
Ile	Asp	Asn	Val	Lys	Tyr	Asp	Thr	Asn	Gln	Asn	Ala	Ser	Arg	Ile	Cys	Tyr	Glu	Ser	Asn	100
ACT	TGG	GCA	AAT	CAA	ACC	GAT	TAC	GGT	TTA	TGT	TCA	GAA	CTA	CAC	ACT	CTT	ACT	TCA	AAC	360
Thr	Trp	Ala	Asn	Gln	Thr	Asp	Tyr	Gly	Leu	Cys	Ser	Glu	Leu	His	Thr	Leu	Thr	Ser	Asn	120
CAA	ATA	TTA	AGC	GAT	GAA	GGT	ATT	ATT	GTA	CAA	TCA	ACT	ATC	TAC	GCT	GTT	GGA	TAT	GGA	420
Gln	Ile	Leu	Ser	Asp	Glu	Gly	Ile	Ile	Val	Gln	Ser	Thr	Ile	Tyr	Ala	Val	Gly	Tyr	Gly	140
TTT	TCA	TTG	ACT	GCT	CTT	GGT	TTG	GCA	GTA	TGG	ATT	TTT	CTT	TAT	TAC	AAA	GAC	TTG	AGG	480
Phe	Ser	Leu	Thr	Ala	Leu	Gly	Leu	Ala	Val	Trp	Ile	Phe	Leu	Tyr	Tyr	Lys	Asp	Leu	Arg	160
TGT	TTG	CGA	AAT	ACA	ATA	CAC	ACG	AAT	CTA	ATG	TGC	ACG	TAC	ATT	CTG	GCT	GAC	TTA	ATG	540
Cys	Leu	Arg	Asn	Thr	Ile	His	Thr	Asn	Leu	Met	Cys	Thr	Tyr	Ile	Leu	Ala	Asp	Leu	Met	180
TGG	ATT	CTT	AGT	TCT	ATT	CAG	GTG	TAC	GTT	AAA	ACA	GAC	CCG	GCA	ATA	TGT	ATG	GTG	CTG	600
Trp	Ile	Leu	Ser	Ser	Ile	Gln	Val	Tyr	Val	Lys	Thr	Asp	Pro	Ala	Ile	Cys	Met	Val	Leu	200
TTC	ATA	CTA	CTA	CAT	TAC	CTA	ATC	CTG	ACT	AAT	TAC	TTT	TGG	ATG	TTT	GTA	GAA	GGG	CTT	660
Phe	Ile	Leu	Leu	His	Tyr	Leu	Ile	Leu	Thr	Asn	Tyr	Phe	Trp	Met	Phe	Val	Glu	Gly	Leu	220
TAC	CTA	TAC	ATG	CTG	GTG	GTT	GAA	ACT	TTT	ACT	AGA	GAG	AAC	ATC	AAT	CTG	CGA	GCT	TAT	720
Tyr	Leu	Tyr	Met	Leu	Val	Val	Glu	Thr	Phe	Thr	Arg	Glu	Asn	Ile	Asn	Leu	Arg	Ala	Tyr	240
TTA	GCC	ATT	GGC	TGG	GGT	ATT	CCA	GTG	ATT	ATA	GTG	ATT	CCC	TCA	TGT	CTG	GCT	AGA	GCA	780
Leu	Ala	Ile	Gly	Trp	Gly	Ile	Pro	Val	Ile	Ile	Val	Ile	Pro	Ser	Cys	Leu	Ala	Arg	Ala	260
TTT	ATT	TCT	GAT	GAC	TAT	GAA	TAT	GGA	TTA	ATT	GGG	CAT	CAC	GAA	GGT	TGT	ACT	TGG	GTC	840
Phe	Ile	Ser	Asp	Asp	Tyr	Glu	Tyr	Gly	Leu	Ile	Gly	His	His	Glu	Gly	Cys	Thr	Trp	Val	280
GTA	TCA	AAT	TCT	AGT	GAT	TGG	ATC	TAT	ATG	ACT	TCA	TCG	ATT	ATA	GTG	TTG	GCT	GTT	AAC	900
Val	Ser	Asn	Ser	Ser	Asp	Trp	Ile	Tyr	Met	Thr	Ser	Ser	Ile	Ile	Val	Leu	Ala	Val	Asn	300
GTC	ACT	TTT	TTA	ATC	ATG	ATC	ATG	TGG	GTG	CTG	ATA	ACC	AAA	TTA	CGT	TCC	AGT	AAC	AAT	960
Val	Ile	Phe	Leu	Ile	Met	Ile	Met	Trp	Val	Leu	Ile	Thr	Lys	Leu	Arg	Ser	Ser	Asn	Asn	320
GCT	GAA	ACC	CAG	CAG	TAC	AGA	AAG	GCG	ACG	AAG	GCT	TTG	CTG	GTG	CTC	ATA	CCA	TTA	TTA	1020
Ala	Glu	Thr	Gln	Gln	Tyr	Arg	Lys	Ala	Thr	Lys	Ala	Leu	Leu	Val	Leu	Ile	Pro	Leu	Leu	340
GGA	GTT	ACA	TAT	ATA	TTG	TTC	ATT	GCT	GGT	CCC	ACC	GAG	GGG	CCT	TAT	GCT	TAT	CTA	TTT	1080
Gly	Val	Thr	Tyr	Ile	Leu	Phe	Ile	Ala	Gly	Pro	Thr	Glu	Gly	Pro	Tyr	Ala	Tyr	Leu	Phe	360
TCA	TAT	ATC	AGA	GCG	TTT	CTA	TTG	TCA	ACG	CAG	GGC	CTG	ATG	GTA	GCG	TTG	TTA	TAC	TGC	1140
Ser	Tyr	Ile	Arg	Ala	Phe	Leu	Leu	Ser	Thr	Gln	Gly	Leu	Met	Val	Ala	Leu	Leu	Tyr	Cys	380
TTT	TTA	AAT	ACC	GAA	GTA	CAG	AAT	ACT	GTG	AGA	CAT	CAT	TTT	ACC	AGA	TGG	AAA	GAA	AGT	1200
Phe	Leu	Asn	Thr	Glu	Val	Gln	Asn	Thr	Val	Arg	His	His	Phe	Thr	Arg	Trp	Lys	Glu	Ser	400
CGT	AAC	CTG	GGA	GCA	AGA	CGC	TAC	ACA	TGC	AGT	AAA	GAT	TGG	TCT	CCA	AAT	ACC	AGA	ACT	1260
Arg	Asn	Leu	Gly	Ala	Arg	Arg	Tyr	Thr	Cys	Ser	Lys	Asp	Trp	Ser	Pro	Asn	Thr	Arg	Thr	420
GAA	AGT	GTC	AGA	TTA	TGT	TCC	AAA	CAT	GAT	GTA	ATG	CCG	TAC	AGA	AAA	AGA	GAA	TCA	GTG	1320
Glu	Ser	Val	Arg	Leu	Cys	Ser	Lys	His	Asp	Val	Met	Pro	Tyr	Arg	Lys	Arg	Glu	Ser	Val	440
GCC	AGC	GAG	AAT	ACA	ACG	ATG	ACT	CTA	GTA	GGA	GGG	AAC	AAT	TCT	AGT	CCA	CAA	AAC	AAA	1380
Ala	Ser	Glu	Asn	Thr	Thr	Met	Thr	Leu	Val	Gly	Gly	Asn	Asn	Ser	Ser	Pro	Gln	Asn	Lys	460
ACA	ATT	AAT	TAC	GAA	TAC	TAG														1401
Thr	Ile	Asn	Tyr	Glu	Tyr	***														467
CTAAGTGGTGAAGTAGAATTTTTGAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTGA 3′																				1481

B

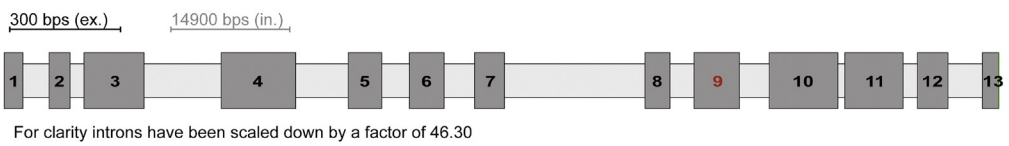

Fig. 1. Rhopr-CRF/DH-R2B cDNA and deduced amino acid sequence. The numbering for both cDNA and amino acid sequence is displayed on the right. Within the amino acid sequence, the initial start codon is capitalized (red) and the six conserved cysteine residues are highlighted in yellow. The 6 potential N-linked glycosylation sites are boxed, the 11 potential phosphorylation sites are shaded in orange and the 7 transmembrane domains are shaded in blue. The stop codon is indicated with asterisks.

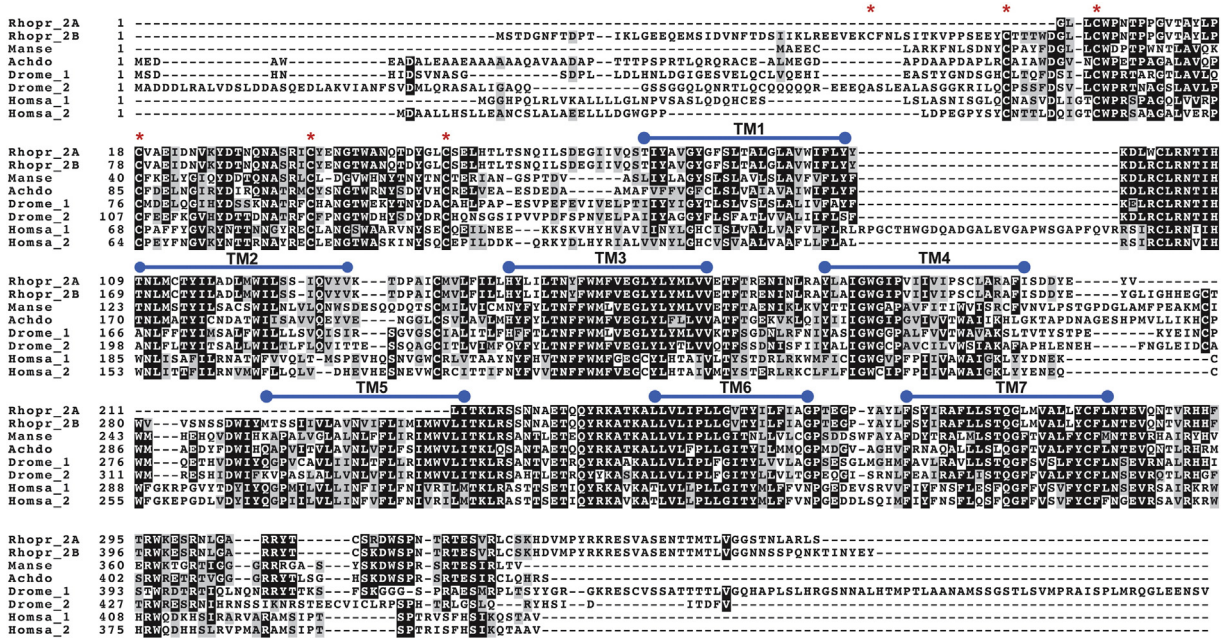

Fig. 2. Multiple sequence alignment of Rhopr-CRF/DH-R2A and 2B protein sequence and select orthologous sequences. Rhopr-CRF/DH-R2A and 2B (Rhopr_2A and 2B) were aligned with CRF/DH receptors of *M. sexta* (Manse), *A. domesticus* (Achdo), *D. melanogaster* (Drome_1 and 2), and *H. sapiens* CRF receptor 1 and 2 (Homsa_1 and 2). Residues that are identical or similar in at least 60% of the sequences have been highlighted in black and gray, respectively. The 6 conserved cysteine residues at the N-terminus are marked with red asterisks and the 7 transmembrane (TM) domains are labeled.

preliminary data revealed that the transcript for Rhopr-CRF/DH-R2, but not Rhopr-CRF/DH-R1, was present in *R. prolixus* MTs (Jean-Paul Paluzzi, Personal communication). Rhopr-CRF/DH-R2 occurs as two variants. The mRNA of the second transcript variant was isolated and fully sequenced (*Rhopr-CRF/DH-R2B*) (Fig. 1A). The partial cDNA sequence of the first transcript variant of the same gene (*Rhopr-CRF/DH-R2A*) encoded only 6 transmembrane domains and was therefore not pursued (data not shown). *Rhopr-CRF/DH-R2B* is 1401 nucleotides long which spans 13 exons and encodes a protein that comprises 467 amino acid residues (Fig. 1A and B). The receptor has the 6 conserved cysteine residues that are found in all family B1 GPCRs. It is also predicted to contain 7 transmembrane domain regions, 6 N-linked glycosylation sites in the extracellular N-terminus and 11 phosphorylation sites in the intracellular domains (Fig. 1).

3.2. Sequence analysis

Multiple sequence alignment of Rhopr-CRF/DH-R2A and B and select orthologous sequences shows that the protein sequence is highly conserved throughout, including the N-terminus which usually forms the ligand binding domain (Figs. 2 and 3). The alignment also reveals that Rhopr-CRF/DH-R2A lacks the fifth transmembrane domain (encoded by exon 9 in Fig. 2) that is present in Rhopr-CRF/DH-R2B. Predicted secondary structure of Rhopr-CRF/DH-R2B reveals several features typical

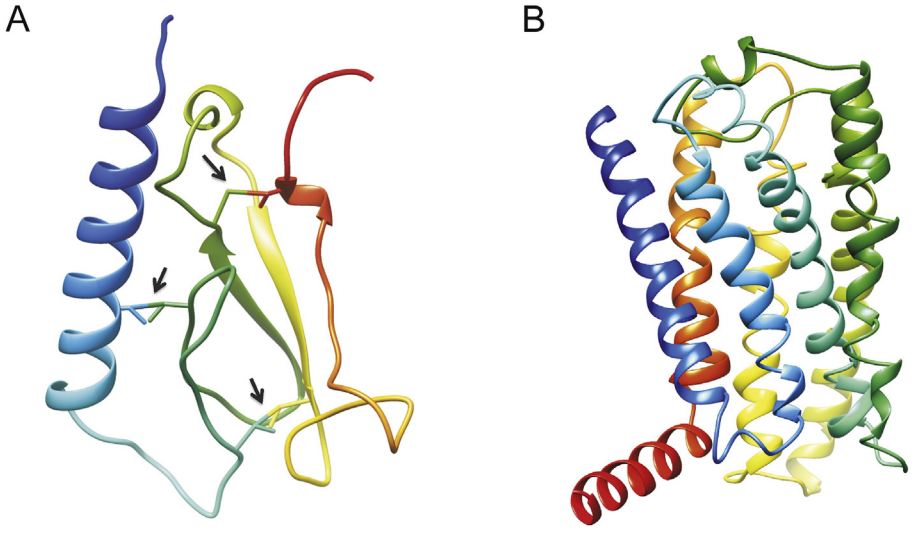

Fig. 3. Predicted tertiary structure protein model of Rhopr-CRF/DH-R2B. Blue colour indicates the 5′ end of the amino acid sequence and follows a rainbow colour pattern to the red, which depicts the 3′ end. Helices represent α-helices and arrows within the structure indicate β-strands. A) Predicted structure of the 5′ end of Rhopr-CRF/DH-R2B from amino acid 21-115. Black arrows show the 3 potential disulphide bonds formed from the 6 conserved residues. B) Predicted structure of amino acids 127-401 of Rhopr-CRF/DH-R2B including the 7 transmembrane domains and part of the cytoplasmic region.

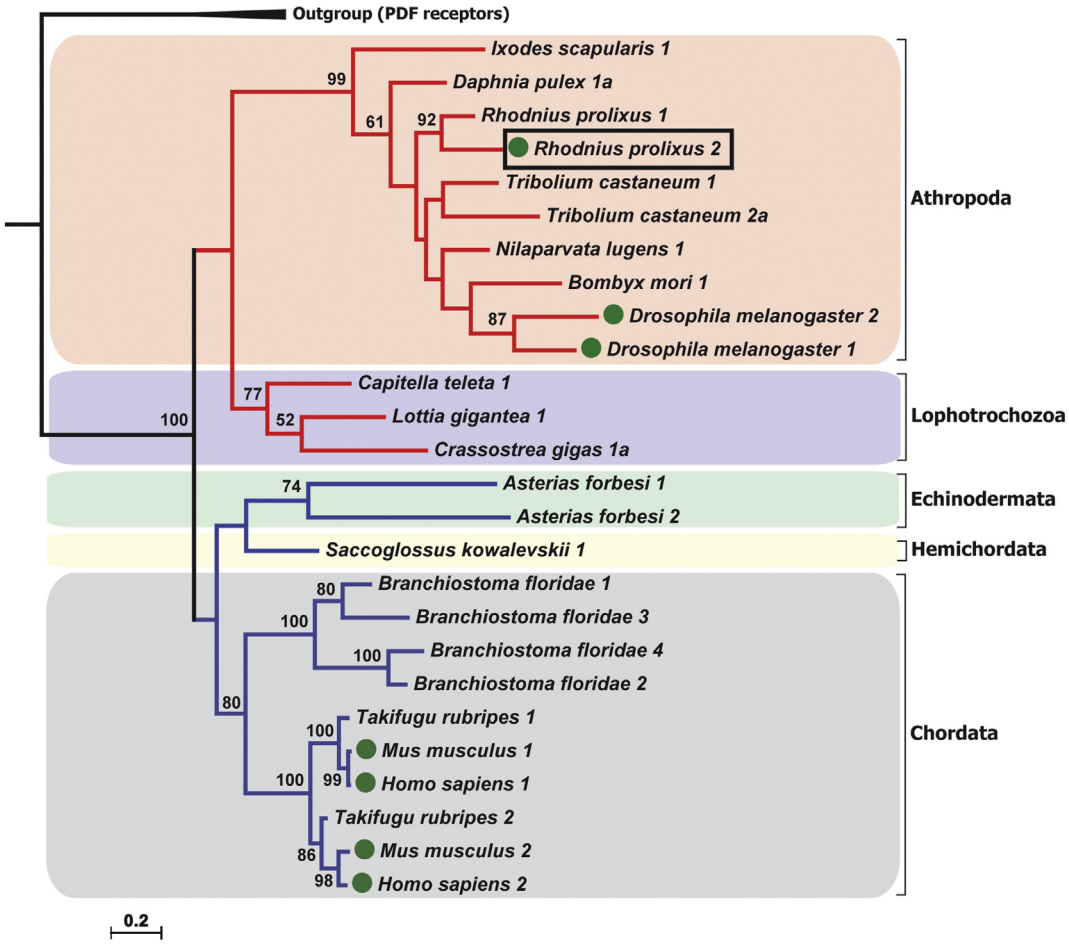

Fig. 4. A maximum-likelihood phylogeny of CRF and CRF/DH receptors. PDF receptors were used as an outgroup (condensed). Bootstrap values are based on 1000 replicates (not indicated at nodes if it is <50). The scale bar indicates amino acid substitutions per site. Species in which the receptor has been functionally characterized are marked using a green circle and the *R. prolixus* receptor characterized in this study is boxed. A partial sequence for Rhopr-CRF/DH-R1 was also included in the analysis (Fig. S1). Note that the protostomian CRF/DH receptors (red branches) and deuterostomian CRF receptors (blue branches) form two distinct clades.

of family B1 GPCRs. Along with the 7 transmembrane domains (Figs. 1 and 2), these features include the 3 disulphide bonds formed between the 6 conserved cysteine residues, two anti-parallel β-sheets and the α-helix with multiple loops that form the N-terminal ectodomain (Fig. 3).

Our phylogenetic analysis of CRF and CRH/DH receptors confirms the previously established relationship between deuterostomian CRF receptors and protostomian CRF/DH receptors (Fig. 4). More importantly, Rhopr-CRF/DH receptors isolated here are orthologous to previously characterized *D. melanogaster* CRF/DH receptors. The analysis also reveals independent expansion of receptors in several lineages. Thus, the CRF receptors have duplicated independently in *R. prolixus*, *Tribolium castaneum*, *D. melanogaster* and *Asterias forbesi*, and the four receptors in *Branchiostoma floridae* have also resulted from a lineage-specific expansion. However, the two receptors in *H. sapiens*, *Mus musculus* and *Takifugu rubripes* arose from a gene duplication in an early vertebrate ancestor.

3.3. Functional characterization of the receptor

To confirm that Rhopr-CRF/DH is the ligand for the Rhopr-CRF/DH-R2B and thereby deorphan the receptor in *R. prolixus*, the receptor was transiently expressed in CHO/G16 cells and ligand-receptor interaction was monitored using a calcium mobilization assay. EC_{50} values for all three biological replicates ranged between 36–47 nM (Fig. 5A). Moreover, Rhopr-CRF/DH produced a rapid response, with the peak response observed between 0 and 5 s (Fig. 5B). Rhopr-CT/DH and Drome-PDF evoked no response (similar values to when only basal media was being tested). These peptides were chosen because they bind to other family B1 type GPCRs. Serotonin was also tested on Rhopr-CRF/DH-R2B to check for any cross-reactivity but it displayed no response. Control cells that were transfected with empty vector showed no response when ligands were added indicating that the calcium mobilization response induced by Rhopr-CRF/DH was due to the presence of Rhopr-CRF/DH-R2B and not due to activation of endogenous receptors within CHO/G16 cells. Rhopr-CRF/DH evoked no response in CHOK1-aeq cells which were transiently transfected with Rhopr-CRF/DH-R2B.

HEK293/CNG cells were used to determine the secondary messenger pathways (the PLC/IP_3 pathway, the cAMP pathway or both) that were stimulated following Rhopr-CRF/DH-R2B activation. To test this, HEK293/CNG cells were transiently transfected with the receptor and split into subsets that were either treated with U73122 (inhibition of response mediated by PLC/IP_3 pathway) or suspended in calcium-free media supplemented with EGTA (inhibition of response mediated by the cAMP pathway). Upon activation of the receptor with Rhopr-CRF/DH, cells treated with U73122 evoked a similar response as untreated cells, with EC_{50} values around 33 nM (Fig. 6A). This indicates that the Rhopr-CRF/DH-R2B does not couple to the PLC/IP_3 pathway. Cells incubated in U73122 reached half the maximal luminescence compared to cells without treatment due to an effect of washing out of cells during the experimental protocol resulting in lower overall bioluminescence. Cells incubated in the absence of extracellular calcium (calcium-free Media with EGTA) failed to evoke any response in the presence of Rhopr-CRF/DH (Fig. 6B) indicating that cAMP is the second messenger.

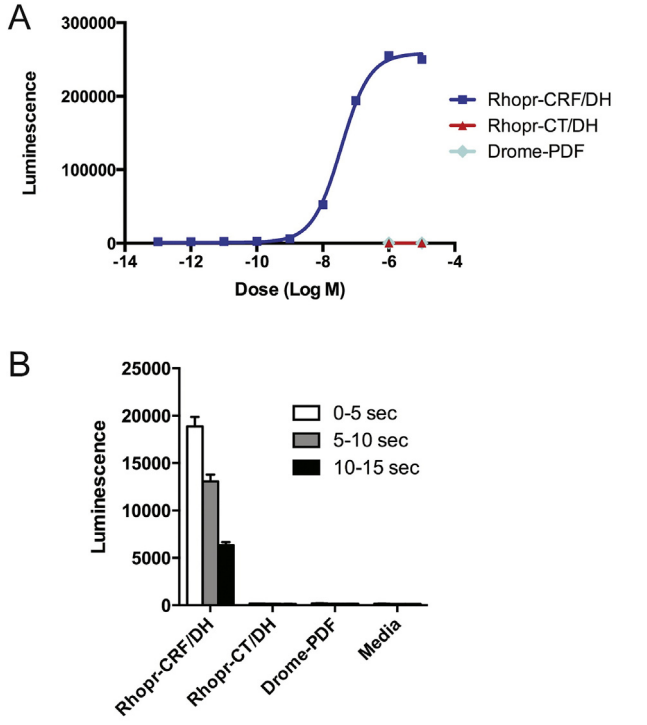

Fig. 5. Functional assay of Rhopr-CRF/DH-R2B transiently expressed in CHO/G16 cells. A) Dose-response curve demonstrating activation of Rhopr-CRF/DH-R2B using Rhopr-CRF/DH as the ligand (EC_{50} = 36 nM). Addition of Rhopr-CT/DH and Drome-PDF at the highest doses (10^{-6} and 10^{-5}) evoked no response from the CHO/G16 cells expressing Rhopr-CRF/DH-R2B. This series of experiments was repeated 4 times and similar results were obtained for each experiment. One representative experiment is shown. B) Kinetics of response (following addition of 10^{-5} M peptide) in 5 s intervals over a period of 15 s. Rhopr-CRF/DH elicits a fast response where as Rhopr-CT/DH, Drome-PDF and media (negative control) show minimal to no response. Each bar represents the mean + S.E. of 3 experiments.

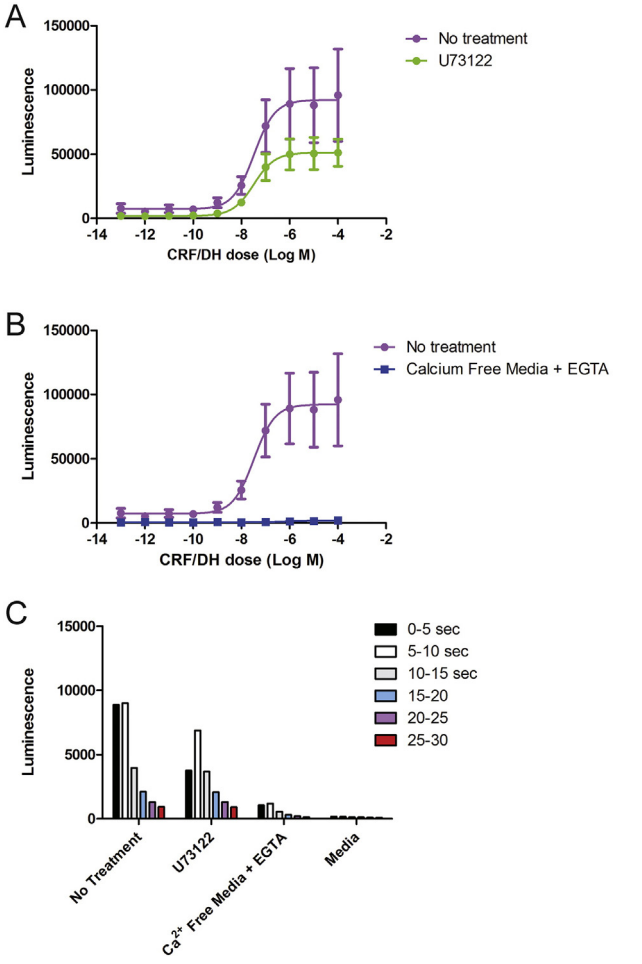

Fig. 6. Functional assay of Rhopr-CRF/DH-R2B transiently expressed in HEK293/CNG cells. A) Dose-response curves showing the effect of Rhopr-CRF/DH on Rhopr-CRF/DH-R2B activation in the presence of 10 μM U73122 (EC_{50} = 33 nM) or in its absence (EC_{50} = 33 nM). B) Dose-response curves showing the effect of Rhopr-CRF/DH on Rhopr-CRF/DH-R2B activation in the absence of extracelluar calcium (achieved using calcium-free media supplemented with EGTA) or in the presence of extracellular calcium (normal media). C) Kinetics of response (following addition of 10^{-4} M Rhopr-CRF/DH or media) in 5 s intervals over a period of 30 s for HEK293/CNG cells expressing Rhopr-CRF/DH-R2B. The cells were either left untreated (no treatment), preincubated in U73122, or suspended in calcium-free media. The response following the addition of media alone (no peptide) was also included. Each symbol represents the mean ± S.E. of 3 experiments.

The response returned upon washing with normal medium (not shown). In this cell line, Rhopr-CRF/DH produced a rapid response, with the peak response observed between 0 and 10 s (Fig. 6C). Interestingly, this response was a bit delayed in the presence of U73122 with maximum response from 5–10s (Fig. 6C). HEK293/CNG cells transfected with empty vector evoked no response upon addition of Rhopr-CRF/DH, indicating that the calcium mobilization response is due to the presence of Rhopr-CRF/DH-R2B in the cell membranes.

3.4. Rhopr-CRF/DH haemolymph titer following feeding

Activation of Rhopr-CRF/DH-R2B in the heterologous receptor assay was used to determine the levels of Rhopr-CRF/DH in the haemolymph of *R. prolixus* following feeding on blood. The receptor was indeed activated by material in the haemolymph, with an increase of Rhopr-CRF/DH seen in the haemolymph by 15 min post-feeding, with the maximum titer of 5 nM Rhopr-CRF/DH by 1 h post-feeding (Fig. 7).

3.5. Spatial expression profile of Rhopr-CRF/DH-R2B transcript

To investigate the physiological targets of Rhopr-CRF/DH, the spatial expression profiling of Rhopr-CRF/DH-R2B was performed using qPCR. Overall, expression of the transcript was seen in all dissected tissues except for very low expression levels in salivary glands (Fig. 8). Within the alimentary canal, highest expression was seen in the foregut which had double the expression of the CNS. The upper MT along with approximately 2/3 of the lower MT, was more enriched in the receptor transcript than the lower 1/3 of the lower MTs (the site of reabsorption). Interestingly, the reproductive tissues of both male and female fifth instar *R. prolixus* showed high expression with an 8-fold higher expression in the immature testes of fifth instars compared to CNS (Fig. 8). Since there was such a high expression of the receptor transcript in the fifth instar reproductive tissues for both sexes, we examined expression levels in adult reproductive tissues relative to the fifth instars. In males, there was no significant difference in expression levels between fifth instar testes and adult testes but the receptor expression was almost negligible in adult male accessory reproductive tissues (Fig. 9A). In females, the transcript levels in adult ovaries was approximately 4-fold higher ($p < 0.01$) compared to both the fifth instar ovaries and female accessory reproductive tissues (Fig. 9B).

4. Discussion

R. prolixus has two genes for CRF/DH receptors, and in this study one isoform, Rhopr-CRF/DH2B was fully isolated and sequenced. The sequence and secondary structure of Rhopr-CRF/DH-R2B is characteristic of secretin GPCRs and has high sequence similarity to human CRH receptors (CRHR2) and *Drosophila* $DH_{44}R2$, both of which act through cAMP [36,45]. Deorphaning of Rhopr-CRF/DH-R2B was done using the CHO/G16 cell line with a promiscuous G-protein (Gα16) to confirm

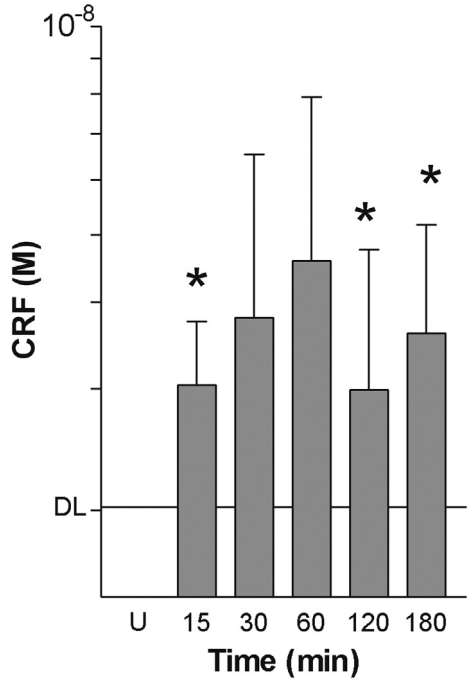

Fig. 7. Rhopr-CRF/DH haemolymph titer following feeding. Rhopr-CRF/DH in the hameolymph was quantified after feeding using the functional receptor assay. The detectable limit (DL) in a 20 μl sample of haemolymph of Rhopr-CRF/DH using this assay is 2.23×10^{-9} M. Samples of haemolymph were analyzed from unfeed (U), as well as, 15, 30, 60, 120 and 180 min after feeding. Histograms are the mean ± S.E. or 3–4 measurements. One tailed *T*-tests compared to Unfed (concentration of 1×10^{-10} M taken from [17]), asterisks indicate $p < 0.05$.

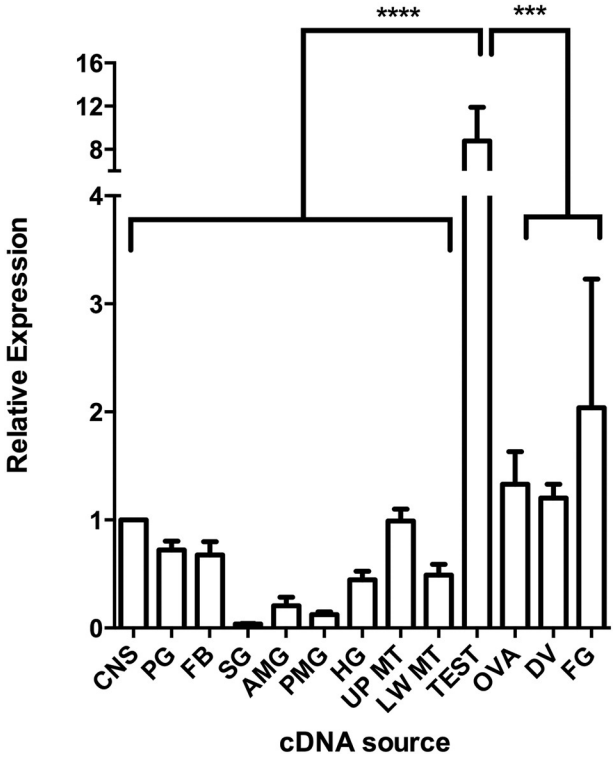

Fig. 8. Rhopr-CRF/DH-R2B receptor transcript spatial expression profile on fifth instar *R. prolixus* tissues using quantitative PCR. Expression was analyzed in the following tissues: central nervous system (CNS), prothoracic gland and associated fat body (PG), fat body (FB), salivary glands (SG), anterior midgut (AMG), posterior midgut (PMG), hindgut (HG), upper MT & 2/3 lower MT (UP MT), lower 1/3 of lower MT (LW MT), immature testes (TEST), immature ovaries (OVA), dorsal vessel (DV) and foregut (FG). Transcript was expressed in all tissues except for salivary glands, with the highest expression being in the immature testes of the male fifth instar. Fold difference in expression is relative to CNS. Values shown are mean ± SEM and the differences among means are statistically significant ($n = 3$, $p < 0.0001$, One-way ANOVA, post hoc Tukey's test).

ligand (Rhopr-CRF/DH) binding without concern for its *in vivo* signal transduction pathway. Rhopr-CRF/DH is a potent ligand for the *R. prolixus* receptor, with an EC_{50} of 36 nM. This value is comparable to the EC_{50}found with the MT secretion bioassay *in vitro* [17]. Two other peptides that also bind to family-B1 GPCRs (namely Rhopr-CT/DH and Drome-PDF) were inactive on the Rhopr-CRF/DH-R2B. Serotonin, which is a diuretic hormone in *R. prolixus*, was also tested to check for receptor promiscuity and thus identify a possible pathway for synergism; however; serotonin did not activate Rhopr-CRF/DH-R2B (data not shown).

We also used this sensitive heterologous cellular system as a bioassay to quantify the hemolymph titer of Rhopr-CRF/DH, which is thought to be released into the haemolymph after blood-feeding. Indeed, the titer of Rhopr-CRF/DH was elevated during gorging, illustrating that it is a true diuretic hormone. The titer of Rhopr-CRF/DH found in the haemolymph 1 h post feeding (5 nM) is similar to that found for Locmi-CRF/DH following 30 min of feeding on wheat grass (1.5 nM) in *L. migratoria* [11]. In addition, the titer is such that Rhopr-CRF/DH would synergize with the low doses of serotonin present at the same time following gorging [29].

HEK293/CNG cells were manipulated to see if this receptor works through the PLC/IP_3 pathway or through cAMP. In short, if receptor binding activates G_q subunit of the heterotrimeric G protein, signal transduction would work through the PLC/IP_3 pathway leading to the release of intracellular calcium, while activation of the G_s and thus the cAMP pathway, would cause extracellular calcium to flow in through the modified CNG channel [46]. The EC_{50} (33 nM) was the same for both control cells, which were stimulated by the ligand Rhopr-CRF/DH, and in cells incubated with U73122 (PLC blocker) prior to addition of the ligand. The cells incubated in the PLC blocker had the same EC_{50}, but reached half the maximal luminescence value as control cells which was most likely due to a loss in the number of cells during washing of the cells necessitated by the experimental protocol. In cells that were kept in calcium-free media containing the chelator, EGTA, addition of the ligand produced no luminescence. When cells in calcium-free media were washed in normal calcium containing medium, receptor activation responses returned (not as high as controls), which shows that the lack of response in calcium-free media did not stem from lack of viability of the cells. Furthermore, there was no response in CHOK1-*aeq* cells transfected with the receptor, which naturally works through the PLC/IP_3 pathway. Taken together, these results indicate that activation of Rhopr-CRF/DH-R2B with Rhopr-CRF/DH stimulates adenylate cyclase to increase cAMP content *in vitro*. The driving force of cAMP signaling is thought to be through the apical V-ATPase, which is believed to elicit the triphasic transepithelial potential (TEP) response in upper MTs along with Cl^- channels and a basolateral Na^+:K^+:$2Cl^-$ (NKCC) co-transporter during diuresis [30,47–49].

RhoprCRF/DH and serotonin are each capable of eliciting maximum secretion rates in MTs *in vitro* when applied at sufficient concentrations [17]. Rhopr-CRF/DH and serotonin applied together act synergistically to increase secretion rates *via* the cAMP signaling pathway [29,50]. There is much evidence which shows that CRF and serotonin both work through cAMP [8,51,52], and indeed the synergism seen for fluid secretion is also reflected in a synergism of adenylate cyclase activity when the peptide and serotonin are applied together [50]. Synerigstic activation of adenylate cyclase has been shown to occur from signals that are received coincidentally from GPCRs and *via* pathways that increase intracellular calcium concentration [53,54,55]. Interestingly, calcium has previously been ruled out as a secondary messenger system used by serotonin in the *R. prolixus* MTs based on evidence that secretion rate of MTs in calcium-free saline with 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), a calcium antagonist, did not change when treated with serotonin (1 nM) compared to

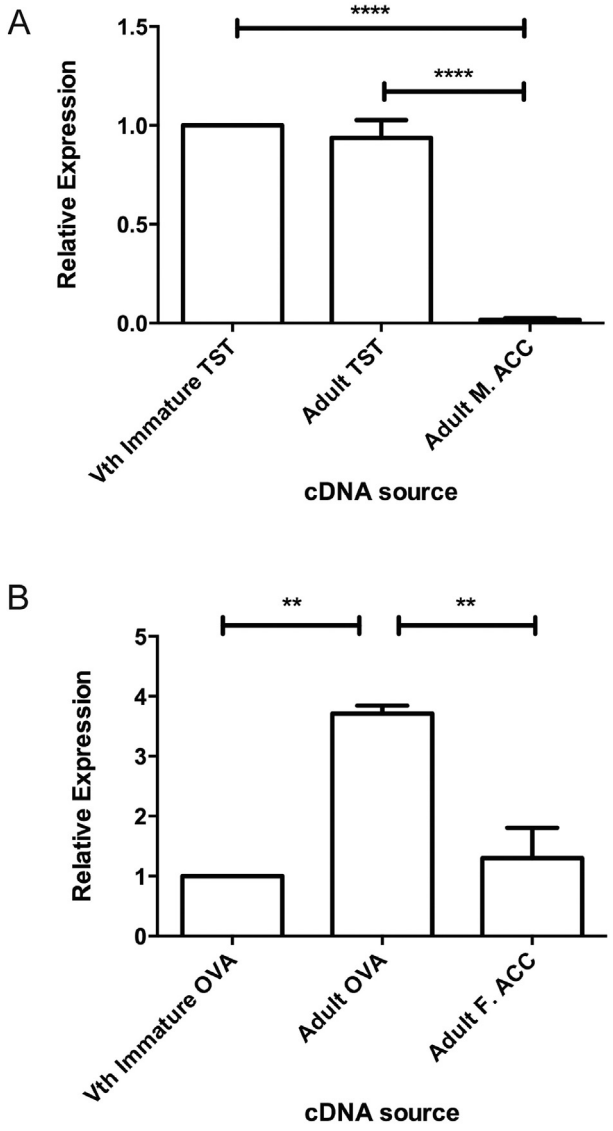

Fig. 9. Spatial expression of Rhopr-CRF/DH-R2B transcript in adult *R. prolixus* reproductive tissues using quantitative PCR. A) Fold difference in expression levels in adult testes (Adult TST) and the male accessory reproductive tissues (Adult M. ACC) are relative to the fifth instar immature testes (Vth Immature TST). Values are mean ± SEM and show statistically significant differences ($n = 3$, $p < 0.001$, One-way ANOVA, post hoc Tukey's test). B) Fold difference in adult ovaries (Adult OVA) and adult female accessory reproductive tissues (Adult F. ACC) are relative to the fifth instar immature ovaries (Vth Immature OVA). Values are mean ± SEM and show statistically significant differences ($n = 3$, $p < 0.01$, one-way ANOVA, post hoc Tukey's test).

controls [43]; however, Paluzzi et al. [29] have subsequently shown that higher doses of TMB-8 (10 μM) significantly decrease serotonin-stimulated secretion rate of MTs in *R. prolixus*. In addition, significant decreases in Rhopr-CRF/DH-stimulated secretion rates were found using 1 μM of TMB-8. No significant changes in serotonin-stimulated secretion rates were seen in the presence of EGTA, suggesting that it is intracellular calcium that acts as a secondary messenger upon activation of serotonin receptors during diuresis. In light of this finding, a recent study by Gioino et al. [56] explored the role of calcium signaling in fluid secretion stimulated by serotonin in *R. prolixus*. They found that intracellular calcium waves were triggered when MTs were treated with serotonin and these waves could be blocked using a cell permeable calcium chelator, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM), in calcium-free saline. Serotonin was not directly releasing intracellular calcium but used cAMP to trigger calcium waves mediated through the protein kinase A (PKA) signaling pathway. Unfortunately, Rhopr-CRF/DH was not tested in these latter assays. A model for serotonin and RhoprCRF/DH in stimulating fluid secretion is shown in Fig. 10. In this model a synergistic stimulation of adenylate cyclase activity is induced by serotonin and Rhopr-CRF/DH leading to a synergistic increase in cAMP [50]. Calcium may contribute to this synergism. The elevated levels of cAMP subsequently activate the PKA-signaling pathway to trigger an increase in inositol 1′, 4′, 5′ – trisphosphate (IP_3) that subsequently increases the amplitude and/or frequency of calcium waves released intracellular from storage sites within the cell [56]. The elevated calcium levels could then modulate the NKCC and Cl^- transporters to drive increases in diuresis that are larger than the sum of activity produced by low concentrations of the ligands working independently (see Fig. 10).

Rhopr-CRF/DH-R2B is expressed in many tissues in the fifth instar of *R. prolixus* including the anterior and posterior midgut, the hindgut and MTs, which are all involved in post-feeding diuresis. Higher expression in the upper portion of the MTs in comparison to the lower portion of the MTs was expected since Rhopr-CRF/DH physiologically acts upon the upper portion of the MTs [17,30,57], but has no effect on potassium reuptake in the very lower MTs [30]. The low levels of expression in the lower portion of the MTs may be somewhat exaggerated by contamination of the upper MTs since it is difficult to accurately separate these areas by dissection. There was also higher expression of the receptor in the hindgut relative to the anterior and posterior midgut. CRF/DH-like immunoreactive processes have been found over the entire hindgut [26] and preliminary results indicate that Rhopr-CRF/DH does indeed stimulate hindgut contractions [31]. The hindgut collects urine and expels it every 2–3 min through the anus during the fast phase of diuresis [26], and so Rhopr-CRF/DH may modulate/control this process.

The dorsal vessel, which has high expression of Rhopr-CRF/DH-R2B, has been shown to be involved in hemolymph flow throughout the insect. At the start of feeding in *R. prolixus*, there are increases in frequency of muscle contractions in the anterior midgut which churn the ingested blood inside the anterior midgut as well as help mix the hemolymph outside. These contractions minimize unstirred layers of hemolymph around the anterior midgut and the MTs but also push the hemolymph towards the posterior of the insect, and with it, the diuretic hormones [2,58]. To circulate the hemolymph back to the anterior of the insect, the heart at the posterior end of the insect expands to draw hemolymph into the heart through ostia and then by a twist-like contraction propels the hemolymph and its neurohormonal contents from the posterior to the anterior end of the dorsal vessel [52,59]. Rhopr-CRF/DH has never been tested on dorsal vessel contractions but one may now anticipate that it increases the frequency of dorsal vessel contractions for hemolymph circulation as does another diuretic hormone, serotonin [60,61]. The foregut in fifth instar *R. prolixus* showed the second highest level of expression of Rhopr-CRF/DH-R2B transcript of all the tissues tested. Rhopr-CRF/DH could target the foregut and induce contractions in a similar manner as Acheta-DP increases frequency and amplitude of contractions in the foregut of *A. domesticus* [62].

High expression of Rhopr-CRF/DH-R2B was found in the male and female reproductive tissues of both fifth instars and adults. Insect CRF/DHs are rarely studied outside the realm of feeding and diuresis, but there are many possibilities for the role of Rhopr-CRF/DH in targeting reproductive tissues. Rhopr-CRF/DH could help in the growth and maintenance of reproductive tissues by mediating ion and water transport. Rhopr-CRF/DH could also be involved in the relationship between feeding and egg production, where nutrients from the blood meal are used to initiate oogenesis [63]. A blood meal is essential to obtain nutrients for egg production and vitellogenin (egg yolk) synthesis for oocyte growth [64]. As mentioned previously, the nutrients of the blood meal are concentrated by diuresis and *R. prolixus* feed as both fifth instars and adults for growth and development. Though fast diuresis lasts for only 3–4 h, the nutrients may take days to start being digested [65], and the highest transfer of lipids for oocyte growth from the anterior midgut occur over the first three days post-feeding. Rhopr-CRF/DH

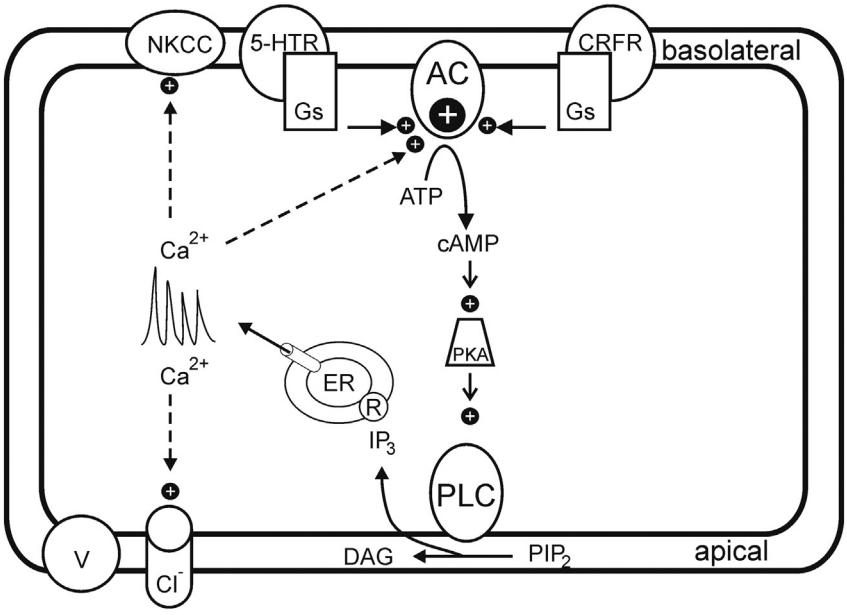

Fig. 10. A hypothetical model for serotonin and RhoprCRF/DH synergistically stimulating fluid secretion from Malpighian tubule cells of R. prolixus. In this model, serotonin (5-HT) and Rhopr-CRF/DH (CRF) at low concentrations bind to their G-protein coupled receptors (R), leading to a synergistic increase in adenylate cyclase (AC) activity and thereby a synergistic increase in cAMP content [50]. The elevated levels of cAMP subsequently activate the protein kinase A (PKA)-signaling pathway to trigger an increase in inositol 1′, 4′, 5′ – trisphosphate (IP3) that subsequently increases the amplitude and/or frequency of calcium waves released intracellular from storage sites (Endoplasmic reticulum – ER) within the cell [56]. The elevated calcium levels could then modulate the $N^+/K^+/Cl^-$ cotransporter (NKCC) and Cl^- channel to drive increases in diuresis that are larger than the sum of activity produced by low concentrations of the ligands working independently. Calcium may contribute to this synergism by enhancing the activity of the adenylate cyclase [53–55]. Abbreviations: Gs – stimulatory G-protein, ATP – adenosine trisphosphate, PLC – protein lipase C, PIP2 – phosphatidylinositol 4,5-bisphosphate, DAG – diacylglycerol, R – IP3 receptor, V – V-type H+-ATPase.

released during feeding could act as an inhibitory signal for reproductive tissues, especially ovaries, to 'hold off' from initiating oocyte maturation before the nutrients are ready to be shuttled to the developing oocytes. In support of this suggestion, it has been shown that injection of CRF/DH led to inhibition of oocyte growth (*in vivo*) in the desert locusts [22]. In vertebrates, CRH has a potential role in reproduction where it has been shown to be involved in the inhibition of the reproductive hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and to affect puberty in mammals [66–70]. Rhopr-CRF/DH might therefore play an integral role in coordinating the timing of oocyte growth and development in *R. prolixus*.

To determine if Rhopr-CRF/DH-2BR is present in the prothoracic gland of *R. prolixus*, thoracic fat body around the prothoracic gland was examined for expression and compared to that of the prothoracic gland with associated fat body. The prothoracic gland lies between two layers of fat in the inner lobe of the thoracic fat body in fifth instars and therefore cannot be dissected out separately [71]. Since the expression of the receptor was the same between these two samples it was concluded that the receptor expression is mainly in the fat body. There is a large growth of fat body cells between fifth instars and adult *R. prolixus*. Rhopr-CRF/DH could be involved in signaling the ingestion of a blood meal for the growth of the fat body in fifth instars in conjunction with oocyte maturation where Rhopr-CRF/DH could signal fat bodies to 'hold off' from shuttling lipids to the developing oocytes [72] until the ovaries are ready to accept the lipid.

5. Conclusions

We have deorphaned the receptor for the neuropeptide Rhopr-CRF/DH and shown it to be a G-protein coupled receptor of the secretin family. This receptor is activated by Rhopr-CRF/DH in a dose-dependent manner with an EC_{50} of 36–47 nM. The transcript for the receptor Rhopr-CRF/DH-R2 is present in the CNS and peripheral tissues, including the MTs. Interestingly, along with the presence of the receptor transcript in feeding-related tissues, the transcript is also present in reproductive tissues. Using a variety of functional receptor assays we have been able to determine that the receptor works *via* the second messenger, cAMP. We have been able to detect Rhopr-CRF/DH in the haemolymph of recently fed *R. prolixus* confirming that Rhopr-CRF/DH is indeed a true diuretic hormone in *R. prolixus*. In addition to altering ion and fluid transport during diuresis Rhopr-CRF/DH could be involved in the control of satiation, muscle contraction, and reproductive processes. It would appear that a basic function of Rhopr-CRF/DH is as a stress hormone that helps maintain homeostasis and proper growth and development in *R. prolixus*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.05.020.

Acknowledgements

We would like to acknowledge Jean-Paul Paluzzi for preliminary data associated with this project and to Natural Sciences and Engineering Council of Canada (NSERC) for funding to ABL (RGPIN 2014-06253) and IO (RGPIN 8522-12).

References

[1] J.H. Spring, Endocrine regulation of diuresis in insects, J. Insect Physiol. 36 (1990) 13–22.
[2] S.H.P. Maddrell, Excretion in the blood-sucking bug, *Rhodnius prolixus*. The control of diuresis, J. Exp. Biol. 40 (1963) 247–256.
[3] S.H.P. Maddrell, Excretion in the blood-sucking bug, *Rhodnius prolixus* Stål. II. The normal course of diuresis and the effect of temperature, J. Exp. Biol. 41 (1964) 163–176.
[4] S.H.P. Maddrell, Excretion in the blood-sucking bug, *Rhodnius prolixus* Stål. III. The control of the release of the diuretic hormone, J. Exp. Biol. 41 (1964) 459–472.
[5] S.H.P. Maddrell, Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. J. Exp. Biol. 51 (1969) 71–97.
[6] S.H.P. Maddrell, J.E. Phillips, Secretion of hypo-osmotic fluid by the lower Malpighian tubules of *Rhodnius prolixus*, J. Exp. Biol. 62 (1975) 671–683.
[7] G.M. Coast, Neuroendocrine control of ionic homeostasis in blood-sucking insects, J. Exp. Biol. 212 (2009) 378–386.
[8] I. Orchard, Peptides and serotonin control feeding-related events in *Rhodnius prolixus*, Front. Biosci. (Elite Ed.) 1 (2009) 250–262.

[9] G.M. Coast, C.S. Garside, Neuropeptide control of fluid balance in insects, Ann. N. Y. Acad. Sci. 1040 (2005) 1–8.
[10] A.B. Lange, I. Orchard, F.M. Barrett, Changes in haemolymph serotonin levels associated with feeding in the blood-sucking bug, *Rhodnius prolixus*, J. Insect Physiol. 35 (1989) 393–399.
[11] N. Audsley, G.J. Goldsworthy, G.M. Coast, Circulating levels of *Locusta* diuretic hormone: the effect of feeding, Peptides 18 (1997) 59–65.
[12] D.A. Lovejoy, D. Barsyte-Lovejoy, Characterization of a corticotropin-releasing factor (CRF)/diuretic hormone-like peptide from tunicates: insight into the origins of the vertebrate CRF family, Gen. Comp. Endocrinol. 165 (2010) 330–336.
[13] H. Kataoka, R.G. Troetschler, J.P. Li, S.J. Kramer, R.L. Carney, D.A. Schooley, Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*, Proc. Natl. Acad. Sci. 86 (1989) 2976–2980.
[14] I. Kay, M. Patel, G.M. Coast, N.F. Totty, A.I. Mallet, G.J. Goldsworthy, Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana* L, Regul. Pept. 42 (1992) 111–122.
[15] E. Lehmberg, R.B. Ota, K. Furuya, D.S. King, S.W. Applebaum, H.J. Ferenz, D.A. Schooley, Identification of a diuretic hormone of *Locusta migratoria*, Biochem. Biophys. Res. Commun. 179 (1991) 1036–1041.
[16] P. Cabrero, J.C. Radford, K.E. Broderick, L. Costes, J.A. Veenstra, E.P. Spana, ... J.A. Dow, The DH gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP, J. Exp. Biol. 205 (2002) 3799–3807.
[17] V.A. Te Brugge, J.P. Paluzzi, D.A. Schooley, I. Orchard, Identification of the elusive peptidergic diuretic hormone in the blood feeding bug *Rhodnius prolixus*: a CRF related peptide, J. Exp. Biol. 214 (2011) 317–381.
[18] M.B. Blackburn, T.G. Kingan, W. Bodnar, J. Shabanowitz, D.F. Hunt, T. Kempe, R.M. Wagner, A.K. Raina, M.E. Schnee, M.C. Ma, Isolation and identification of a new diuretic peptide from the tobacco hornworm, *Manduca sexta*, Biochem. Biophys. Res. Commun. 181 (1991) 927–932.
[19] F.L. Clottens, G.M. Holman, G.M. Coast, N.F. Totty, T.K. Hayes, I. Kay, A.I. Mallet, M.S. Wright, J.-S. Chung, O. Truong, D.L. Bull, Isolation and characterization of a diuretic peptide common to the house fly and stable fly, Peptides 15 (1994) 971–979.
[20] K. Furuya, M.A. Harper, K.M. Schegg, D.A. Schooley, Isolation and characterization of CRF-related diuretic hormones from the white-lined sphinx moth *Hyles lineata*, Insect Biochem. Mol. Biol. 30 (2000) 127–133.
[21] L.L. Keeley, J.S. Chung, T.K. Hayes, Diuretic and antifeedant actions by *Manduca sexta* diuretic hormone in lepidopteran larvae, Experientia 48 (1992) 1145–1148.
[22] P. Van Wielendaele, S. Dillen, E. Marchal, L. Badisco, J.V. Broeck, CRF-like diuretic hormone negatively affects both feeding and reproduction in the desert locust, *Schistocerca gregaria*, PLoS One 7 (2012) e31425.
[23] Y.-J. Kim, D. Žitňan, K.-H. Cho, D.A. Schooley, A. Mizoguchi, M.E. Adams, Central peptidergic ensembles associated with organization of an innate behavior, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 14211–14216.
[24] D.J. Cavanaugh, J.D. Geratowski, J.R.A. Wooltorton, J.M. Spaethling, C.E. Hector, X. Zheng, E.C. Johnson, J.H. Eberwine, A. Sehgal, Identification of a circadian output circuit for rest: activity rhythms in *Drosophila*, Cell 157 (2014) 689–701.
[25] K.-M. Lee, I. Daubnerová, R.E. Isaac, C. Zhang, S. Choi, J. Chung, Y.-J. Kim, A neuronal pathway that controls sperm ejection and storage in female *Drosophila*, Curr. Biol. 25 (2015) 790–797.
[26] V.A. Te Brugge, S.M. Miksys, G.M. Coast, D.A. Schooley, I. Orchard, The distribution of a CRF-like diuretic peptide in the blood-feeding bug *Rhodnius prolixus*, J. Exp. Biol. 202 (1999) 2017–2027.
[27] S.H.P. Maddrell, D.E.M. Pilcher, B.O.C. Gardiner, Pharmacology of the Malpighian tubules of *Rhodnius* and *Carausius*: the structure-activity relationship of tryptamine analogues and the role of cyclic AMP, J. Exp. Biol. 54 (1971) 779–804.
[28] F.M. Barrett, I. Orchard, Serotonin-induced elevation of cAMP levels in the epidermis of the blood-sucking bug, *Rhodnius prolixus*, J. Insect Physiol. 36 (1990) 625–633.
[29] J.P. Paluzzi, C. Yeung, M.J. O'Donnell, Investigations of the signaling cascade involved in diuretic hormone stimulation of Malpighian tubule fluid secretion in *Rhodnius prolixus*, J. Insect Physiol. 59 (2013) 1179–1185.
[30] A. Donini, M.J. O'Donnell, I. Orchard, Differential actions of diuretic factors on the Malpighian tubules of *Rhodnius prolixus*, J. Exp. Biol. 211 (2008) 42–48.
[31] G. Bhatt, R. da Silva, R.J. Nachman, I. Orchard, The molecular characterization of the kinin transcript and the physiological effects of kinins in the blood-gorging insect, *Rhodnius prolixus*, Peptides 53 (2014) 148–158.
[32] M. Zandawala, S. Li, F. Hauser, C.J. Grimmelikhuijzen, I. Orchard, Isolation and functional characterization of calcitonin-like diuretic hormone receptors in *Rhodnius prolixus*, PLoS One 8 (2013) e82466.
[33] J.D. Reagan, Molecular cloning of a putative Na^+-K^+-2Cl^- cotransporter from the Malpighian tubules of the tobacco hornworm, *Manduca sexta*, Insect Biochem. Mol. Biol. 25 (1995) 875–880.
[34] J.D. Reagan, Molecular cloning and function expression of a diuretic hormone receptor from the house cricket, *Acheta domesticus*, Insect Biochem. Mol. Biol. 26 (1996) 1–6.
[35] E.C. Johnson, L.M. Bohn, P.H. Taghert, *Drosophila* CG8422 encodes a functional diuretic hormone receptor, J. Exp. Biol. 207 (2004) 743–748.
[36] C.E. Hector, C.A. Bretz, Y. Zhao, E.C. Johnson, Functional differences between two CRF-related diuretic hormone receptors in *Drosophila*, J. Exp. Biol. 212 (2009) 3142–3147.
[37] J.P. Paluzzi, W.K. Russell, R.J. Nachman, I. Orchard, Isolation, cloning, and expression mapping of a gene encoding an antidiuretic hormone and other CAPA-related peptides in the disease vector, *Rhodnius prolixus*, Endocrinology 149 (2008) 4638–4646.
[38] M. Zandawala, Y. Lytvyn, D. Taiakina, I. Orchard, Cloning of the cDNA, localization, and physiological effects of FGLamide-related allatostatins in the blood-gorging bug, *Rhodnius prolixus*, Insect Biochem. Mol. Biol. 42 (2012) 10e21.
[39] F. Odronitz, H. Pillmann, O. Keller, S. Waack, M. Kollmar, WebScipio: an online tool for the determination of gene structures using protein sequences, BMC Genomics 9 (2008) 422.
[40] K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform, Nucleic Acids Res. 30 (2002) 3059–3066.
[41] A. Criscuolo, S. Gribaldo, BMGE (block mapping and gathering with entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments, BMC Evol. Biol. 10 (2002) 210.
[42] J. Stables, A. Green, F. Marshall, N. Fraser, E. Knight, M. Sautel, G. Milligan, M. Lee, S. Rees, A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor, Anal. Biochem. 252 (1997) 115–126.
[43] J.P.V. Paluzzi, P. Young, M.S. Defferrari, I. Orchard, C.R. Carlini, M.J. O'Donnell, Investigation of the potential involvement of eicosanoid metabolites in anti-diuretic hormone signaling in *Rhodnius prolixus*, Peptides 34 (2012) 127–134.
[44] S.H.P. Maddrell, B.O.C. Gardiner, Diuretic hormone in adult *Rhodnius prolixus*: total store and speed of release, Physiol. Entomol. 1 (1976) 265–269.
[45] J.C. Cardoso, R.C. Félix, C.A. Bergqvist, D. Larhammar, New insights into the evolution of vertebrate CRH (corticotropin-releasing hormone) and invertebrate DH44 (diuretic hormone 44) receptors in metazoans, Gen. Comp. Endocrinol. 209 (2014) 162–170.
[46] A. Visegrády, A. Boros, Z. Némethy, B. Kiss, G.M. Keserű, Application of the BD ACTOne technology for the high-throughput screening of Gs-coupled receptor antagonists, J. Biomol. Screen. 12 (2007) 1068–1073.
[47] M.J. O'Donnell, S.H.P. Maddrell, Secretion by the Malpighian tubules of *Rhodnius prolixus* stal — electrical events, J. Exp. Biol. 110 (1984) 275–290.
[48] T.M. Clark, T.K. Hayes, G.M. Holman, K.W. Beyenbach, The concentration-dependence of CRF-like diuretic peptide: mechanisms of action, J. Exp. Biol. 201 (1998) 1753–1762.
[49] J.P. Ianowski, M.J. O'Donnell, Transepithelial potential in Malpighian tubules of *Rhodnius prolixus*: lumen-negative voltages and the triphasic response to serotonin, J. Insect Physiol. 47 (2001) 411–421.
[50] S.H.P. Maddrell, W.S. Herman, J.A. Farndale, J.A. Riegel, Synergism of hormones controlling epithelial fluid transport in an insect, J. Exp. Biol. 174 (1993) 65–80.
[51] G.M. Coast, I. Orchard, J.E. Phillips, D.A. Schooley, Insect diuretic and antidiuretic hormones, J. Insect Physiol. 29 (2002) 279–409.
[52] I. Orchard, Serotonin: a coordinator of feeding-related physiological events in the blood-gorging bug, *Rhodnius prolixus*, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 144 (2006) 316–324.
[53] M.S. Livingston, Genetic dissection of *Drosophila* adenylate cyclase, Proc. Natl. Acad. Sci. 82 (1985) 5992–5996.
[54] T.W. Abrams, K.A. Karl, E.R. Kandel, Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: dual regulation of adenylate cyclase by Ca^{2+}/calmodulin and transmitter, J. Neurosci. 11 (1991) 2655–2665.
[55] R.K. Sunahara, C.W. Dessauer, A.G. Gilman, Complexity and diversity of mammalian adenylyl cyclases, Annu. Rev. Pharmacol. Toxicol. 36 (1996) 461–480.
[56] P. Gioino, B.G. Murray, J.P. Ianowski, Serotonin triggers cAMP and PKA-mediated intracellular calcium waves in Malpighian tubules of *Rhodnius prolixus*, Am. J. Physiol. Regul. Integr. Comp. Physiol. 307 (2014) R828–R836.
[57] V.A. Te Brugge, D.A. Schooley, I. Orchard, The biological activity of diuretic factors in *Rhodnius prolixus*, Peptides 23 (2002) 671–681.
[58] K.A. Collier, M.J. O'Donnell, Analysis of epithelial transport by measurement of K^+, Cl^- and pH gradients in extracellular unstirred layers: ion secretion and reabsorption by Malpighian tubules of *Rhodnius prolixus*, J. Exp. Biol. 200 (1997) 1627–1638.
[59] R.G. Chiang, J.A. Chiang, K.G. Davey, Morphology of the dorsal vessel in the abdomen of the blood-feeding insect *Rhodnius prolixus*, J. Morphol. 204 (1990) 9–23.
[60] R.G. Chiang, J.A. Chiang, K.G. Davey, A sensory input inhibiting heart rate in an insect, *Rhodnius prolixus*, Experientia 48 (1992) 1122–1125.
[61] J.-P.V. Paluzzi, A.S. Haddadd, L. Sedra, I. Orchard, A.B. Lange, Functional characterization and expression analysis of the myoinhibiting peptide receptor in the Chagas disease vector, *Rhodnius prolixus*, Mol. Cell. Endocrinol. 399 (2015) 143–153.
[62] P.D. Blake, I. Kay, G.M. Coast, Myotropic activity of Achetadiuretic peptide on the foregut of the house cricket, *Acheta domesticus*, J. Insect Physiol. 42 (1996) 1053–1059.
[63] P.A. Buxton, The biology of a blood-sucking bug, *Rhodnius prolixus*, Trans. Royal Entomol. Soc. London 78 (1930) 227–256.
[64] K. Davey, The interaction of feeding and mating in the hormonal control of egg production in *Rhodnius prolixus*, J. Insect Physiol. 53 (2007) 208–215.
[65] H.S. Coelho, G.C. Atella, M.F. Moreira, K.C. Gondim, H. Masuda, Lipophorin density variation during oogenesis in *Rhodnius prolixus*, Arch. Insect Biochem. Physiol. 35 (1997) 301–313.
[66] J.S. Kinsey-Jones, X.F. Li, A.M.I. Knox, Y.S. Lin, S.R. Milligan, S.L. Lightman, K.T. O'Byrne, Corticotrophin-releasing factor alters the timing of puberty in the female rat, J. Neuroendocrinol. 22 (2010) 102–109.
[67] X.F. Li, A.M.I. Knox, K.T. O'byrne, Corticotrophin-releasing factor and stress-induced inhibition of the gonadotrophin-releasing hormone pulse generator in the female, Brain Res. 1364 (2010) 153–163.
[68] D. Chand, D.A. Lovejoy, Stress and reproduction: controversies and challenges, Gen. Comp. Endocrinol. 171 (2011) 253–257.
[69] D.A. Lovejoy, B.S. Chang, N.R. Lovejoy, J. del Castillo, Molecular evolution of GPCRS: CRH/CRH receptors, J. Mol. Endocrinol. 52 (2014) T43–T60.
[70] D. Lovejoy, B. Chang, N. Lovejoy, J. del Castillo, Origin and functional evolution of the corticotrophin-releasing hormone receptors, J. Mol. Endocrinol. JME-13 (2014).
[71] V.B. Wigglesworth, The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting, J. Exp. Biol. 29 (1952) 561–570.
[72] G.C. Atella, K.C. Gondim, H. Masuda, Transfer of phospholipids from fat body to lipophorin in *Rhodnius prolixus*, Arch. Insect Biochem. Physiol. 19 (1992) 133–144.