Urbilaterian origin of paralogous GnRH and corazonin neuropeptide signalling pathways

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Gonadotropin-releasing hormone (GnRH) is a key regulator of reproductive maturation in humans and other vertebrates. Homologs of GnRH and its cognate receptor have been identified in invertebrates – for example, the adipokinetic hormone (AKH) and corazonin (CRZ) neuropeptide pathways in arthropods. However, the precise evolutionary relationships and origins of these signalling systems remain unknown. Here we have addressed this issue with the first identification of both GnRH-type and CRZ-type signalling systems in a deuterostome - the echinoderm (starfish) *Asterias rubens*. We have identified a GnRH-like neuropeptide (pQIHYKNPGWGPG-NH_2) that specifically activates an *A. rubens* GnRH-type receptor and a novel neuropeptide (HNTFTMGGQNRWKAG-NH_2) that specifically activates an *A. rubens* CRZ-type receptor. With the discovery of these ligand-receptor pairs, we demonstrate that the vertebrate/deuterostomian GnRH-type and the protostomian AKH systems are orthologous and the origin of a paralogous CRZ-type signalling system can be traced to the common ancestor of the Bilateria (Urbilateria).

Introduction

Neuropeptides are important regulators of physiological processes and behaviour in humans and other animals [1,2]. One of the most widely known and well-studied neuropeptide signalling pathways is the gonadotropin-releasing hormone (GnRH) system, which controls reproductive maturation and function in humans and other vertebrates. Thus, GnRH stimulates release of the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland [3,4].

Homologs of GnRH have been identified in invertebrates, including adipokinetic hormone (AKH), corazonin (CRZ) and AKH/CRZ-related peptide (ACP) in arthropods. AKH is a lipid-mobilizing hormone in insects that is released during flight and other energy utilizing activities [5]. CRZ was discovered on account of its stimulatory effect on heart rate in cockroaches [6] but it also has other functions that range from initiating ecdysis in moths to triggering gregarization-associated dark-pigmentation in locusts [7,8]. The recently discovered ACP signalling system is a paralog of the AKH system that arose in a common ancestor of arthropods [9,10], but its functions remain unclear [11].

Although GnRH-related neuropeptides have been studied extensively in chordates and arthropods, their evolutionary relationships are a matter of debate, largely due to lack of information from other phyla. It is well-established that AKH, ACP, CRZ and GnRH form a superfamily and that protostomian AKH/ACP and deuterostomian GnRH are orthologs. However, the relationship of CRZ to AKH, ACP and GnRH is less clear. Some studies consider AKH/ACP and CRZ neuropeptides to both be orthologous to the vertebrate GnRH system [1,12,13], whilst other studies were inconclusive regarding the evolutionary origins of the CRZ system [2,10].

Informed by analysis of genome sequence data, a candidate neuropeptide (pQILCARAFTYTHTW-NH_2) in the cephalochordate *Branchiostoma floridae* that activates

one of two CRZ-type receptors but neither of two GnRH-type receptors has been reported [14]. It is not known, however, if this predicted mature peptide actually exists in *B. floridae.* Furthermore, a previous report from the same group [15] showed that the *B. floridae* CRZ-type receptor could also be activated by an insect AKH with equal effectiveness. Thus, it remains to be established whether or not distinct GnRH-type and CRZ-type signalling pathways occur in deuterostomes. Here we have addressed this issue in a non-chordate deuterostomian phylum – the echinoderms.

Results and Discussion

Four GnRH/CRZ-type receptors have been identified in the sea urchin *Strongylocentrotus purpuratus* based on analysis of genome sequence data [2,14] but the ligands for these receptors have not been discovered. Here we set out to identify and characterise GnRH/CRZ-type receptors in another echinoderm species - the common European starfish *Asterias rubens* - utilizing neural transcriptome sequence data that has been obtained recently [16,17]. BLAST analysis of *A. rubens* neural transcriptome sequence data using an *S. purpuratus* GnRH-type receptor as the query sequence identified two candidate GnRH/CRZ-type receptor transcripts, which we cloned and sequenced as cDNAs (Supplementary Figures S1 and S2). Phylogenetic analysis of the relationships of the two *A. rubens* receptors with GnRH-, AKH-, ACP- and CRZ-type receptors, using Bayesian and maximum-likelihood methods, generated trees with well-supported topologies. With both methods, one receptor grouped with GnRH/AKH/ACP-type receptors and the other grouped with protostomian and *B. floridae* CRZ-type receptors (Fig. 1 and Supplementary Figure S3). Henceforth we will refer to the two receptors as ArGnRHR and ArCRZR, respectively.

Having identified a GnRH-type receptor and a CRZ-type receptor in *A. rubens*, we sought to identify the neuropeptides that act as ligands for these receptors. Analysis of

86 neural transcriptome sequence data has revealed the occurrence of two GnRH/CRZ-type
87 precursors in *A. rubens* [17] and here we cloned cDNAs encoding these precursors to confirm
88 their sequences (Fig. 2a; Supplementary Figures S4 and S5). Precursor 1 comprises a single
89 copy of the putative GnRH-like peptide pQIHYKNPGWGPG-NH_2 (peptide 1) whereas
90 precursor 2 comprises a single copy of the putative peptide HNTFTMGGQNRWKAG-NH_2
91 (peptide 2). LC-MS-MS analysis of radial nerve cord extracts demonstrated that both of these
92 predicted neuropeptides occur in *A. rubens* (Supplementary Figures S6 and S7).
93 We hypothesized that the GnRH-like peptide 1 is the ligand for ArGnRHR and
94 peptide 2 is the ligand for ArCRZR and to test this hypothesis the receptors were expressed in
95 a heterologous cellular system. Neither peptide 1 nor peptide 2 elicited any response when
96 tested on cells transfected with an empty vector (not shown) but, consistent with our
97 hypothesis, peptide 1 caused dose-dependent activation of ArGnRHR (EC_{50} = 0.603 nM; Fig.
98 2b), and peptide 2 caused dose-dependent activation of ArCRZR (EC_{50} = 115 nM; Fig. 2d).
99 Importantly, peptide 1 did not activate ArCRZR (Fig. 2b, c) and likewise peptide 2 did not
100 activate ArGnRHR (Fig. 2d, e), demonstrating the existence of two distinct signalling
101 systems. Neither receptor was activated by other GnRH/CRZ-type peptides (*Drosophila*
102 AKH and CRZ) or by other starfish neuropeptides (NGFFYamide, SALMFamide-1 and
103 SALMFamide-2), providing further evidence of the specificity of peptides 1 and 2 as ligands
104 for ArGnRHR and ArCRZ, respectively (Supplementary Figure S8). Therefore, henceforth
105 we will refer to peptide 1 (pQIHYKNPGWGPG-NH_2) as ArGnRH and peptide 2
106 (HNTFTMGGQNRWKAG-NH_2) as ArCRZ.
107 ArCRZ is the first ligand for a CRZ-type receptor to be biochemically identified in a
108 deuterostome. Furthermore, precursors of ArCRZ-like peptides can be identified in the sea
109 urchin *S. purpuratus* and the hemichordate *Saccoglossus kowalevskii* (Supplementary Figure
110 S9). Discovery of these ambulacrarian corazonins prompted us to examine the reported

precursor of a putative CRZ-type receptor ligand (pQILCARAFTYTHTW-NH_2) in the cephalochordate *B. floridae* [14]. Analysis of the precursor sequence using the signal peptide prediction tool SignalP 4.1 [18] reveals the presence of a signal peptide cleavage site between the alanine (A) and phenylalanine (F) residues in the middle of the QILCARAFTYTHTW sequence (Supplementary Figure S9). Therefore, the neuropeptide derived from this *B. floridae* precursor protein is predicted to be FTYTHTW-NH_2. This peptide shares modest sequence similarity with the ambulacrarian corazonins but a feature that unifies deuterostomian CRZ-type precursor genes are two introns that interrupt the protein-coding sequence (Supplementary Figure S9). Furthermore, this feature distinguishes deuterostomian CRZ-type precursor genes from deuterostomian GnRH-type precursor genes, which have a single conserved intron (Supplementary Figure S9).

In conclusion, our discovery of ArCRZ, ArGnRH and their cognate receptors in the starfish *A. rubens*, a deuterostomian invertebrate, indicates that these paralogous signalling systems originated by gene duplication in a common ancestor of the Bilateria (Urbilateria) (Fig. 3). Evidence in support of this conclusion has been obtained previously by Roch *et al*. [14,19] in phylogenetic analyses of GnRH/CRZ-type receptor sequences. Consistent with our findings, trees generated by these authors contain two distinct receptor clades – one comprising GnRH/AKH/ACP-type receptors and another comprising CRZ-type receptors, with receptors from protostomes and deuterostomes in both clades. Furthermore, to enable comparison with the findings of Roch *et al*., in Supplementary Figures S10 and S11 we show neighbour joining and maximum likelihood trees, respectively, that were generated using the same sequences analysed by Roch *et al*. [14], but with the addition of ArGnRHR and ArCRZR (boxed in red). Consistent with our findings (Fig. 1; Supplementary Figure S3), ArGnRHR is positioned in the GnRHR clade and ArCRZR is positioned in the CRZR clade (Supplementary Figures S10, S11).

136 Interestingly, comparison of the sequences of GnRH/AKH/ACP/CRZ neuropeptides
137 and precursor proteins throughout the Bilateria does not reveal any structural characteristics
138 that distinguish CRZ-type from GnRH/AKH/ACP-type neuropeptides/precursors. An
139 explanation for this may be that the gene duplication that gave rise to GnRH/AKH/ACP-type
140 neuropeptides/precursors on the one hand and CRZ-type neuropeptides/precursors on the
141 other occurred just prior to the divergence of protostomes and deuterostomes. Thus, there
142 may have been little or no sequence divergence in the paralogous precursor proteins at the
143 time of the protostome-deuterostome split. An alternative, but less parsimonious, explanation
144 would be that the gene duplications that gave rise to ligands for GnRH-type receptors and
145 CRZ-type receptors occurred independently in both the protostome and deuterostome
146 lineages after the protostome-deuterostome split.
147 Surveying the occurrence of the GnRH-type and CRZ-type signalling systems
148 throughout the Bilateria reveals that the GnRH-type signalling system appears to have been
149 retained throughout the Bilateria whereas the CRZ-type signalling system has been lost in
150 vertebrates, nematodes and some arthropods (Fig. 3; [2,10]). In this context, our discovery of
151 both GnRH-type and CRZ-type signalling in an echinoderm is interesting because it has, for
152 the first time, provided a basis for comparison of the physiological roles of these paralogous
153 systems in a deuterostome. Investigation of the actions of GnRH-type neuropeptides has
154 revealed roles in regulation of reproductive processes in chordates [3,4] and in the nematode *C.*
155 *elegans* [13]. In arthropods, duplication of the GnRH-type signalling system to give rise the
156 AKH-type and ACP-type signalling systems complicates the picture. AKH regulates lipid-
157 mobilisation in insects [5] but the physiological roles of the more recently discovered ACP
158 have yet to be well characterised [11]. In this context, it will be interesting to determine the
159 physiological roles of a GnRH-type neuropeptide in an echinoderm, as this will serve as a
160 "bridge" between vertebrates and protostomes in our knowledge and understanding of the

161 evolution of GnRH function. Likewise, whilst much is now known about the physiological
162 roles of the CRZ-type signalling in arthropods [6-8], nothing is known about physiological roles
163 of this signalling system in deuterostomes. Our discovery of ArCRZ and ArCRZR provides a
164 unique opportunity to address this issue.

Materials and Methods

Identification and cloning of GnRH/CRZ-type receptors and neuropeptide precursors in *A. rubens*

Two putative GnRH-type neuropeptide precursors have been identified recently in *A. rubens* [17] and two candidate GnRH/CRZ-type receptors for peptides derived from these precursors were identified by BLAST analysis of *A. rubens* radial nerve cord transcriptome sequence data using a *S. purpuratus* GnRH-type receptor as the query. Then cDNAs encoding these precursor proteins and receptors were cloned and sequenced, using specific primers (see Supplementary Figures S1 and S2) designed using Primer3 online tool (http://primer3.ut.ee).

Phylogenetic Analysis

Phylogenetic analysis of the relationship of *A. rubens* GnRH/CRZ-type receptors with GnRH-, AKH-, ACP- and CRZ-type receptors from other species was accomplished using Bayesian and maximum-likelihood methods [11]. The sequences were aligned using the MAFFT v7.017 plugin in Geneious 8.0.5 (slow iterative, maximum 1000 iterations, BLOSUM30) [21]. The alignment was then trimmed using BMGE with the following options: BLOSUM30, max –h =1, -b = 1 [22]. The maximum-likelihood tree was produced using PhyML 3.0 (LG substitution model, 1000 Bootstrap) [23]. The Bayesian tree was produced using Mr Bayes version 3.2.1 (WAG model, + I + G + F, 2 runs, 1000000 trees; burn-in 10%). The consensus tree was created in Geneious 8.0.5.

Mass spectrometry

Two different methods were used to prepare extracts of radial nerve cords from *A. rubens*, with radial nerve cords from two animals used for each method. For an acetic acid based

extraction, nerve cords were dissected and transferred to a micro-centrifuge tube containing 3% acetic acid (in ddH_2O). The tube was incubated in a boiling water bath for 10 minutes. The nerve cords were then sonicated and homogenized to lyse cells. The extract was centrifuged and the supernatant transferred to a glass vial. For a second method, nerve cords were dissected and transferred to a 90% methanol and 9% acetic acid solution. The nerve cords were sonicated and homogenized to lyse cells. The extract was centrifuged and the supernatant transferred to a glass vial. Finally, the solvent was bubbled-off using nitrogen gas. The extract was analysed by means of nanoflow liquid chromatography with electrospray ionisation quadrupole time-of-flight tandem mass spectrometry (nanoLC-ESI-MS/MS) using a nanoAcquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters Corporation, Milford, MA, USA) and MassLynx v4.1 SCN 908 software (Waters Corporation, Milford, MA, USA). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.0) and Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. [For a more detailed account of the mass spectrometry methods employed, see Supplementary Figure S6.]

Functional characterization of ArGnRHR and ArCRZR

ArGnRHR and ArCRZR were cloned as described previously [24] and the positions of the primers used are indicated in Supplementary Figures S1 and S2. Next, the ORF of ArGnRHR was amplified using the oligos 5'-aagcttCACCATGGCGACTACATC-3' and 5'-ctcgagTTATACACATTTCTCAG-3' and subcloned into the eukaryotic expression vector pcDNA 3.1+ (Invitrogen) that was cut with *Hind*III and *Xho*I; The ORF of ArCRZR was amplified using the oligos 5'- gatatcCACCATGAGTGTTCAAT-3' and 5'-tctagaTCAGGTTGTTGTTGTGA-3'and subcloned into pcDNA 3.1+ that was cut with

215 *EcoR*V and *Xba*I. A partial Kozak translation initiation sequence (CACC) was also
216 introduced in the upstream primer. Chinese hamster ovary (CHO)-K1 cells stably
217 overexpressing the human Gα16 protein and mitochondrial targeted apo-aequorin were used
218 as a heterologous expression system to functionally characterize the receptors. Cells were
219 cultured, transfected and a bioluminescence assay performed as described previously [24]. The
220 *A. rubens* neuropeptides pQIHYKNPGWGPG-NH_2 and HNTFTMGGQNRWKAG-NH_2
221 were custom synthesized by PPR Ltd (Fareham, UK) and were tested as candidate ligands for
222 ArGnRHR and ArCRZR at concentrations ranging from 10^{-4} M to 10^{-14} M. Ca^{2+} responses
223 were normalized to the total Ca^{2+} response monitored after addition of Triton X-100 (0.1%).
224 Dose–response data were determined as a % of the highest response (100% activation). EC_{50}
225 values were calculated from dose–response curves based on at least three independent
226 transfections (Prism 6.0). Other GnRH/CRZ-type neuropeptides (*Drosophila* AKH and
227 *Drosophila* AKH) and other starfish neuropeptides (NGFFYamide, SALMFamide-1 and
228 SALMFamide-2 [16,25]) were also tested (at 10 μM) to assess the specificity of receptor
229 activation.

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Author contributions

M.R.E., S.T. and M.Z. conceived the study; S.T. cloned and sequenced cDNAs and created expression vectors; M.Z. did the receptor assays, with assistance from I.B. and E.B.; S.E.S. and J.H.S. did the LC-MS-MS; S.T., M.Z. and M.R.E. wrote the paper, with input from other authors.

Competing interests

The authors declare no competing financial interests

Figure legends

Figure 1. Phylogenetic analysis of GnRH/AKH/ACP/CRZ-type receptors using a Bayesian method reveals two distinct clades – a GnRH/AKH/ACP-type receptor clade and a CRZ-type receptor clade. Single representatives of both clades are present in the starfish *A. rubens* (*A. rub*., black boxes). GnRH-type receptors are labelled using red squares, AKH-type receptors using orange squares, ACP-type receptors using pink squares and CRZ-type receptors using purple circles. Neuropeptide S and CCAP receptors were used as an outgroup (condensed). The stars represent posterior probabilities and the pastel coloured backgrounds represent different groups of animals (see legend). The scale bar indicates amino acid substitutions per site. Species for which receptor-ligand interactions have been experimentally characterized are coloured in green, including the *A. rubens* receptors characterized in this study (boxed). Species names are as follows: A.rub, *Asterias rubens*; S.pur, *Strongylocentrotus purpuratus*; B.flo, *Branchiostoma floridae*; H.sap, *Homo sapiens;* D.rer, *Danio rerio*; G.gal, *Gallus gallus*; C.tel, *Capitella teleta*, C.gig, *Crassostrea gigas*; L.gig, *Lottia gigantea*; S.mar, *Strigamia maritima*; D.pul, *Daphnia pulex*; B.mor, *Bombyx mori*; R.pro, *Rhodnius prolixus*; A.gam, *Anopheles gambiae*; I.sca, *Ixodes scapularis*; S.kow, *Saccoglossus kowalevskii*; O.vul, *Octopus vulgaris*. [accession numbers and references for the receptor sequences are included the legend of Supplementary Figure S3].

Figure 2. Identification of GnRH-type and Corazonin-type (CRZ)-type signalling systems in the starfish *Asterias rubens.* (a) Amino acid sequences of two *A. rubens* GnRH/CRZ-type neuropeptide precursor proteins – precursor 1 and precursor 2. Signal peptides are highlighted in blue, putative neuropeptides (without post-translational modifications) are highlighted in red (peptide 1) or purple (peptide 2) and dibasic cleavage

sites are highlighted in green. Peptides 1 and 2 with post-translational N-and C-terminal modifications, determined by mass spectrometry, are shown below the precursor sequences. (b) Peptide 1 causes dose-dependent stimulation of a bioluminescence response in CHO-K1 cells stably expressing aequorin and Gα16 and transfected with ArGnRHR; EC_{50} = 6.03 x 10^{-10} M. Peptide 2 has no effect when tested over the same concentration range as peptide 1, demonstrating the specificity of the activation of ArGnRHR by peptide 1, which is therefore designated as "ArGnRH". (c) Comparison of the total bioluminescent responses of ArGnRHR-expressing cells for 30 seconds after the addition of BSA media (control), peptide 1 (10^{-5} M) or peptide 2 (10^{-5} M). (d) Peptide 2 causes dose-dependent stimulation of a bioluminescence response in CHO-K1 cells stably expressing aequorin and Gα16 and transfected with ArCRZR; EC_{50} = 1.15 x 10^{-7} M. Peptide 1 has no effect when tested over a similar concentration range as peptide 2, demonstrating the specificity of the activation of ArCRZR by peptide 2, which is therefore designated as "ArCRZ". (e) Comparison of the total bioluminescent responses of ArCRZR-expressing cells for 30 seconds after the addition of BSA media (control), peptide 1 (10^{-5} M) or peptide 2 (10^{-5} M).

Figure 3. Schematic showing the evolution of GnRH-type and CRZ-type receptors in the Bilateria. GnRH-type receptors (red) and CRZ-type receptors (purple) arose by gene duplication in a common ancestor of the Bilateria. A second gene duplication of a GnRH-type receptor in a common ancestor of the Arthropoda gave rise to AKH-type receptors (orange) and ACP-type receptors (pink). CRZ-type receptors have been lost in multiple lineages (purple crosses), including vertebrates, and the ACP-type receptor has been lost in *Drosophila* (pink cross). The occurrence of each receptor type in species belonging to different phyla are shown on the right (white box denotes loss of a receptor). Species where neuropeptide ligands for receptors have been identified are labelled with a yellow asterisk.

376 Note that, as reported in this paper, the starfish *Asterias rubens* is the first and only
377 deuterostome in which the neuropeptide ligands for a GnRH-type receptor and a CRZ-type
378 receptor have been identified. The ? in CRZR box for *Branchiostoma floridae* indicates
379 uncertainty regarding the structure of a candidate ligand, as discussed in the main text of this
380 paper. Images of representative animals from each phylum were created by the authors, with
381 the exception of the sea urchin image, which was obtained from
382 https://openclipart.org/detail/170807/sea-urchin-silhouette.

Outgroup

A. rub
S. pur - 1
S. pur - 2
S. pur - 3
B. flo - 1
B. flo - 2
H. sap - 1
D. rer - 1
G. gal - 1
D. rer - 2
G. gal - 2
C. tel
C. gig - 2
L. gig
S. mar
D. pul
R. pro
B. mor
A. gam
S. mar
I. sca - 1
B. mor - 1
R. pro
A. gam
B. flo - 3
B. flo - 4
A. rub
S. pur 4
S. kow
C. tel
O. vul
C. gig
L. gig
I. sca
S. mar - 1
D. pul
B. mor
A. gam

GnRHR / AKHR / ACPR

CRZR

Legend

100%
>95%
>90%
>80%
>70%
>50%

Ambulacraria
Chordata
Lophotrochozoa
Arthropoda

0.2

a

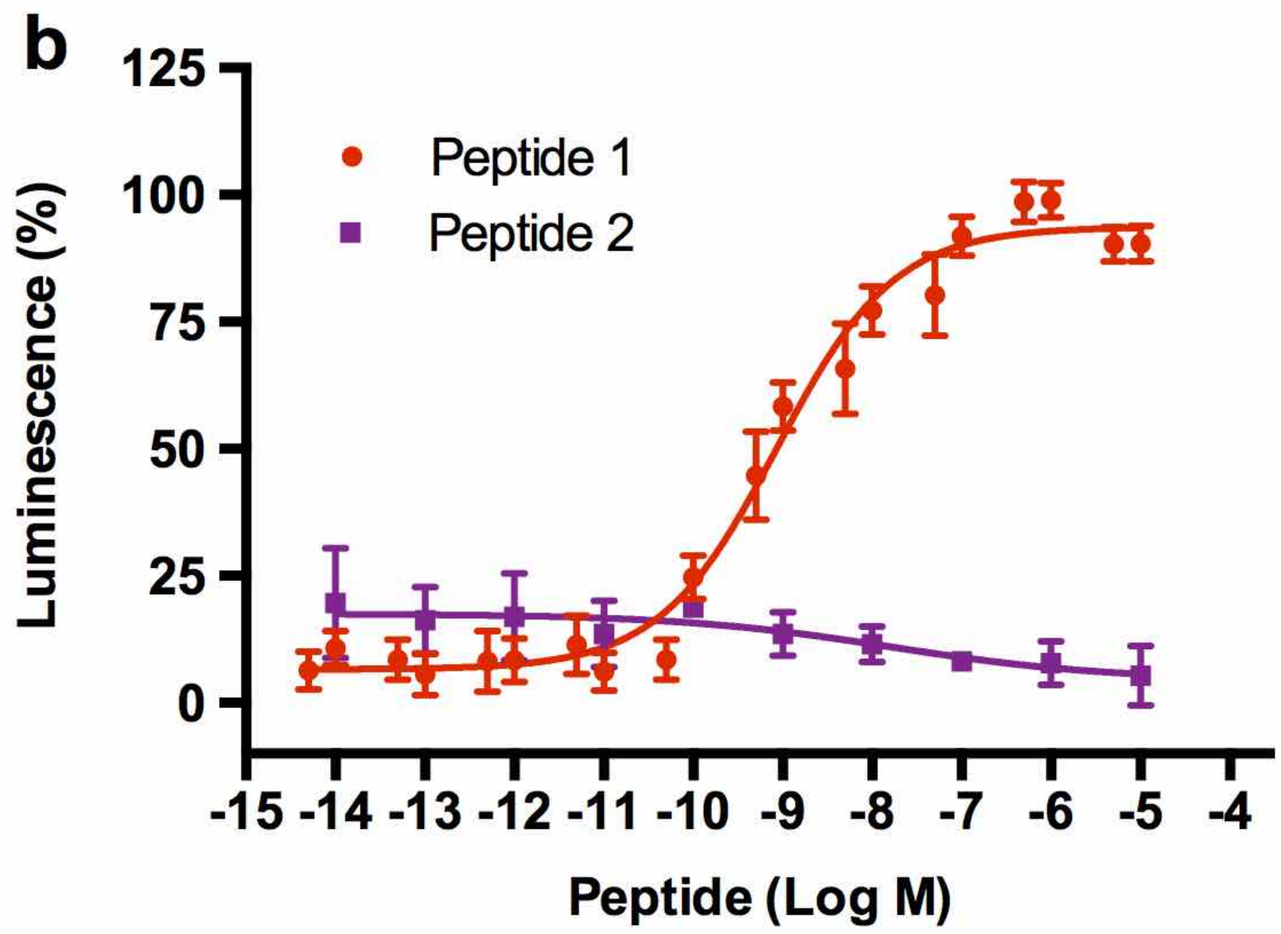
Precursor 1

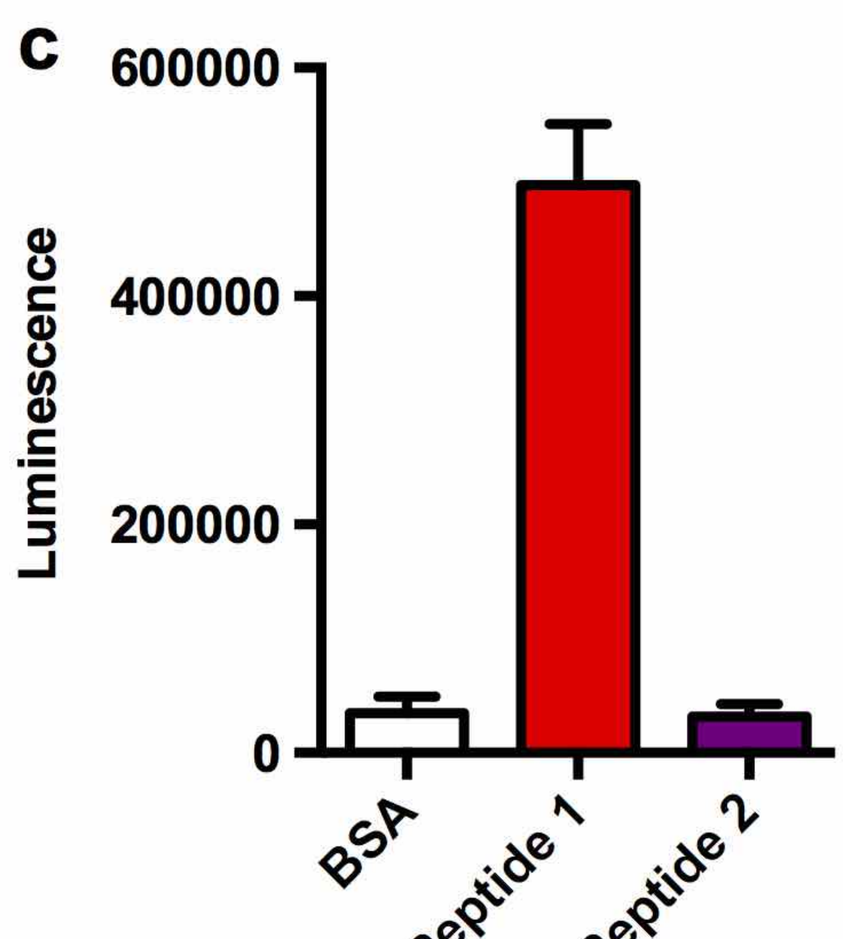
MADMRMLTLTSVLVSLLFMAEIQRCQGQIHYKNPGWGPGGKRSSHMTGSNVLRKRHWRVES
DQMGTDSMQKERNLIMLQEIAKSLAKQLVVPTSEDDTVLDQLTVDQWRQEADEINDNGWN

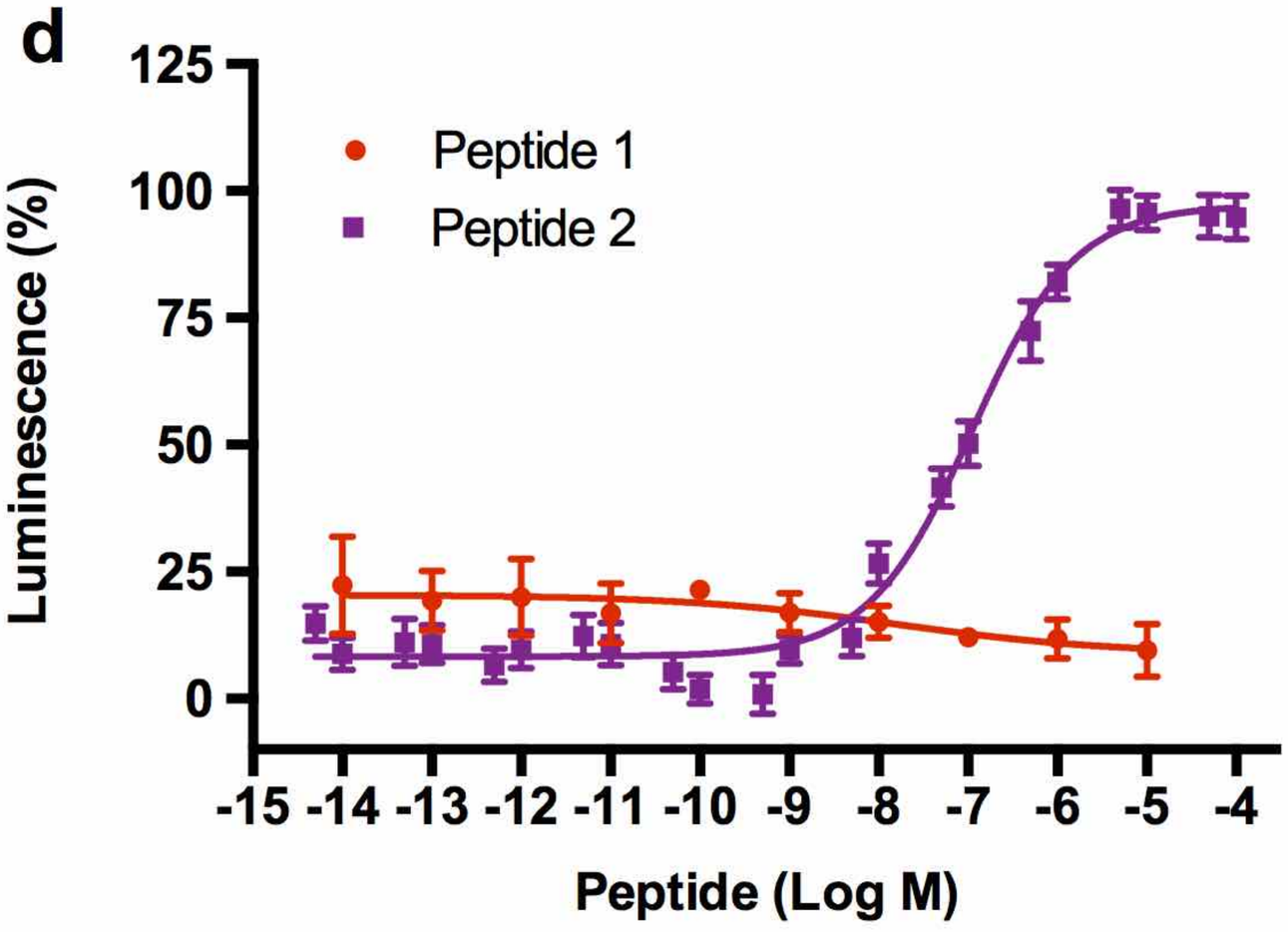
Precursor 2

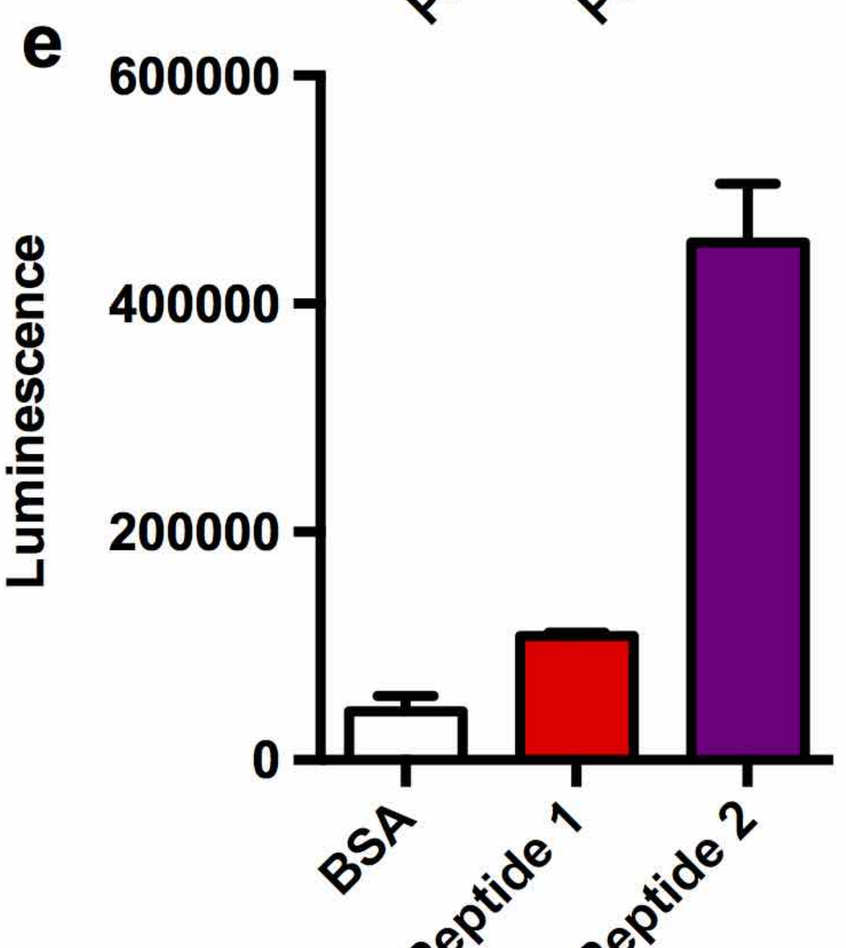
MGSYSVTATIYLALGSLVCSAHNTFTMGGQNRWKAGGKRSAPAGRPQQTFLDPSSFSEDQQ
GETTITLREMLVDMRDYCSFLLKLLDNVRLPQTERK

Peptide 1 **pQIHYKNPGWGPG-NH_2** **Peptide 2** **HNTFTMGGQNRWKAG-NH_2**









GnRHR
AKHR
ACPR
CRZR
Deuterostomia
Chordata
Vertebrata
Homo sapiens
Danio rerio
Cephalochordata
Branchiostoma floridae
Hemichordata
Saccoglossus kowalevskii
Ambulacraria
Echinodermata
Asterias rubens
Strongylocentrotus purpuratus
Protostomia
Lophotrochozoa
Annelida
Capitella teleta
Mollusca
Lottia gigantea
Ecdysozoa
Nematoda
Caenorhabditis elegans
Arthropoda
Drosophila melanogaster
Bombyx mori
Tribolium castaneum
GnRHR
CRZR
AKHR
ACPR
GnRHR