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Abstract

Although tachykinin-like neuropeptides have been identified in molluscs more than two decades ago, knowledge on their function and signalling has so far remained largely elusive. We developed a cell-based assay to address the functionality of the tachykinin G-protein coupled receptor (Cragi-TKR) in the oyster *Crassostrea gigas*. The oyster tachykinin neuropeptides that are derived from the tachykinin precursor gene Cragi-TK activate the Cragi-TKR in nanomolar concentrations. Receptor activation is sensitive to Ala-substitution of critical Cragi-TK amino acid residues. The Cragi-TKR gene is expressed in a variety of tissues, albeit at higher levels in the visceral ganglia (VG) of the nervous system. Fluctuations of Cragi-TKR expression is in line with a role for TK signalling in *C. gigas* reproduction. The expression level of the Cragi-TK gene in the VG depends on the nutritional status of the oyster, suggesting a role for TK signalling in the complex regulation of feeding in *C. gigas*.

Keywords: Mollusc, neuropeptide, Tachykinin signalling, feeding

Introduction

Tachykinins (TKs) represent a large family of evolutionarily conserved brain/gut peptides in bilaterian animals. In mammals, the TK peptide family derives from alternate processing of three TAC genes [1] (for review). TAC1 encodes substance P (SP), neurokinin A (NKA) as well as neuropeptide K (NPK) and neuropeptide γ (Npγ) [2]. TAC3 (designated as TAC2 in rodents) only encodes neurokinin B (NKB) [3]. A third gene, TAC4 encodes endokinins A, B, C and D (EKA-D) as well as hemokinin-1 (HK-1) [4]. These genes are conserved from mammals to teleosts [5] and a gene encoding two TK peptides was also characterized in the urochordate *Ciona intestinalis* [6]. Outside the chordate phylum, TKs have also been characterized in insects, crustaceans, molluscs and annelids [7,8] (for review).

Chordate TK sequences display the conserved C-terminal pentapeptide signature FXGLM-amide, whereas protostome TKs share the C-terminal consensus sequence FX_1GX_2R-amide. Interestingly, some vertebrate-type TKs, derived from a distinct gene, have been identified in the salivary glands of cephalopod molluscs [9,10] and insects [11] serving respectively as neurotoxins [12] and as vasodilatory agents that act on vertebrate prey TK receptors (TKR) but not on endogenous receptors [13].

TKs are widely distributed in the nervous systems of all bilaterian animal species. They have been shown to display regulatory roles in an extraordinarily diverse range of physiological processes. In addition to their modulatory role in the central control of respiration and cardiovascular activity, TKs, mainly via SP, also mediate pain, anxiety and motor coordination in the CNS of mammals [14,15]. In arthropods, TKs are involved in odour perception and locomotion as shown in *Drosophila* [16] and in visual processing as suggested in crustaceans [17]. In bilateria, TKs have been shown to participate in the control of the activity of a wide array of peripheral organs and tissues. *In vitro* studies on organ preparations of protostome

species suggest that TK signalling plays a role in the regulation of gut activity and visceral and skeletal muscle contractions [18–21]. Deficient TK functioning contributes to multiple disease processes in humans [1].

In contrast to Ecdysozoa [22], which comprises arthropods and nematodes as major phyla, TK signalling has so far been largely unexplored in Lophotrochozoa, the protostome sister group of the Ecdysozoa. Only two studies, respectively in *Octopus* [13] and in the lophotrochozoan worm *U. unitinctus* [23], reported on the identification of a TKR in Lophotrochozoa. In bivalve molluscs, TK peptides have been molecularly characterized more than two decades ago in the mussel *Anodonta cygnea* [24] and more recently in the oyster *Crassostrea gigas* [25]. The recent development of an extended transcriptomic database of *C. gigas* [26] offers the opportunity to characterize neuropeptide receptors and thus establish their physiological role(s). The present study reports on the characterization of a TKR in the oyster *C. gigas* and shows that it is functionally activated by oyster TKs. In addition, we investigated the structure-activity relationship of ligand-receptor pairs by assessing the potency of a series of synthetic TK analogues. In order to further explore TK signalling in *C. gigas*, we determined the expression patterns of the genes encoding the TK precursor and the TKR at successive reproduction stages as well as in distinct nutritional conditions.

Material and methods

Peptide synthesis

All peptides were custom synthesized by GeneCust (Luxemburg). The sequences of *C. gigas* peptides were obtained from an in-house peptide database yielded by mass spectrometry analyses of tissue extracts and data mining [25].

In silico analyses

Multiple sequence alignment was performed with TKR from various species (supplementary table 1) using Clustal W [27]. To determine the relationship between Cragi-TKR and TKRs from other species (supplementary table 2), a phylogenetic tree was generated by the maximum likelihood method using the phylogeny pipeline (www.phylogeny.fr) [28] connecting the following programs: MUSCLE for multiple alignment (full processing mode), Gblocks for alignment curation (minimum length of a block after gap cleaning: 10, no gap positions allowed in the final alignment, all segments with contiguous non-conserved positions higher than 8 rejected, minimum number of sequences for a flank position: 85%.), PhyML for phylogeny (the default substitution model was chosen assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data Model). The reliability of internal branches was evaluated using an approximate likelihood-ratio test (aLRT). TreeDyn was used for tree drawing.

Reverse endocrinology

Molecular cloning of the *Cragi-TK*R and transfection of mammalian cells:

In silico screening of the oyster transcriptomic database "GigaTon" [26] resulted in the identification of a full length cDNA encoding *Cragi-TK*R (CHOYP_LOC100744404.1.1). The CDS of the *Cragi-TK*R gene was amplified by PCR (Pfu DNA polymerase, Promega) using gene-specific sense primer (5'-CACCATGGAGGGGAACAATTCAACAAAAG-3') harbouring a Kozak consensus sequence and antisense primer (5'-TCATAAATATTCAGCACTAGTTCTCCGCCC-3'). Ten nanogram of plasmid DNA (Pal 17.3 vector, Evrogen) from a *C. gigas* "all developmental stages and adult central nervous system" directional and normalized cDNA library [29] was used as template. The resulting PCR product was directionally cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) and the correct insertion confirmed by sequencing. Human embryonic kidney (HEK293T) cells

were transiently transfected with the *Cragi-TK*R/pcDNA3.1 construct using Fugene HD (Promega) according to the manufacturer's instructions. As a first step, co-transfection was done with an expression construct for the human $G\alpha_{16}$ subunit, a promiscuous G protein that can direct intracellular signalling of GPCRs to the release of calcium via the phospholipase C_β pathway, regardless of the endogenous G protein coupling of the receptor (Mertens et al, 2004). To assess receptor activity independent of $G\alpha_{16}$, calcium responses were measured in cells expressing only *Cragi-TK*R. Cells for negative control experiments were transfected with empty pcDNA3.1 and $G\alpha_{16}$/pcDNA3.1 constructs.

Calcium fluorescence assay:

Activation of *Cragi-TK*R by oyster TK synthetic peptides was monitored using a fluorescence-based calcium mobilization assay. Briefly, transfected HEK293T cells were loaded with Fluo-4 Direct plus probenecid (qsp 2.5mM final in the cell) (Invitrogen / Molecular Probes) for 1 hour (45min at 37°C and 15min at room temperature). Excitation of the fluorophore was done at 488 nm. The calcium response was measured for 2 min at 525 nm using the FLEXstation 3 (Molecular Devices) at 37°C. Data were analysed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} M. Concentration-response measurements of activating ligands were conducted in triplicate and for at least three independent experiments. Half maximal effective concentrations (EC_{50} values) were calculated from concentration-response curves that were constructed using nonlinear regression analysis with a sigmoidal dose-response equation using Prism 5.0 (GraphPad software, USA).

cAMP luminescence assay.

Cragi-TKR transfected HEK 293T cells were incubated with Glosensor cAMP reagent (qsp 4% final in the medium) (Promega) for 2 hours at room temperature prior to the injection of the

candidate ligands. .cAMP luminescence response was measured for 30 min after injection using a FLEX station 3 (Molecular Devices) at room temperature. Data were analysed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} M.

Animals and tissue sampling

Two-year old adult oysters *C. gigas*, purchased from a local farm (Normandie, France), were used for peptide characterization and transcription analyses. Stages of reproduction (Stage 0: resting undifferentiated stage, Stage 1: gonial multiplication stage, Stage 2: maturation stage, Stage 3: sexual maturity) were determined by histological analysis of gonad sections as described previously [31]. To study the influence of trophic conditions, one-year-old adult oysters were reared in water tanks either in absence of food or in presence of *Isochysis galbana* (clone T-Iso) maintained at a concentration of 6 million of cells/mL during 4 weeks. Adult tissues (mantle, gill, labial palps, digestive gland, gonad, hemolymph, adductor muscle) were sampled, the visceral ganglia (VG) were carefully dissected out, thus limiting any contamination from the adjacent adductor muscles. All the samples were either placed in TriReagent (Sigma) or stored at -80°C until use. For expression studies, adult tissues or VG and gonads during gametogenesis from 6 animals were mixed to generate 5 pools of each tissue. Individual VG from 19 and 17 animals were used to study gene expression in fed and starved animals respectively.

*Reverse transcription quantitative PCR (*RT-qPCR)

RT-qPCR analysis was performed using the iCycler iQ© apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Recovered RNA was further purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment during 20 min at 37°C with 1 U of DNase I (Sigma) to prevent genomic

DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200 U MMuLV Reverse Transcriptase (Promega) at 37°C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70°C for 10 min. The GoTaq® qPCR Master Mix (Promega) was used for real time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95°C/15 s, 60°C/15 s) with the following primers: Qs-Cragi-TKR (5′-ATGGCCCACAAGCGGATG-3′) and Qa-Cragi-TKR (5′-GGTGGACACAAACGCCGT-3′) as sense (Qs) and antisense (Qa) primers for Cragi-TKR cDNA and Qs-Cragi-TK (5′-GCATACCAGAATCATCAA-3′) and Qa-Cragi-TK (5′-GTTTATTGTTCCGAACTAAT -3′) for Cragi-TK precursor cDNA. Accurate amplification of the target amplicon was checked by performing a melting curve analysis. Using Qs-*Cg*-EF (5′-ACCACCCTGGTGAGATCAAG-3′) and Qa-*Cg*-EF (5′-ACGACGATCGCATTTCTCTT-3′) primers, a parallel amplification of oyster Elongation Factor 1α (EF1 α) transcript (BAD15289) was carried out to normalize the expression data of *Cragi-TK*R and *Cragi-TK* transcripts. EF1 α was found as a reliable normalization gene as no significant difference ($p<0.05$) of Ct values was observed between the different samples compared. Coefficient of variation of EF1 α was less than 5%. Thus, the relative level of each gene expression was calculated for one copy of the EF1 α reference gene by using the following formula: $N = 2^{(Ct\ EF1\alpha - Ct\ Cg\text{-cDNA})}$. The PCR amplification efficiency (E; $E = 10^{(-1/slope)}$) for each primer pair was determined by linear regression analysis of a dilution series to ensure that E ranged from 1.98 to 2.02. The specificity of the primer pairs was confirmed by melting curve analysis at the end of each RT-qPCR run.

Statistical analysis

Gene expression levels between different tissues and between samples at different reproduction stages were compared using one-way ANOVA followed by a Tukey post hoc test. Expression levels between fed and starved animals were compared using an unpaired Student’s t test.

Significance was set at $p < 0.05$.

Results:

Molecular characterization of an oyster tachykinin receptor (Cragi-TKR).

The unique sequence displaying homology with vertebrate and protostome TKRs was retrieved from GigaTON, an oyster comprehensive transcriptomic database [26]. Alignment of *C. gigas* receptor (Cragi-TKR) with other receptors of the family displays an overall identity of 42% with *Octopus* TKRPR and 32% with *Drosophila* DTKR and human TKR1 (Fig. 1). A phylogenetic analysis clearly showed that Cragi-TKR clustered with predicted or functionally characterized mollusc TKRs and as a separate branch from the insect TKRs. Annotated orphan nematode TKRs appeared more distant and emerged as a separate branch. All vertebrate TK-related receptors including the three distinct classes of NK receptors (NK1R, NK2R and NK3R) formed a distinct clade (Fig.2). Alignment of the Cragi-TKR cDNA with *C. gigas* genomic sequence (http://www.oysterdb.com) identified a gene (CGI_10007698) organized into 5 exons with 4 introns shared at conserved positions and with the same intron phasing with the receptors from vertebrate and protostome species [23,32] suggesting an evolution from a gene already present in the bilaterian common ancestor.

Oyster TKs specifically activate Cragi-TKR.

A calcium mobilization assay was used to identify the cognate ligands of Cragi-TKR [33]. Transiently transfected HEK293T cells expressing the oyster receptor and the promiscuous G protein $G\alpha_{16}$ were challenged with the three oyster synthetic TKs (Cragi-TK1: FGFAPMR-amide, Cragi-TK2: ARFFGLR-amide and Cragi-TK3: FRFTALR-amide). These TKs are derived from the Cragi-TK neuropeptide precursor by posttranslational processing (Fig.3A) and have previously been characterized as part of *C. gigas'* repertoire of neuropeptides [25]. Since Cragi-TKR was equally activated with high doses (10^{-5}M) of all three Cragi-TK peptides in

198 presence or absence of the promiscuous $G\alpha_{16}$ protein (supplementary Figure 1), a dose-
199 dependent activation of Cragi-TKR was recorded by omitting the $G\alpha_{16}$ protein (Fig.3B). Half
200 maximal effective concentrations (EC_{50}) were of 4.1 nM for Cragi-TK2, 4.6 nM for Cragi-TK1
201 and 11.5 nM for Cragi-TK3. No signal was observed with cells transfected with an empty vector
202 or with high concentrations (10^{-5} M) of the oyster GALRF-amide unrelated peptide used as
203 negative control [33].
204 To determine the residues that are critical for receptor activation, a series of alanine-substituted
205 analogues of Cragi-TK2 were assessed (Fig.4). The activity of the different analogues can be
206 ranked into three main groups, a first one including the peptides displaying a high EC_{50}
207 corresponding to the [Arg^7] and [Phe^3] alanine-substituted peptides, a second group comprising
208 [Arg^2] and [Phe^4] alanine-substituted peptides for which the modification only moderately
209 affected the potency and a third group including the [Gly^5] and [Leu^6] alanine-substituted
210 peptides displaying a higher potency than the naturally occurring peptides (Table 1). All these
211 agonists displayed the same efficacy. None of the three naturally occurring peptides or the
212 alanine substituted peptides activate the cAMP signalling pathway even at concentrations as
213 high as 10^{-5}M.
214 *Gene expression of Cragi-TKR and Cragi-TK.*
215 The expression of Cragi-TKR and of Cragi-TK genes was analysed by RT-qPCR. Cragi-TKR
216 was found to be mainly expressed in the visceral ganglia and to a lower level in a majority of
217 adult tissues including, the gills, the adductor muscle, the heart, the mantle, the gonads, the
218 labial palps and the digestive gland (Fig.5A). To determine a possible involvement of TK
219 signalling in the regulation of oyster reproduction, Cragi-TKR gene expression was assayed in
220 the visceral ganglia and in the gonads along the reproductive cycle (Fig.5B). Except a slight
221 peak of expression in females during vitellogenesis (stage2), Cragi-TKR gene expression did
222 not fluctuate significantly in the visceral ganglia. In the gonads, Cragi-TKR gene expression

was maximal in undifferentiated gonads (Stage 0) and gradually declined along the reproductive cycle in both males and females. Besides, Cragi-TK gene was chiefly expressed in the visceral ganglia and at basal levels in the mantle, the adductor muscle and the labial palps (Fig.5C). No significant differential expression of Cragi-TK gene was noticed along the reproductive cycle in the visceral ganglia (Fig.5D). Interestingly, Cragi-TK gene, but not Cragi-TKR gene, was significantly more expressed in four weeks starved animals than in fed animals (Fig.5E and F).

Discussion.

TK signalling systems have been extensively studied in a vast number of animal species. Mature TKs were first biochemically isolated and identified and as a result of the development of molecular biology approaches and genomics, the characterization of their precursor as well as their cognate receptors has become accessible. By mining *C. gigas* comprehensive transcriptomic [26] and genomic [34] databases, a unique receptor (Cragi-TKR) displaying consistent homology and phylogenetic proximity with vertebrate and insect TKRs has been identified. In contrast, vertebrate [1] and *Drosophila* [35,36] genomes encode respectively three and two TKR types. Diverse TKs (SP, NKA, NKB) derived from distinct peptide precursor genes, activate vertebrate TKR with distinct potencies [1]. In *Drosophila,* all TK peptides derived from the TK-related neuropeptide precursor gene activate the DTKR with different potencies [37]. The other *Drosophila* receptor (NKD), is activated *in vitro* by only one of these six TKs (DTK-6) albeit at high concentrations [38]. Finally, NKD turned out to represent the *bona fide* receptor for *Drosophila* natalisins, a family of insect neuropeptides that are derived from a distinct neuropeptide precursor gene. Natalisins promote insect reproduction and also display the C-terminal FXXXRamide motif common to all protostomian TKs [39].

In all lophotrochozoan species investigated so far, only one specific TKR has been identified [13,23]. The occurrence of a natalisin type of receptor is unlikely in *C. gigas* since no homologous neuropeptide has been found among the exhaustive neuropeptide repertoires of

oyster [25] and other Lophotrochozoa [40,41]. Moreover, our phylogenetic study suggests that arthropod-specific natalisin receptors may have arisen from a recent duplication during the evolution of arthropods.

Cragi-TKR behaves as a genuine TKR. Similar to its vertebrate and protostome counterparts, it is specifically and slightly selectively activated by all three oyster TKs encoded by the oyster TK precursor at concentration ranges similar to those required for the activation of TKRs in other species [23,37,42]. Similar to the *Octopus* TKR [13], Cragi-TKR triggers *in vitro* only the phospholipase Cβ-mediated calcium transduction pathway, a feature distinct to some insect and vertebrate receptors which additionally transduce their signal via an increase in cAMP levels [36,43–45]. Although distinct in sequence, Cragi-TKs exhibit only minor potency differences suggesting that they may be functionally redundant. To determine the essential amino acids of Cragi-TKs, a structure activity relationship analysis was performed using a series of synthetic analogues of Cragi-TK2 (ARFFGLR-amide) in which each amino acid was sequentially replaced by the neutral alanine residue. Considering the C-terminal consensus FX_1GX_2Ramide sequence of protostome TKs, only the replacement of the terminal Arginine or the first Phenylalanine showed drastic negative effects consistent with the high conservation of these two residues possibly due to a strong selective pressure during the evolution of protostome TKs. This also reflects the low activity reported for chordate-type TKs (harbouring a C-terminal methionine instead of an arginine) on protostome receptors [13,23,46]. Change of the N-terminal extension of Cragi-TK2 did not alter significantly the neuropeptide activity. As expected, change of the flexible residue (X_1) of the consensus sequence resulted in only limited effects on the activation of Cragi-TKR. Surprisingly, Cragi-TKR showed higher sensitivity to peptide analogues with an alanine replacing either the conserved Glycine or the penultimate (X_2) residue. The glycine residue does not appear to be crucial since the three naturally occurring oyster TKs display a variability of residues at this position. Interestingly both Cragi-

TK3 and the bivalve mollusc *Anodonta cygnea* TK hold an alanine at this position [24]. Such naturally occurring alanine-containing TKs also exist in insects [47,48], and were proven more potent than their glycine-containing counterparts but behave as partial agonists due to reduced maximal calcium mobilisation efficacy [49]. Partial agonistic activity and transduction pathway plasticity in insect and mammalian neurokinin signalling were suggested to reflect the existence of multiple receptor conformation states [49,50] that may disclose a potential fine-tuning of physiological processes. Unexpectedly such a situation does not appear to exist in oyster since all peptides show equivalent efficacy.

The expression of Cragi-TKR in a wide variety of oyster organs and tissues clearly conforms with the pleiotropic regulatory role of TKs in other animal groups. As for other molluscs, the central nervous system represents the unique source of TKs, a situation different from insects where gut endocrine cells also contribute to the production of this family of peptides [51] suggesting a possible link with the digestive processes and feeding. The increased expression level of the Cragi-TK gene in the CNS of starved oysters suggests a role in feeding behaviour. However, it is not well-defined whether this activity is exerted centrally, likely initiated through nutrient sensing, via the control of neuronal feeding circuits or peripherally at the level of the gills and labial palps -the main food collector organs- or the digestive tract. In mice, Tac1 (SP/neurokinin A) controls circadian feeding behaviour and metabolism [52]. Likewise in insects, TKs injected in starved *Bombyx mori* larvae induce a stimulatory effect in feeding behaviour by reducing the period of latency to the first bite [53]. The content of mature TKs was also affected in the brain of honey bees in association with nectar and pollen foraging suggesting a role in this social behaviour [54]. Given the involvement of TKs in olfactory and locomotion behaviour in *Drosophila* [16], it was proposed that TK signalling could play a role in the perception, the localisation of a food source and its collection [54]. Such hypothesis fits the presence of Cragi-TKR in the gills and the labial palps, the oyster organs implicated in the

collection and sieving of food particles. In vertebrates and insects, the digestive tract also represents an important target for TKs. In the mammalian intestine, TKs mainly released from neurons control the activity of neuronal networks, influence fluid secretion and act on smooth muscles [55]. Similarly, TKs stimulate *in vitro* contractions of the gut in insects [20] and also regulate enterocyte lipid production and systemic lipid homeostasis in *Drosophila* [56]. However, this later activity is mainly controlled by TKs released from enteroendocrine cells, the peptide content of which increases in starved animals. The weak expression of Cragi-TKR in the oyster digestive gland is consistent with a role of TKs in lipid metabolism. However, the lack of TK gene expression and the absence of endogenous TKs in this organ implies a regulation by TKs released as a circulating neurohormone. It is intriguing that, with the singular exception of the AKH signalling system [57], most oyster neuroendocrine systems investigated so far appear sensitive to the nutritional status [33,58]. This reflects the complexity of the feeding control in animals and emphasizes the requirement of a fine regulation to support constant energy needs in a context of sporadic food availability. That TK signalling also regulates the activity of oyster gonad cells was suggested by the fluctuating Cragi-TKR expression during the reproductive cycle. This is reminiscent of the role of TKs in the regulation of reproduction-associated processes in both vertebrate and protostome species. Indeed, TKs were shown to participate in the neuroendocrine control of reproduction in mammals [59,60] and fish [61], in the regulation of oocyte growth in the ascidian *C. intestinalis* [62] and in the oviducal myotropic activity in the locust [20].

Conclusion

We have characterized in the oyster *C. gigas*, a TK signalling system that appears to share common features with that of other animal species: (1) an involvement in the regulation in a variety of physiological processes implied by a distribution of the TK receptors in diverse organs (2) a potential feeding modulating activity of TK peptides suggested by a marked

increase in expression of their encoding gene in the CNS of starved oysters, (3) a likely role in regulating reproduction processes in line with the variability of expression of TK signalling components along the reproductive cycle. In contrast to vertebrates and insects, oyster and other protostome species [8] express their TK gene in the central nervous system but not in the gut.

Acknowledgments.

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Figure legends:

Figure 1: Sequence alignment of the Cragi-TKR and TKR family members.

The amino acid sequence of *Crassostrea gigas* (Cragi-TKR: MF320350) was aligned with those of *Octopus vulgaris* (Ov-TRR: Q58A49), *Drosophila melanogaster* (Dm-DTKR: P30975), *Homo sapiens* (Hs-NK1R: P25103), *Caenorhabditis elegans* (Ce-TKRF: O44148) and *Ciona intestinalis* (Ci-TKR: Q60GS8) using CLUSTALW.

Bars indicate the seven putative TM domains. Identical amino acid residues are highlighted in dark grey and similar residues in light grey. Putative N-linked glycosylation sites (NXS/T) or S, T and Y potential phosphorylated residues are underlined with a dotted line. Amino acid residues in boxes are believed to play a pivotal role in GPCR activation. Arrow heads indicate the position of introns. * indicates functionally characterized receptors.

Figure. 2: Phylogenetic representation of the relationship between the *Cragi-TK*R and other TKR family members. The tree was generated by a maximum likelihood method using the phylogeny pipeline **(www.phylogeny.fr)** [28]**.** *Crassostrea gigas* TKR (MF320350) *Ancyclostoma ceylanicum* TKR (A0A016WHR5), *Apis mellifera* TRP-R (A0A141CIU0), *Aplysia californica* TKR (XP_012936179.1), *Caenorhabditis elegans* TKR (O44148), *Ciona intestinalis* TKR (Q60GS8), *Danio rerio* TACR1a (E9QCW0)*;* TACR1b (I6UDB5)*;* TACR2 (F1QPL8)*;* TACR3-like (F1R3V0) and TACR3a (H6A6A7), *Drosophila melanogaster* DTKR (P30975)and NKDR (P30974), *Gallus gallus* TACR3 (F1NJ82)*;* SPR (Q9W6I3) and TACR2 (E1BRR8), *Homo sapiens* NK1R (P25103), NK2R (P21452) *and* NK3R (P29371), *Limulus polyphemus* TKR (XP_013772923.1), *Lottia gigantea* (V4BE54), *Mus musculus* TACR1 (P30548)*;* TACR2 (Q3KP20) and TACR3 (EDL12172.1), *Nilaparata lugens* GPCR (U3U967), *Octopus vulgaris* TKR (Q58A49), *Octopus bimaculoides* TKR (XP_014785645.1) *Parasteatoda tepidariorum* TKLPR (XP_015910841.1), *Stomoxys calcitrans* TKLPR (A0A1I8PID0), *Toxocara canis* TAKR (A0A0B2V4Q7), *Varoa destructor* TRP-R (A0A141CIT9) were the sequences used to construct the tree. The Cg-sNPFR-like receptor (MF320349) was chosen as outgroup. * indicates functionally characterized receptors. Branch node labels correspond to likelihood ratio test values.

Figure 3: Dose-dependent activity of Cragi-TK peptides on Cragi-TKR expressed in HEK293T cells. A: schematic representation of Cragi-TK precursor (SP: Signal peptide). B: Concentration–response data evoked by Cragi-TK peptides are shown as relative (%) to the highest value (100% activation) for a given peptide. Data are the means of three independent experiments done in triplicate. The *C. gigas* GALRF-amide peptide was used as negative control. Vertical bars represent the standard error of the mean (SEM).

Figure 4: Comparison of dose-response relationships of a series of Cragi-TK2 single amino acid replacement analogues. Fluorescent signal induced by Cragi-TKR expressed in HEK293T cells and challenged by a series of alanine-substituted analogues of Cragi-TK2**.** Grey shading represents the position of the Cragi-TK2 amino acids replaced by an alanine residue. Data are shown as relative (%) to the highest value (100% activation) for a given peptide, and were performed at least in triplicate. Vertical bars represent the SEM.

Figure 5: **Expression of Cragi-TKR and Cragi-TK genes**. (A) Distribution of mRNAs encoding Cragi-TKR in adult tissues, (B) level of expression of Cragi-TKR mRNA in visceral ganglia (VG) and gonads (GO) during gametogenesis, (C) Distribution of mRNAs encoding Cragi-TK precursor in adult tissues, (D) level of expression of Cragi-TK mRNA in visceral ganglia (VG) along an annual reproductive cycle, (E and F) expression levels of Cragi-TKR and Cragi-TK mRNA respectively in VG of four weeks *Isochysis galbana* fed or starved oysters, Each value is the mean + SEM of 5 pools of 6 animals (in adult tissues); 5 pools of 6 animals (VG during gametogenesis) and 19 or 17 independent animals (VG after conditioning with or without food). Expression levels were calculated as the number of copies of Cragi-TKR / Cragi-TK transcripts per 10^3 copies of elongation factor 1α (EF1α) mRNA. Results were statistically tested with a one-way ANOVA (A, B, D and E) or student's t test (C and F), $p<0,05$. Significantly different means are indicated by different letters (A, B and D) or *** ($p<0.001$) (B). No significant statistical difference was observed for (C and E). M: Mantle; G: Gills; LP: Labial Palps; DG: Digestive Gland; Go Gonad; H: heart; AM: Adductor Muscle; VG: Visceral Ganglia; F: Female; M: Male; 0: stage 0 (sexual resting stage); 1: stage 1 (gonial multiplication stage); 2: stage 2 (tubule development and maturation stage); 3: stage 3 (sexual maturity stage)

Table 1: **Amino acid sequences of Cragi-TK2 analogues and their respective EC50 for receptor activation.** Grey shading represents the position of the Cragi-TK2 amino acids replaced by an alanine residue.

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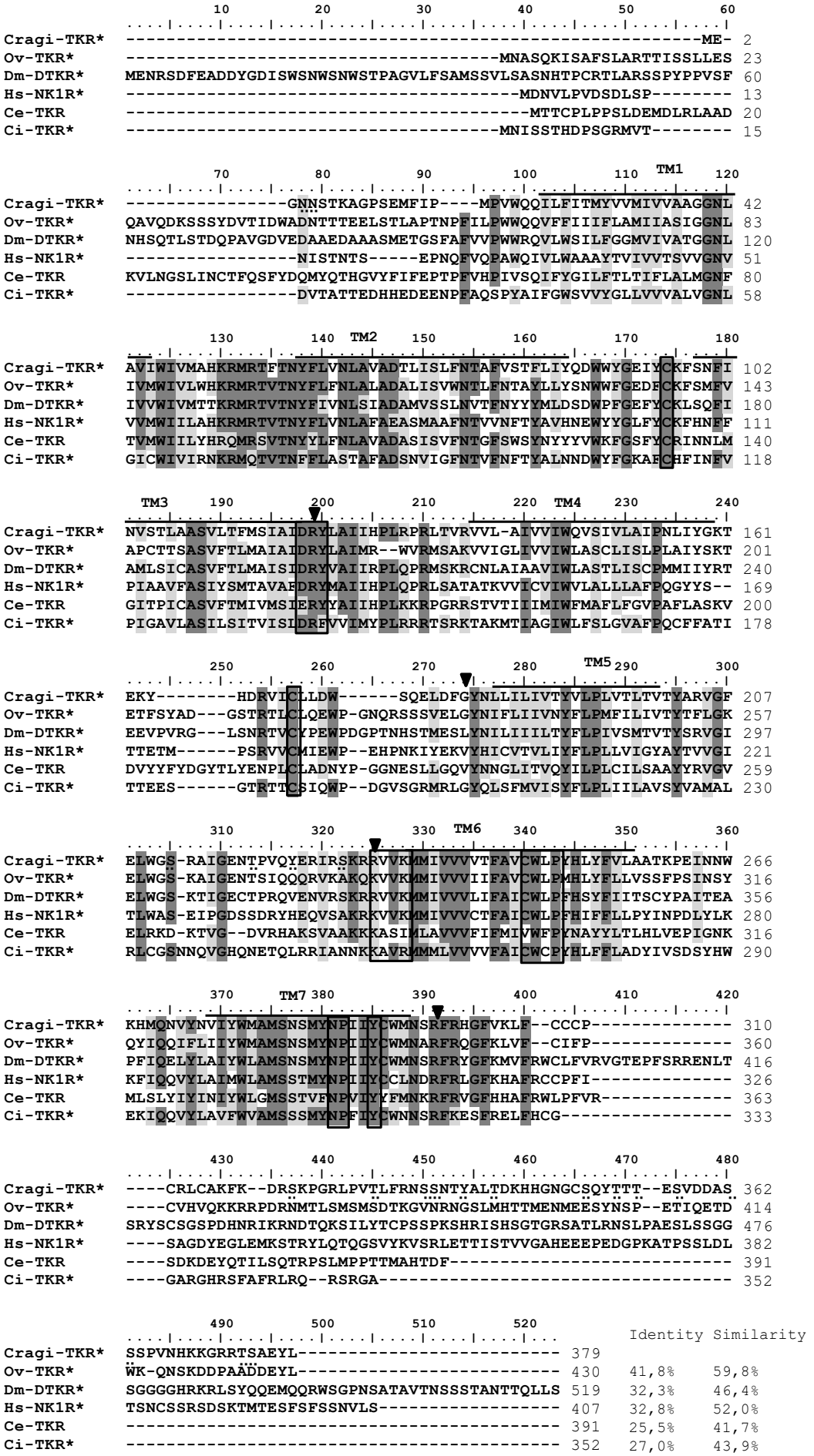
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TM2
TM3
TM4
TM5
TM6
TM7
Cragi-TKR*
Ov-TKR*
Dm-DTKR*
Hs-NK1R*
Ce-TKR
Ci-TKR*
Identity Similarity
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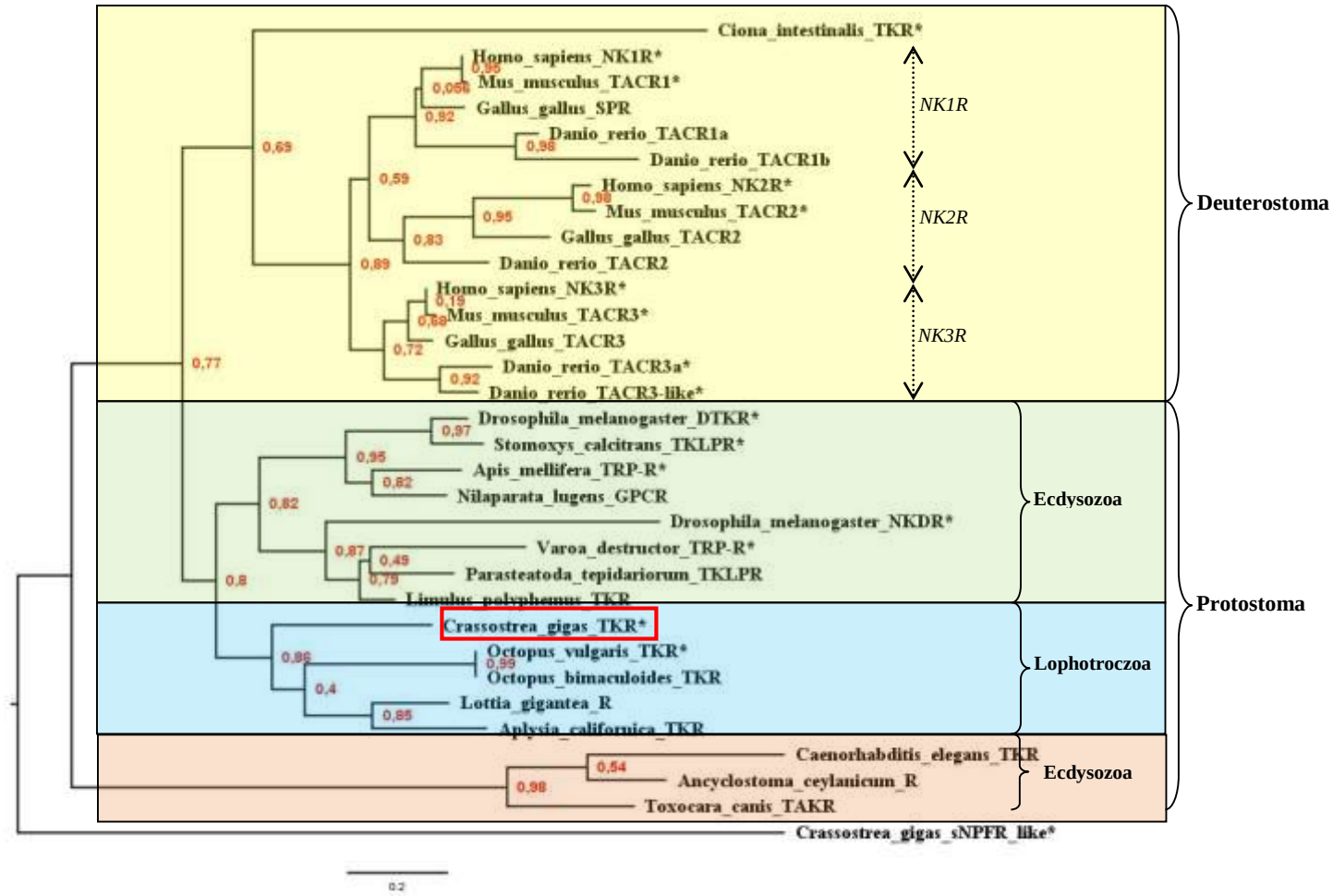
32,3% 46,4%
32,8% 52,0%
25,5% 41,7%
27,0% 43,9%

Figure 1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Danio_rerio_TACR3-like*
NK1R
NK2R
NK3R
Deuterostoma
Drosophila_melanogaster_DTKR*
Stomoxys_calcitrans_TKLPR*
Apis_mellifera_TRP-R*
Nilaparata_lugens_GPCR
Drosophila_melanogaster_NKDR*
Varoa_destructor_TRP-R*
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Protostoma
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Lottia_gigantea_R
Aplysia_californica_TKR
Lophotroczoa
Caenorhabditis_elegans_TKR
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Figure. 2

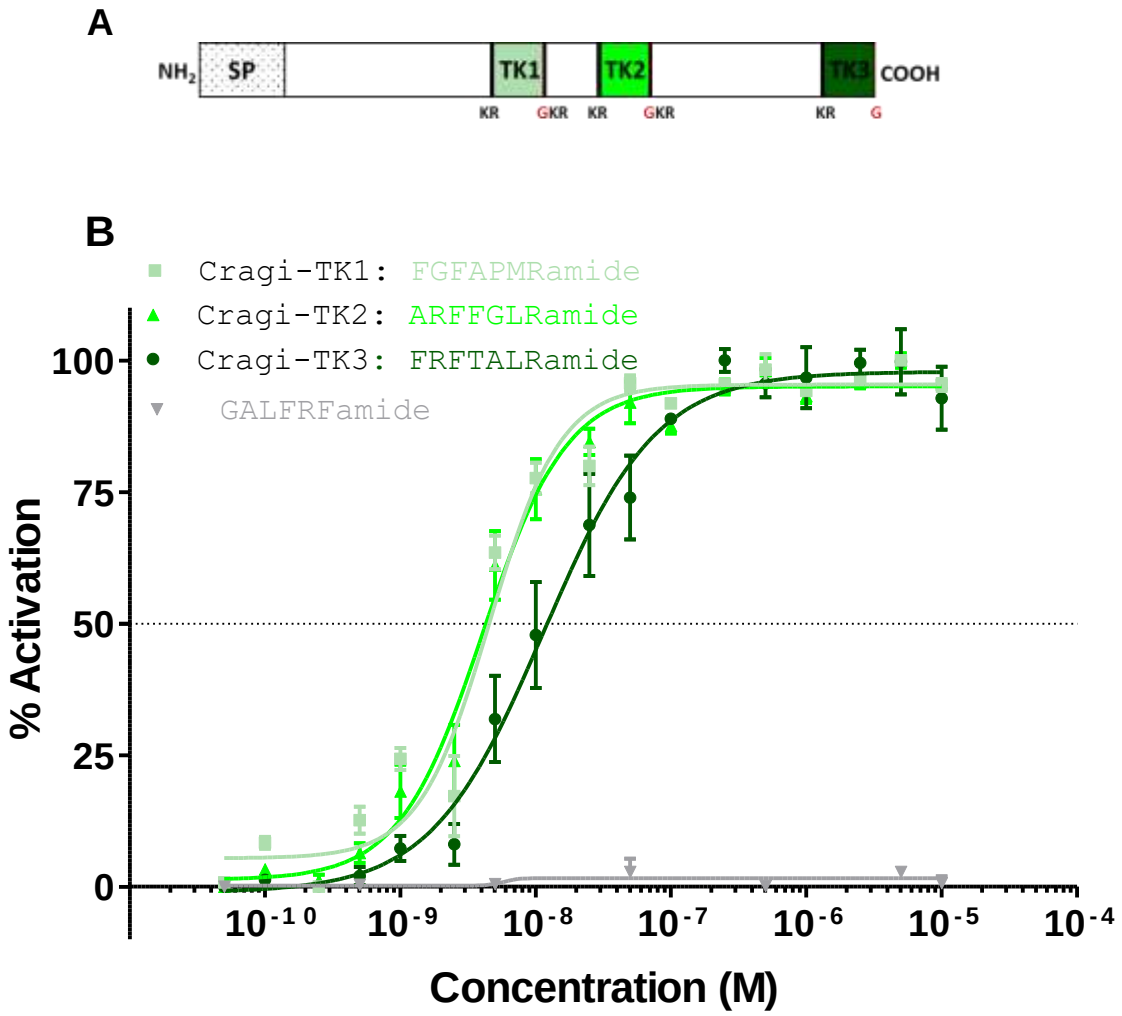


Figure 3

Table 1.

									EC_{50} (nM)
Name	**Peptide sequence**								**EC_{50} (nM)**
TK Vertebrate consensus	-	-	F	X	G	L	M	amide	
TK Protostome consensus	-	-	F	X	G	X	R	amide	
Cragi-TK2	A	R	F	F	G	L	R	amide	4.48
Cragi -TK2-A2	A	A	F	F	G	L	R	amide	7.43
Cragi -TK2-A3	A	R	A	F	G	L	R	amide	906.3
Cragi -TK2-A4	A	R	F	A	G	L	R	amide	14.2
Cragi -TK2-A5	A	R	F	F	A	L	R	amide	0.83
Cragi -TK2-A6	A	R	F	F	G	A	R	amide	0.74
Cragi -TK2-A7	A	R	F	F	G	L	A	amide	4276
Cragi -TK1	F	G	F	A	P	M	R	amide	4.6
Cragi -TK3	F	R	F	T	A	L	R	amide	11.5

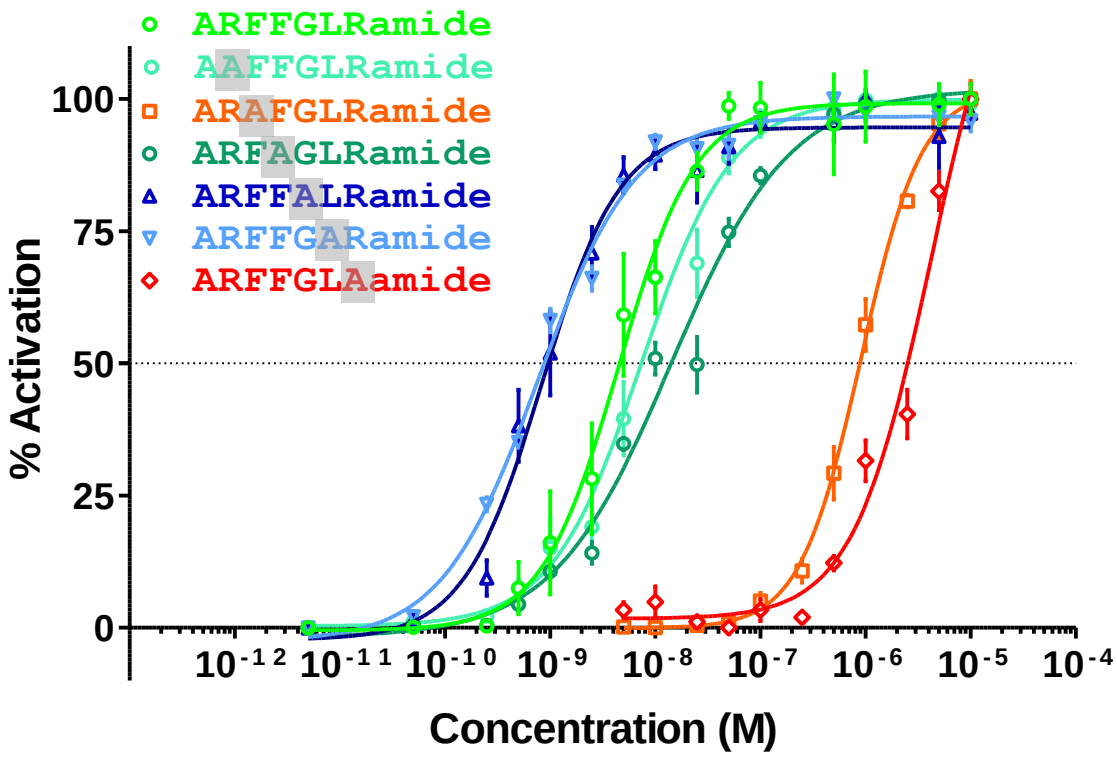
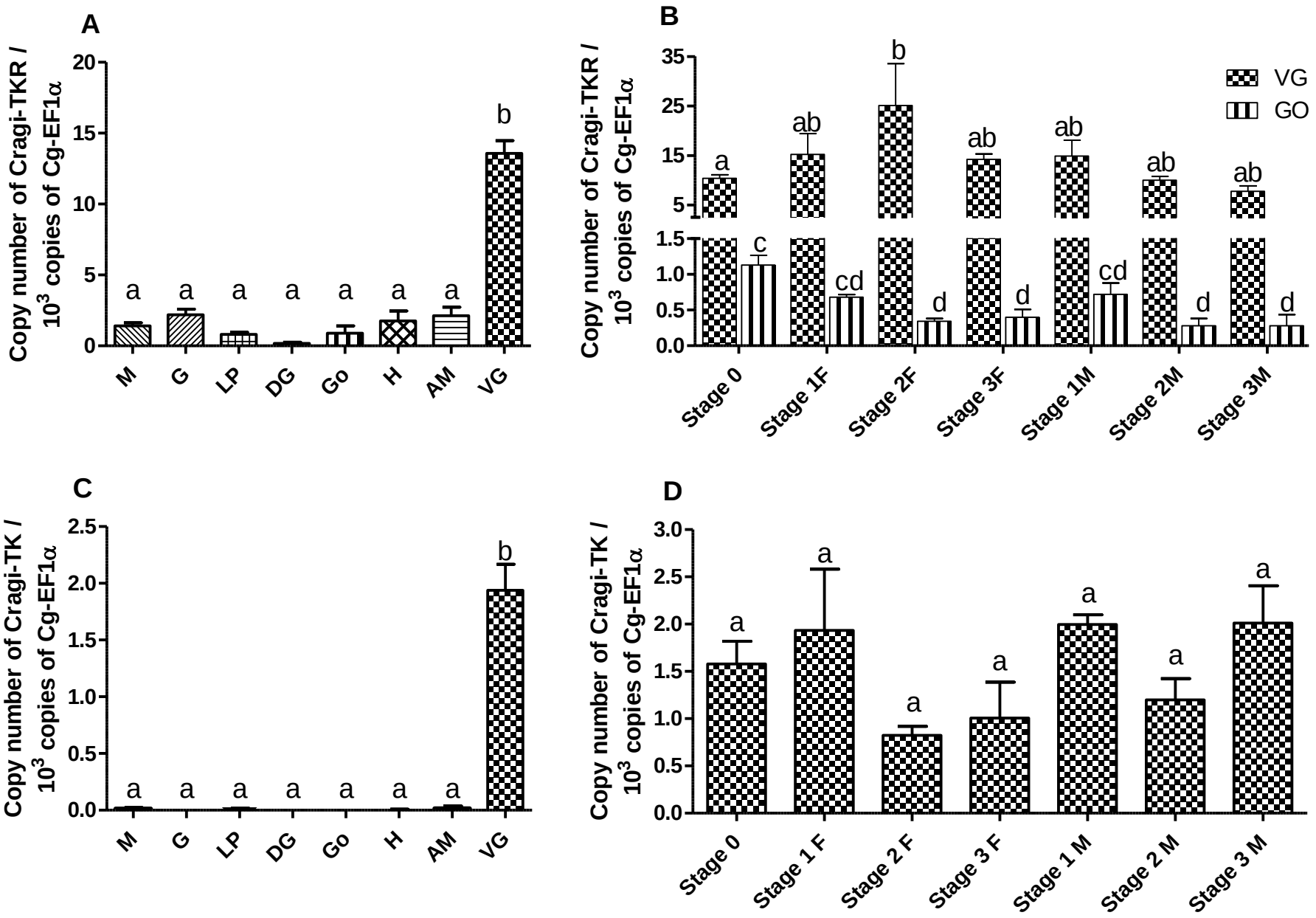

ARFFGLRamide
AAFFGLRamide
ARAFGLRamide
ARFAGLRamide
ARFFALRamide
ARFFGARamide
ARFFGLAamide
% Activation
100
75
50
25
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
10-5
10-4
Concentration (M)

Figure 4.


A
Copy number of Cragi-TKR / 10^3 copies of Cg-EF1α
20
15
10
5
0
a a a a a a a b
M G LP DG Go H AM VG
B
Copy number of Cragi-TKR / 10^3 copies of Cg-EF1α
35
25
15
5
1.5
1.0
0.5
0.0
a c ab cd b d ab d ab cd ab d ab d
VG
GO
Stage 0 Stage 1F Stage 2F Stage 3F Stage 1M Stage 2M Stage 3M
C
Copy number of Cragi-TK / 10^3 copies of Cg-EF1α
2.5
2.0
1.5
1.0
0.5
0.0
a a a a a a a b
M G LP DG Go H AM VG
D
Copy number of Cragi-TK / 10^3 copies of Cg-EF1α
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a a a a a a a
Stage 0 Stage 1 F Stage 2 F Stage 3 F Stage 1 M Stage 2 M Stage 3 M

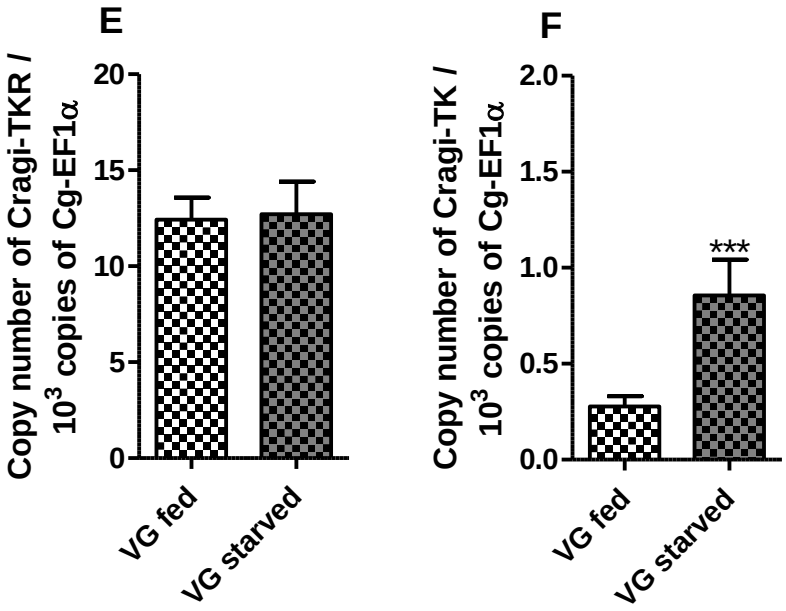

E
Copy number of Cragi-TKR / 10^3 copies of Cg-EF1α
20
15
10
5
0
VG fed
VG starved
F
Copy number of Cragi-TK / 10^3 copies of Cg-EF1α
2.0
1.5
1.0
0.5
0.0
***
VG fed
VG starved

Figure 5

Supplementary table 1: Accession numbers and references of the TKR sequences used for the alignment in Figure1

Abbreviation	Species name	Accession number	Phylum	reference
Cragi-TKR*	*Crassostrea gigas*	MF320350	Lophotrochozoa (Mollusc)	Present paper
Ov-TKR*	*Octopus vulgaris*	Q58A49	Lophotrochozoa (Mollusc)	A. Kanda, K. Takuwa-Kuroda, M. Aoyama, H. Satake, A novel tachykinin-related peptide receptor of *Octopus vulgaris* - Evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide, FEBS J. 274 (2007) 2229–2239.
Dm-DTKR*	*Drosophila melanogaster*	P30975	Ecdysozoa (Arthropod)	X.J. Li, W. Wolfgang, Y.N. Wu, R.A. North, M. Forte, Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides., EMBO J. 10 (1991) 3221–9.
Ci-TKR*	*Ciona intestinalis*	Q60GS8	Urochordate	H. Satake, M. Ogasawara, T. Kawada, K. Masuda, M. Aoyama, H. Minakata, T. Chiba, H. Metoki, Y. Satou, N. Satoh, Tachykinin and tachykinin receptor of an ascidian , *Ciona intestinalis*, J. Biol. Chem. 279 (2004) 53798–53805.
Hs-NK1R*	*Homo sapiens*	P25103	Vertebrate	N.P. Gerard, L.A. Garraway, R.L. Eddy, T.B. Shows, H. Iijima, J.L. Paquet, C. Gerard, Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones., Biochemistry. 30 (1991) 10640–6.
Ce-TKRF	*Caenorhabditis elegans*	O44148	Ecdysozoa (Nematode)	No reference

*** functionally characterized receptors.**

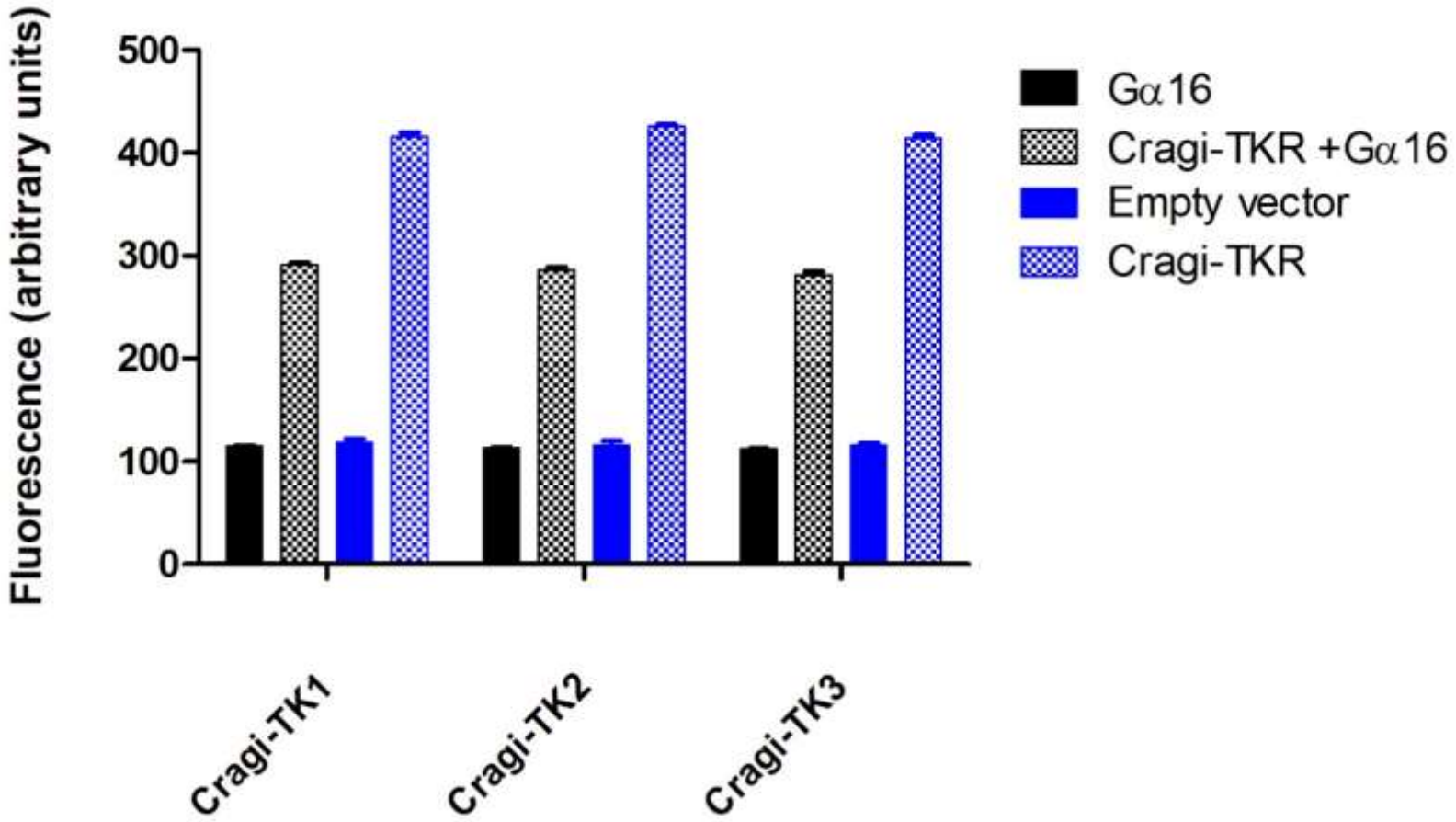
Supplementary table 2: Accession numbers and references of the TKR sequences used for the phylogenetic tree in Figure2

Abbreviation	Species name	Accession number	Phylum	reference
Hs-NK1R*	*Homo sapiens*	P25103	Vertebrate	N.P. Gerard, L.A. Garraway, R.L. Eddy, T.B. Shows, H. Iijima, J.L. Paquet, C. Gerard, Human substance P receptor (NK-1): organization of the gene, chromosome localization, and functional expression of cDNA clones., Biochemistry. 30 (1991) 10640–6.
Mm-TACR1*	*Mus musculus*	P30548.2	Vertebrate	J.B. Sundelin, D.M. Provvedini, C.R. Wahlestedt, H. Laurell, J.S. Pohl, P.A. Peterson, Molecular cloning of the murine substance K and substance P receptor genes., Eur. J. Biochem. 203 (1992) 625–31.
Gg-SPR	*Gallus galus*	Q9W6I3	Vertebrate	No reference
Dr-TACR1a	*Danio rerio*	E9QCW0	Vertebrate	R. Lopez-Bellido, K. Barreto-Valer, R.E. Rodriguez, Expression of tachykinin receptors (tacr1a and tacr1b) in zebrafish: influence of cocaine and opioid receptors, J. Mol. Endocrinol. 50 (2013) 115–129.
Dr-TACR1b	*Danio rerio*	I6UDB5	Vertebrate	R. Lopez-Bellido, K. Barreto-Valer, R.E. Rodriguez, Expression of tachykinin receptors (tacr1a and tacr1b) in zebrafish: influence of cocaine and opioid receptors, J. Mol. Endocrinol. 50 (2013) 115–129.
Hs-NK2R*	*Homo sapiens*	P21452.3	Vertebrate	S. Arkinstall, I. Emergy, D. Church, A. Chollet, E. Kawashima, Calcium influx and protein kinase C alpha activation mediate arachidonic acid mobilization by the human NK-2 receptor expressed in Chinese hamster ovary cells., FEBS Lett. 338 (1994) 75–80.
Mm-TACR2*	*Mus musculus*	Q3KP20	Vertebrate	J.B. Sundelin, D.M. Provvedini, C.R. Wahlestedt, H. Laurell, J.S. Pohl, P.A. Peterson, Molecular cloning of the murine substance K and substance P receptor genes., Eur. J. Biochem. 203 (1992) 625–31.
Gg-TACR2	*Gallus galus*	E1BRR8	Vertebrate	No reference
Dr-TACR2	*Danio rerio*	F1QPL8	Vertebrate	No reference
Hs-NK3R*	*Homo sapiens*	P29371.1	Vertebrate	Y. Takeda, K.B. Chou, J. Takeda, B.S. Sachais, J.E. Krause, Molecular cloning, structural characterization and functional expression of the human substance P receptor., Biochem. Biophys. Res. Commun. 179 (1991) 1232–40.

Mm-TACR3*	*Mus musculus*	EDL12172.1	Vertebrate	H.M. Sarau, J.A. Feild, R.S. Ames, J.J. Foley, P. Nuthulaganti, D.B. Schmidt, P.T. Buckley, N.A. Elshourbagy, M.E. Brawner, M.A. Luttmann, G.A. Giardina, D.W. Hay, Molecular and pharmacological characterization of the murine tachykinin NK(3) receptor., Eur. J. Pharmacol. 413 (2001) 143–50.
Gg-TACR3	*Gallus galus*	F1NJ82	Vertebrate	No reference
Dr-TACR3a	*Danio rerio*	H6A6A7	Vertebrate	J. Biran, O. Palevitch, S. Ben-dor, B. Levavi-sivan, Neurokinin Bs and neurokinin B receptors in zebrafish- potential role in controlling fish reproduction, Proc Natl Acad Sci U S A. 109 (2012) 10269–10274.
Dr-TACR3-like	*Danio rerio*	F1R3V0	Vertebrate	W. Zhou, S. Li, Y. Liu, X. Qi, H. Chen, C.H.K. Cheng, X. Liu, Y. Zhang, H. Lin, Molecular and Cellular Endocrinology The evolution of tachykinin / tachykinin receptor (TAC / TACR) in vertebrates and molecular identification of the TAC3 / TACR3 system in zebrafish (*Danio rerio*), Mol. Cell. Endocrinol. 361 (2012) 202–212.
Dm-DTKR*	*Drosophila melanogaster*	P30975	Ecdysozoa (Arthropod)	X.J. Li, W. Wolfgang, Y.N. Wu, R.A. North, M. Forte, Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides., EMBO J. 10 (1991) 3221–9.
Sc-TKLPR*	*Stomoxys calcitrans*	A0A1I8PID0	Ecdysozoa (Arthropod)	H. Torfs, R. Shariatmadari, F. Guerrero, M. Parmentier, J. Poels, W. Van Poyer, E. Swinnen, A. De Loof, K. Åkerman, J. Vanden Broeck, Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line, J. Neurochem. 74 (2000) 2182–2189.
Am-TRP-R	*Apis mellifera*	A0A141CIU0	Ecdysozoa (Arthropod)	H. Jiang, D. Kim, S. Dobesh, J.D. Evans, R.J. Nachman, K. Kaczmarek, J. Zabrocki, Y. Park, Ligand selectivity in tachykinin and natalisin neuropeptidergic systems of the honey bee parasitic mite *Varroa destructor*, Sci. Rep. 6 (2016) 19547.
Nl-GPCR	*Nilaparvata-lugens*	U3U967	Ecdysozoa (Arthropod)	No reference
Dm-NKDR*	*Drosophila melanogaster*	P30974.2	Ecdysozoa (Arthropod)	J. Poels, R.T. Birse, R.J. Nachman, J. Fichna, A. Janecka, J. Vanden Broeck, D.R. Nässel, Characterization and distribution of NKD, a receptor for *Drosophila* tachykinin-related peptide 6, Peptides. 30 (2009) 545–556.

Vd-TRP-R*	*Varroa destructor*	A0A141CIT9	Ecdysozoa (Arthropod)	H. Jiang, D. Kim, S. Dobesh, J.D. Evans, R.J. Nachman, K. Kaczmarek, J. Zabrocki, Y. Park, Ligand selectivity in tachykinin and natalisin neuropeptidergic systems of the honey bee parasitic mite *Varroa destructor*, Sci. Rep. 6 (2016) 19547.
Pt-TKLPR	*Parasteatoda tepidariorum*	XP_015910841.1	Ecdysozoa (Arthropod)	No reference
Lp-TKR	*Limulus polyphemus*	XP_013772923.1	Ecdysozoa (Arthropod)	No reference
Cragi-TKR*	*Crassostrea gigas*	MF320350	Lophotrochozoa (Mollusc)	Present paper
Ov-TKR*	*Octopus vulgaris*	Q58A49	Lophotrochozoa (Mollusc)	A. Kanda, K. Takuwa-Kuroda, M. Aoyama, H. Satake, A novel tachykinin-related peptide receptor of *Octopus vulgaris* - Evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide, FEBS J. 274 (2007) 2229–2239.
Ob-TKR	*Octopus bimaculoides*	XM_014930159.1	Lophotrochozoa (Mollusc)	No reference
Lg-R	*Lottia gigantea*	V4BE54	Lophotrochozoa (Mollusc)	No reference
Ac-TKR	*Aplysia californica*	XP_012936179.1	Lophotrochozoa (Mollusc)	No reference
Ce-TKRF	*Caenorhabditis elegans*	O44148	Ecdysozoa (Nematode)	No reference
Ac-R	*Ancyclostoma ceylanicum*	A0A016WHR5	Ecdysozoa (Nematode)	No reference
Tc-TAKR	*Toxocara canis*	A0A0B2V4Q7	Ecdysozoa (Nematode)	No reference
Cg-sNPFR-like	*Crassostrea gigas*	MF320349	Lophotrochozoa (Mollusc)	L. Bigot, I. Beets, M.-P. Dubos, P. Boudry, L. Schoofs, P. Favrel, Functional characterization of a short neuropeptide F-related receptor in a lophotrochozoan, the mollusk *Crassostrea gigas*, J. Exp. Biol. 217 (2014) 2974–2982.

*** functionally characterized receptors.**



Supplementary Figure 1 : Fluorescent signal induced by Cragi-TKR expressed in HEK293T cells and challenged by Cragi-TKs at the concentration of 10^{-5}M in absence (Cragi-TKR) or presence (Cragi-TKR + $G_{\alpha 16}$) of the promiscuous protein $G_{\alpha 16}$. $G_{\alpha 16}$ expressed alone or cells transfected with an empty vector were used as negative controls. Vertical bars represent the standard error of the mean (SEM), number of replicates n=3.