The *Brugia malayi* neuropeptide receptor-4 is activated by FMRFamide-like peptides and signals via $G\alpha_i$

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Abstract

Genetic studies undertaken in the model organism *Caenorhabditis elegans* have demonstrated the importance of neuropeptidergic signalling in nematode physiology. Disruption of this signalling may have deleterious phenotypic consequences, including altered locomotion, feeding behaviour, and reproduction. Neuropeptide G protein-coupled receptors (GPCRs) that transduce many of these signals therefore represent cogent drug targets. Recently published genomic sequencing data for a number of parasitic helminths of medical and veterinary importance has revealed the apparent conservation of a number of neuropeptides, and neuropeptide receptors between parasitic and free-living species, raising the intriguing possibility of developing broad-spectrum anthelmintic therapeutics. Here, we identify and clone a neuropeptide receptor, NPR-4, from the human filarial nematode *Brugia malayi* and demonstrate its activation *in vitro*, by FMRFamide-like peptides of the FLP-18 family, and intracellular signalling via $G\alpha_i$ mediated pathways. These data represent the first example of deorphanization of a neuropeptide GPCR in any parasitic helminth species.

Keywords

- G protein-coupled receptor
- *Brugia malayi*
- FMRFamide-like peptides
- Neuropeptide
- Neuropeptide receptor
- Lymphatic filariasis

Results

Human lymphatic filariasis (LF), caused by infection with the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, is a major health burden representing one of the leading causes of physical disability in the world [1,2]. Recent estimates suggest that up to 1.34 billion people living in endemic regions may be at risk, with 120 million currently infected [1,3]. Infective filarial larvae are transmitted to humans via the bites of mosquitos, and undergo maturation and sexual reproduction in the host lymphatic vessels. The result of reproduction is the release of sheathed pre-larvae (microfilariae) into the blood, which subsequently pass back to the mosquitos during blood meals [4]. Lymphatic function is often compromised in infected individuals, due to obstruction and fibrosis of lymphatic vessels, and clinical manifestations include lymphedema, hydroceles and the highly debilitating condition elephantiasis [3,5].

While the current treatments, including diethylcarbamazine (DEC), albendazole (ABZ) and the macrocyclic lactone ivermectin (IVM), have potent microfilaricidal activity, the effects on adult nematodes (which can be reproductively active for 4-9 years) are less pronounced [6-8]. Additionally, severe adverse reactions to IVM and DEC have been described in patients suffering from polyparasitism, or high microfilarial loads [9-11]. Furthermore, anthelmintic resistance in animals of veterinary importance [12], reports of reduced efficacy in the treatment of human patients infected with *W. bancrofti* to DEC [13], and sub-optimal responses of the filarial nematode *Onchocerca volvulus* to IVM [14-16], and hookworms to mebendazole and pyrantel [17-19], highlight the need for development of novel therapeutic interventions.

G protein-coupled receptors (GPCRs) are a large family of seven transmembrane domain proteins that are responsible for about 80% of signal transduction across eukaryotic cell membranes [20]. They transduce extracellular signals (ligands) to intracellular signalling pathways through interaction with a family of intracellular heterotrimeric G proteins, consisting of Gα, Gβ and Gγ subunits. Upon activation, the receptor acts as a guanine exchange factor (GEF) and catalyses the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the Gα subunit. This causes dissociation of the G protein complex from the receptor and dissociation of the Gα subunit from the Gβγ dimer. The dissociated G protein entities can then activate various intracellular signalling pathways. There are up to 20 known subtypes of the Gα subunit in humans, broadly arranged into one of 4 groups; $G\alpha_s$, $G\alpha_q$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$ [21]. The different subtypes can be classified based on the intracellular signalling pathways that they activate; $G\alpha_s$ and $G\alpha_i$ activate or inhibit adenylyl cyclase respectively; $G\alpha_q$ activates phospholipase C (PLC) resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and production of inositol trisphosphate (IP_3) and diacyl glycerol (DAG); $G\alpha_{12/13}$ has been implicated in the regulation of Rho signalling via the activation of Rho GTPase nucleotide exchange factors (RhoGEFs), and the regulation of PLC and phospholipase D (PLD) activity [22]. GPCR signalling is responsible for regulating many important biological processes in a wide variety of species and cell types and consequently, GPCRs are appealing drug targets, with approximately 40-50% of all marketed therapeutics targeting GPCR signalling pathways [23].

GPCRs are highly prevalent in the phyla nematoda, with the genome of the prototypical model nematode, *Caenorhabditis elegans,* containing over 1,100

predicted GPCRs, representing approximately 6% of the total predicted protein-coding genes [24]. It has been predicted that almost 100 of these receptors may represent neuropeptide GPCRs (GPCRs with cognate peptide ligands) [25]. Neuropeptidergic signalling in parasitic helminths has been advocated as a valid target for therapeutic intervention [26], since the nematode neuropeptide receptors regulate important biological processes including reproduction, chemosensation, feeding and behavioural responses, locomotion, and energy homeostasis [27], and disruption of these systems would clearly have detrimental effects on nematode viability. Furthermore, the conservation of many GPCRs across nematode species raises the possibility of developing therapeutics with broad spectrum anthelmintic activity.

The *C. elegans* neuropeptide receptor NPR-4 (CeNPR-4, Genbank ID: NM_077700.4) is activated by FMRFamide-like peptide (FLP) ligands [28]. *C. elegans npr-4* null mutants display phenotypes including reduced chemotaxis, impaired foraging behaviour, and deregulated fat storage [29] while knockdown of NPR-4 mRNA in an RNAi screen suggested a possible role in reproduction [30], thus suggesting that homologous gene products in parasitic nematodes may provide potential targets for novel anthelmintic development.

In the present study, the protein sequence of CeNPR-4 (Genbank ID: NP_510101.2) was used as a query to BLASTp search the Genbank non-redundant protein sequence database. Two highly ranked hits were proteins from the parasitic filarial nematodes *B. malayi* (XP_001897991.1, 57% identity), and *W. bancrofti* (EJW83575.1, 57% identity).

Hydrophobicity plots (http://www.cbs.dtu.dk/services/TMHMM/) indicated that both proteins had the predicted 7 transmembrane domains (TMs), indicative of GPCRs (data not shown). The proteins also contained conserved motifs associated with rhodopsin family (class A) GPCR activation; NPXXY at the cytosolic end of TM7, and a conservative variation of the E/DRY motif, DRF, at the cytosolic end of TM3 (Figure 1a). The conservative substitution of tyrosine to phenylalanine in the E/DRY motif has previously been described for a number of GPCRs [31]. Phylogenetic comparison of these proteins with *C. elegans* GPCRs activated by FLP peptides revealed that both proteins clustered with CeNPR-4, and may have originated from a common ancestral gene (Figure 1b). In summary, we predict these proteins to be putative homologues of CeNPR-4, and contain motifs associated with receptor activation.

To confirm NPR-4 mRNA expression in *B. malayi* nematodes, the published coding sequence of *B. malayi* NPR-4 (BmNPR-4; NCBI XM_001897956.1) was used to design primers. PCR was performed on cDNA prepared from adult *B. malayi* tissue (kindly provided by Dr Simon Babayan, University of Edinburgh). An amplicon of the expected size (~1.1kb) was produced (Figure 1c, indicated as PCR+) confirming the presence of BmNPR-4 mRNA in adult *B. malayi*. The BmNPR-4 amplicon was subsequently sequenced. The gene contains 7 annotated exons and 6 introns. Interestingly, sequencing analysis revealed the presence of an additional short 60bp exon in intron 4, which had been incorrectly annotated as intronic, during contig prediction and assembly (Figure 1d).

The ligands for *C. elegans* NPR-4 have been previously identified as FLP-18 peptides utilising an *in vitro* electrophysiological assay in which *Xenopus laevis* oocytes were co-injected with cRNAs for NPR-4 and G protein-coupled inwardly-rectifying potassium channels (GIRKs) [29]. The ability of nematode FLP-18 neuropeptides to activate NPR-4 was then measured by analysing changes in cell membrane potential, as a result of GIRK activation, by dissociated Gβγ dimer. The FLP-18 peptides EMPGVLRF-amide and SEVPGVLRF-amide were identified as the most potent. GIRKs are predominantly activated through the stimulation of GPCRs that couple to pertussis toxin (PTX) sensitive $G\alpha_i$ subunits. Interestingly, activation of another *C. elegans* neuropeptide receptor, CeNPR-5, by FLP-18 peptides was also demonstrated in the same assay, but unlike CeNPR-4, in addition to activation of potassium channels, an inward chloride current was observed, indicative of signalling through $G\alpha_{q/11}/Ca^{2+}/IP_3$ mediated pathways [32]. FLP-18 peptides therefore appear to be capable of activating different signal transduction pathways, via different receptors. In addition, both FLP-1 and FLP-4 peptides were also identified as ligands for CeNPR-4, by GTPγS assay, albeit with reduced potency in comparison to FLP-18 peptides [28].

To date, approximately 30 flp genes have been identified in *C. elegans*, coding for over 70 distinct FLP peptides. Many *flp* precursor genes appear to be conserved between free-living and parasitic nematodes [33,34], supporting the notion that homologous receptors between different species may utilise homologous ligands, and therefore similar mechanisms of activation. Indeed, previously published data identified ESTs for 4 putative *flp* precursor genes (*flp-6*, *flp-14*, *flp-21*, and *flp-24*) in *B. malayi* [33], and following the publication of the draft genome of *B. malayi* [35],

other putative FLP peptides have been annotated, including a possible FLP-1 proprotein (GenBank ID: XP_001899127.1). A bioinformatics search of the draft genome of *B. malayi* failed to identify FLP-18 peptide homologues. This cannot exclude the possibility of endogenous *B. malayi* FLP-18 peptides existing, as the *B. malayi* draft genome is only partially annotated, and additionally, short peptides often have to be isolated biochemically, due to the complexities of utilising bioinformatics as a sole means of identification.

In order to characterise the biochemical pharmacology of CeNPR-4 and the putative *B. malayi* NPR-4 homologue, receptors were cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) and heterologously expressed in a mammalian cell-line, HEK293-T, and their signalling response to neuropeptide ligands examined. HEK293-T have been previously utilised to express functional nematode GPCRs, and additionally contain the requisite intracellular signalling components necessary to examine GPCR signalling mechanisms. The cells were transiently transfected with vector containing cDNA encoding either CeNPR-4, BmNPR-4, empty vector as a negative control or the *C. elegans* GNRR-1 receptor (GenBank ID: NP_491453) which is known to activate $G\alpha_q$ signalling [36] as a positive control. After 48hr the cells were stimulated with *C. elegans* FLP-18 peptides DVPGVLRF-amide, EMPGVLRF-amide, or SEVPGVLRF-amide, previously shown to have high potency at the CeNPR-4 receptor [29] and then an inositol phosphate (IP) accumulation assay was conducted (Figure 2a).

Both the CeNPR-4 and BmNPR-4 receptors failed to elicit an IP response following stimulation with FLP-18 peptides when compared to the empty vector negative

control, indicating a lack of $G\alpha_q$ signalling (Figure 2a). In contrast, cells expressing the *C. elegans* GNRR-1 receptor and stimulated with the cognate AKH/GnRH-like peptide ligand (pGlu-MTFTDQWT) generated a robust IP response (9±1.9-fold over basal).

Stimulation of CeNPR-4 and BmNPR-4 with the FLP-18 peptides also failed to increase cAMP accumulation (measured as expression of a luciferase reporter gene under the control of a cAMP response element promoter), indicating that these receptors do not couple to $G\alpha_s$ G proteins (data not shown). However, stimulation of CeNPR-4 and BmNPR-4 with the FLP-18 peptides resulted in an inhibition of cAMP production when the receptor expressing cells were co-stimulated with forskolin (FSK), a direct activator of adenylyl cyclase [37], indicating activation of $G\alpha_i$ (Figures 2b and 2c). The FLP-18 ligand EMPGVLRF-amide elicited the largest statistically significant ($p \leq 0.001$) response for CeNPR-4 and BmNPR-4 with 55.3±0.75% and 69.1±3.5% decreases in relative light units (RLU), respectively, followed by SEVPGVLRF-amide (36.17±6.2%, and 68.99±5.8% decreases in RLU, respectively; SEVPGVLRF-amide only elicited a statistically significant response at the BmNPR-4 receptor), and DVPGVLRF-amide (21.95±17.4% and 38.89±17.8% decreases in RLU, respectively which were not statistically significant, $p > 0.05$). Frequently, individual *C. elegans* neuropeptide receptors, including CeNPR-4, are activated in response to stimulation with multiple different FLP ligands [28]. The activity of the FLP-1 peptide KPNFLRF-amide at the CeNPR-4 and BmNPR-4 receptors was also examined in this assay but did not elicit statistically significant responses for $G\alpha_s$ (data not shown) or $G\alpha_i$ (Figures 2b and 2c). A non-FLP neuropeptide (AKH/GnRH-like peptide) was also examined by luciferase assay, to determine the specificity of

the NPR-4 receptors for the FLP-18 ligands. This peptide was unable to inhibit FSK-induced cAMP production through the NPR-4 receptors (Figures 2b and 2c). Dose response analyses with BmNPR-4 confirmed that EMPGVLRF-amide and SEVPGVLRF-amide were able to inhibit FSK-induced cAMP production in HEK293-T cells in a concentration-dependent manner. Both ligands elicited similar maximal response (E_{max}) and had similar nanomolar potencies (EMPGVLRF-amide EC_{50} = 2nM, and SEVPGVLRF-amide EC_{50} = 6nM; data not shown).

In order to confirm that the actions of these peptides at CeNPR-4 and BmNPR-4 receptors are mediated through coupling to $G\alpha_i$, the effects of pertussis toxin (PTX) was examined. PTX ADP-ribosylates the $G\alpha_i$ subunit of the G protein heterotrimer, thus inhibiting members of this G protein family. Treatment of CeNPR-4 or BmNPR-4 transfected cells with PTX significantly reversed the inhibitory effects of the FLP-18 peptides on FSK-induced cAMP production (Figures 2b and 2c) confirming $G\alpha_i$ coupling.

Genetic studies have established the importance of neuropeptides in *C. elegans*. However, in order to examine the potential of neuropeptidergic signalling as a target for therapeutic intervention in nematodes, these genetic studies must be complemented with molecular and pharmacological studies. Functional FLP signalling in the parasitic nematodes of animals has been demonstrated in the helminth *Ascaris suum* [38,39], and previously, the FLP-14 homologue (AF2) was biochemically isolated from *Haemonchus contortus* [40]. In this paper we have cloned and expressed a neuropeptide GPCR, NPR-4 of the filarial parasite *B. malayi*. We have also demonstrated that both CeNPR-4 and BmNPR-4 signal upon

stimulation with a subset of FLP-18 ligands through a pertussis toxin-sensitive $G\alpha_i$-mediated pathway, representing the first example of GPCR signalling in the *B. malayi* parasite. Interestingly, a FLP-1 peptide, KPNFLRF-amide, and a FLP-4 peptide, (ASPSFIRF-amide), were previously shown to stimulate CeNPR-4 by GTPγS assay [28]. We have demonstrated that FLP-1 fails to elicit a $G\alpha_i$ (Figures 2b and 2c) or $G\alpha_s$ response (data not shown) by luciferase assay. This may reflect a markedly reduced potency of FLP-1 for NPR-4 when compared to the FLP-18 peptides. However, it would be prudent to examine the activity of FLP-1 and FLP-4 peptides further given that a FLP-18 homologue has yet to be identified in *B. malayi*, but a possible FLP-1 proprotein (GenBank ID: XP_001899127.1) is present in this species. This study further highlights the conservation of FLP-GPCR signalling components between free-living and parasitic nematodes and validates the use of biochemical and genomic approaches in *C. elegans* to identify potential targets for therapeutic intervention in parasitic helminths. Furthermore, the discovery that FLP-18 can activate BmNPR-4 provides a platform for the conceptualisation and development of ligands to regulate the activity of this receptor.

Acknowledgements

This work was supported by research grants from MRC South Africa and the University of Cape Town awarded to Arieh A. Katz and Robert P. Millar.

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Figure Legends

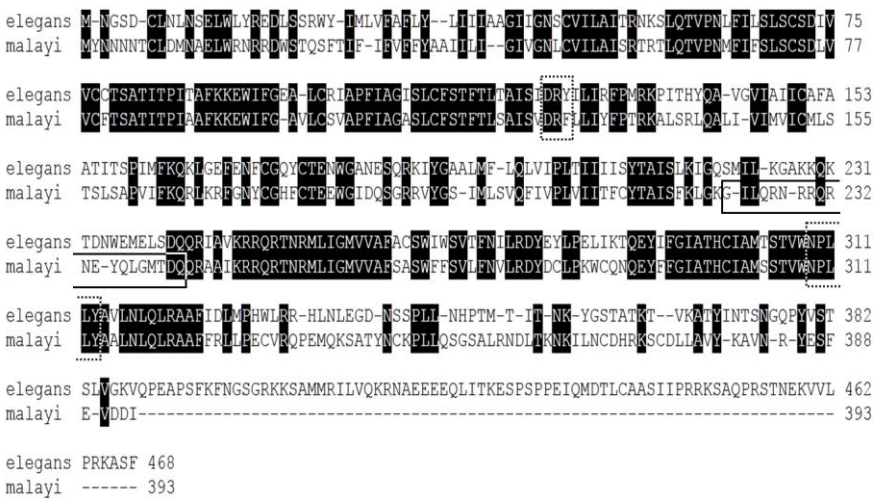
Figure 1). Characterisation and cloning of the putative *B. malayi* NPR-4 homologue. A sequence alignment of CeNPR-4 and the putative *B. malayi* NPR-4 homologue **(a)** were generated using Clustal Omega (https://www.ebi.ac.uk/). Black shading denotes identical amino acids. Dotted line boxes represent conserved class A GPCR motifs implicated in receptor activation. Solid line box denotes the 20 amino acid addition representing the corrected annotation of exon 5, identified by sequencing of the cloned receptor. Phylogram of known FLP-activated *C. elegans* (Ce) NPRs, and putative Wuchereria bancrofti (Wb) and B. malayi (Bm) NPRs **(b)** was constructed using Clustal Omega. The 2 database annotated NPR's from *B. malayi* and *W. bancrofti* cluster with CeNPR-4 (solid line box). A BmNPR-4 amplicon was produced by PCR performed on cDNA prepared from adult *B. malayi* tissue (PCR+ lane) but not in the control PCR (PCR-). Primers used were 5'-ATGTACAATAACAATAATACC-3' (F) and 5'-CAGCTCGAGTTAAATGTCATCTACT TCAA-3' (R) **(c)**. The resulting product was cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). Sequencing of the cloned product revealed the presence of a 60bp exon within intron 4 **(d)**.

Figure 2). BmNPR-4 is activated by FLP-18 peptides. To assay for $G\alpha_q$ activation, inositol phosphate (IP) accumulation assays were performed as described previously [41] **(a)**. HEK293-T cells were transfected with empty pcDNA3.1(+), BmNPR-4, CeNPR-4, or CeGNRR-1 (GenBank: CCD68969.1), and incubated in media containing 2μCi/ml [^{3}H]-myoinositol before stimulation with 1μM peptides or vehicle only for 1 hour at 37^{0}C (pGlu; pyroglutamate). Cell lysates were transferred to columns containing DOWEX ion-exchange resin, washed, then eluted into scintillation vials containing liquid scintillant, and activity measured in a β-counter. Results are expressed as fold over vehicle only controls, and represent 2 independent experiments. To assay for $G\alpha_i$ activation, dual-luciferase assays were performed **(b and c)**. HEK293-T cells were seeded onto 24-well plates then co-transfected with plasmid containing receptor or empty pcDNA3.1(+) cDNA, and pGL4 and pRL firefly and renilla luciferase constructs (Promega), at a ratio of [1]:[1]:[0.06] respectively. The cells were then cultured for 24 hours, and the media replaced with starving media +/- 100ng/ml pertussis toxin. Cells were cultured for a further 24 hours before stimulation with 2μM peptides and/or 1μM forskolin in starving media for 6 hours. Cells were assayed for luciferase production via Dual-Luciferase Reporter Assay (Promega) according to manufacturer's instructions using a Glomax Multiplate Reader (Promega). Results represent at least 3 independent experiments. One-way ANOVA followed by Bonferroni's multiple comparison test

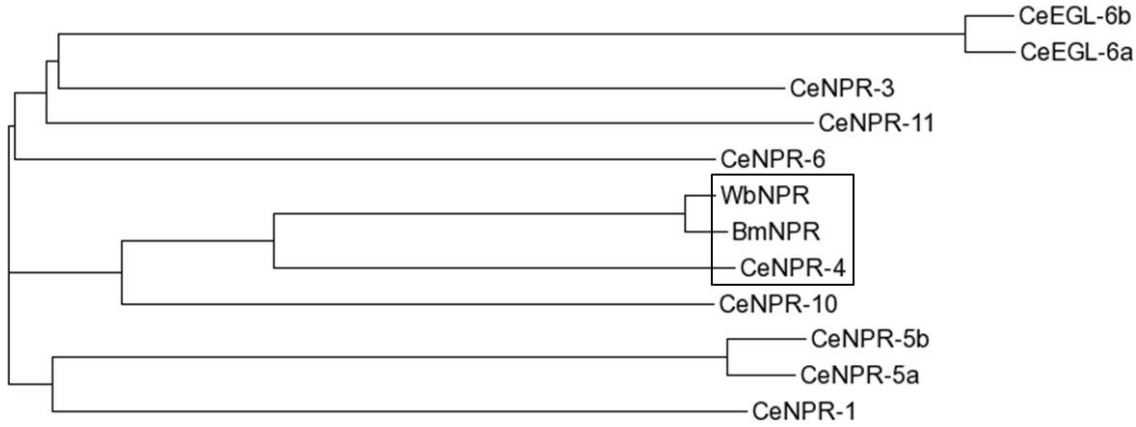
was performed on the data to determine experimental significance * = vehicle vs peptide, $^{\phi}$ = peptide PTX(-) vs peptide PTX (+). */$^{\phi}$ = p≤0.05, **/$^{\phi\phi}$ = p≤0.01, ***/$^{\phi\phi\phi}$ = p≤0.001.

Fig 1.

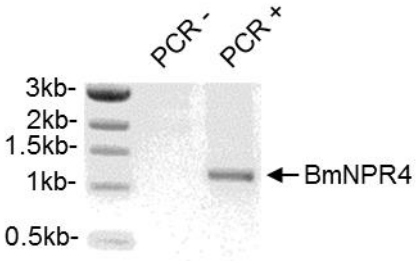
a)


elegans
malayi

b)


CeEGL-6b
CeEGL-6a
CeNPR-3
CeNPR-11
CeNPR-6
WbNPR
BmNPR
CeNPR-4
CeNPR-10
CeNPR-5b
CeNPR-5a
CeNPR-1

c)


PCR-
PCR+
3kb-
2kb-
1.5kb-
1kb-
0.5kb-
BmNPR4

d)

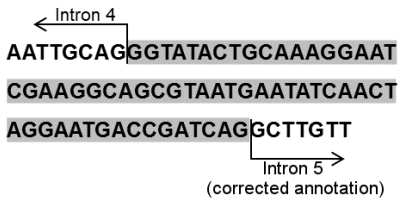
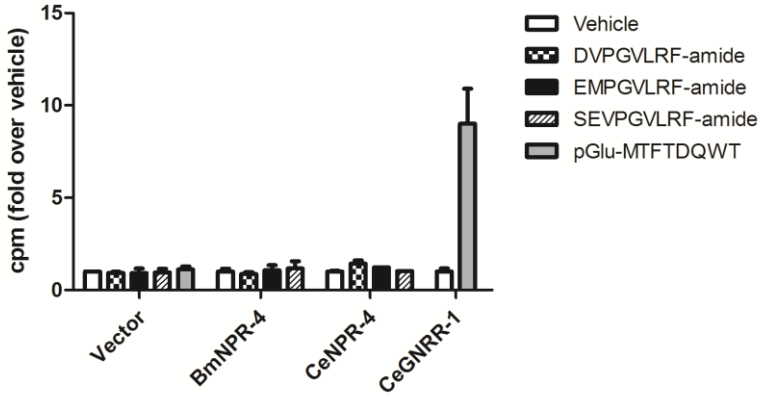
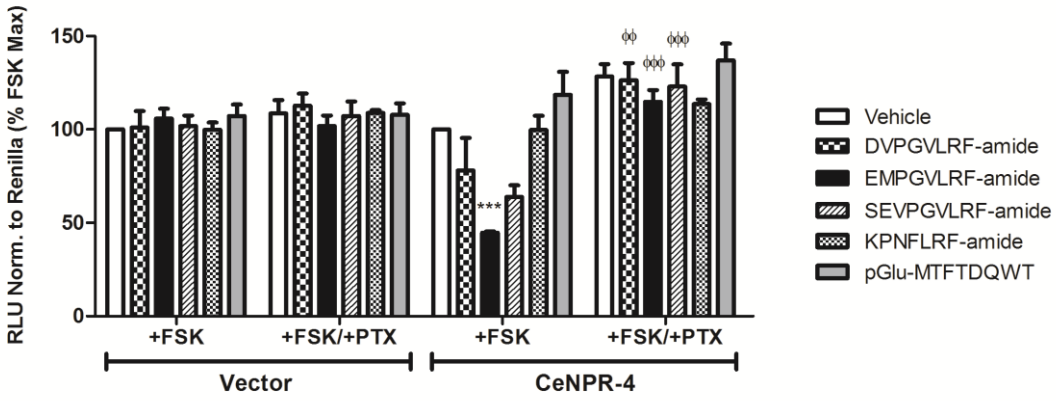

Intron 4
AATTGCAGGGTATACTGCAAAGGAAT
CGAAGGCAGCGTAATGAATATCAACT
AGGAATGACCGATCAGGCTTGTT
Intron 5
(corrected annotation)

Fig 2.

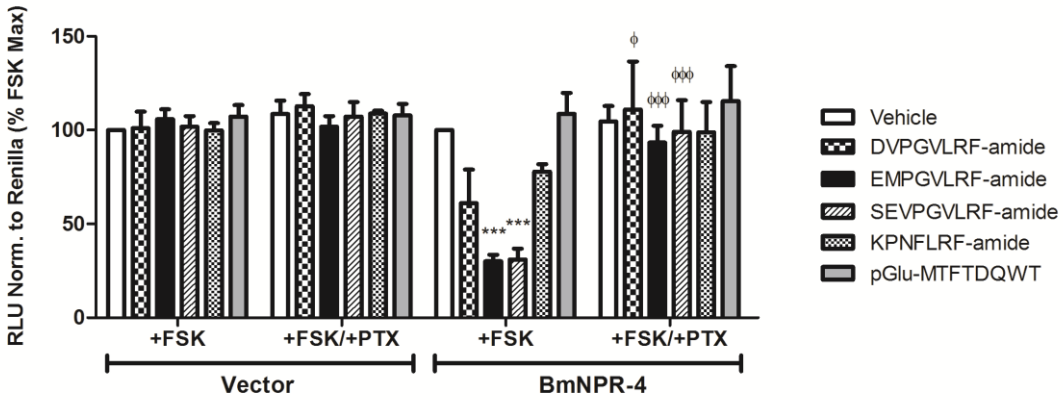
a)


cpm (fold over vehicle)
15
10
5
0
Vector
BmNPR-4
CeNPR-4
CeGNRR-1
Vehicle
DVPGVLRF-amide
EMPGVLRF-amide
SEVPGVLRF-amide
pGlu-MTFTDQWT

b)


RLU Norm. to Renilla (% FSK Max)
150
100
50
0
***
ΦΦ
ΦΦΦ
ΦΦΦ
+FSK
+FSK/+PTX
+FSK
+FSK/+PTX
Vector
CeNPR-4
Vehicle
DVPGVLRF-amide
EMPGVLRF-amide
SEVPGVLRF-amide
KPNFLRF-amide
pGlu-MTFTDQWT

c)


RLU Norm. to Renilla (% FSK Max)
150
100
50
0
***
***
Φ
ΦΦΦ
+FSK
+FSK/+PTX
+FSK
+FSK/+PTX
Vector
BmNPR-4
Vehicle
DVPGVLRF-amide
EMPGVLRF-amide
SEVPGVLRF-amide
KPNFLRF-amide
pGlu-MTFTDQWT