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1 **Characterization of a tachykinin signalling system in the bivalve mollusc**

2 ***Crassostrea gigas*.**

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5

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11

12 **Abstract**

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

24

25 **Keywords:** Mollusc, neuropeptide, Tachykinin signalling, feeding

26

27 **Introduction**

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

37 Chordate TK sequences display the conserved C-terminal pentapeptide signature FXGLM-
38 amide, whereas protostome TKs share the C-terminal consensus sequence FX₁GX₂R-amide.
39 Interestingly, some vertebrate-type TKs, derived from a distinct gene, have been identified in
40 the salivary glands of cephalopod molluscs [9,10] and insects [11] serving respectively as
41 neurotoxins [12] and as vasodilatory agents that act on vertebrate prey TK receptors (TKR) but
42 not on endogenous receptors [13].

43 TKs are widely distributed in the nervous systems of all bilaterian animal species. They have
44 been shown to display regulatory roles in an extraordinarily diverse range of physiological
45 processes. In addition to their modulatory role in the central control of respiration and
46 cardiovascular activity, TKs, mainly via SP, also mediate pain, anxiety and motor coordination
47 in the CNS of mammals [14,15]. In arthropods, TKs are involved in odour perception and
48 locomotion as shown in *Drosophila* [16] and in visual processing as suggested in crustaceans
49 [17]. In bilateria, TKs have been shown to participate in the control of the activity of a wide
50 array of peripheral organs and tissues. *In vitro* studies on organ preparations of protostome

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

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

68

69 **Material and methods**

70 *Peptide synthesis*

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

74 *In silico analyses*

75 Multiple sequence alignment was performed with TKR from various species (supplementary
76 table 1) using Clustal W [27]. To determine the relationship between *Cragi*-TKR and TKRs
77 from other species (supplementary table 2), a phylogenetic tree was generated by the maximum
78 likelihood method using the phylogeny pipeline (www.phylogeny.fr) [28] connecting the
79 following programs: MUSCLE for multiple alignment (full processing mode), Gblocks for
80 alignment curation (minimum length of a block after gap cleaning: 10, no gap positions allowed
81 in the final alignment, all segments with contiguous non-conserved positions higher than 8
82 rejected, minimum number of sequences for a flank position: 85%), PhyML for phylogeny (the
83 default substitution model was chosen assuming an estimated proportion of invariant sites and
84 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma
85 shape parameter was estimated directly from the data Model). The reliability of internal
86 branches was evaluated using an approximate likelihood-ratio test (aLRT). TreeDyn was used
87 for tree drawing.

88 *Reverse endocrinology*

89 Molecular cloning of the *Cragi*-TKR and transfection of mammalian cells:

90 *In silico* screening of the oyster transcriptomic database “GigaTon” [26] resulted in the
91 identification of a full length cDNA encoding *Cragi*-TKR (CHOYP_LOC100744404.1.1). The
92 CDS of the *Cragi*-TKR gene was amplified by PCR (Pfu DNA polymerase, Promega) using
93 gene-specific sense primer (5’-CACCATGGAGGGGAACAATTCAACAAAAG-3’)
94 harbouring a Kozak consensus sequence and antisense primer (5’-
95 TCATAAATATTCAGCACTAGTTCTCCGCCC-3’). Ten nanogram of plasmid DNA (Pal
96 17.3 vector, Evrogen) from a *C. gigas* “all developmental stages and adult central nervous
97 system” directional and normalized cDNA library [29] was used as template. The resulting PCR
98 product was directionally cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen)
99 and the correct insertion confirmed by sequencing. Human embryonic kidney (HEK293T) cells

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

108 Calcium fluorescence assay:

109 Activation of *Cragi-TKR* by oyster TK synthetic peptides was monitored using a fluorescence-
110 based calcium mobilization assay. Briefly, transfected HEK293T cells were loaded with Fluo-
111 4 Direct plus probenecid (qsp 2.5mM final in the cell) (Invitrogen / Molecular Probes) for 1
112 hour (45min at 37°C and 15min at room temperature). Excitation of the fluorophore was done
113 at 488 nm. The calcium response was measured for 2 min at 525 nm using the FLEXstation 3
114 (Molecular Devices) at 37°C. Data were analysed using SoftMax Pro (Molecular Devices).
115 Candidate peptide ligands were first tested at a final concentration of 10^{-5} M. Concentration-
116 response measurements of activating ligands were conducted in triplicate and for at least three
117 independent experiments. Half maximal effective concentrations (EC_{50} values) were calculated
118 from concentration-response curves that were constructed using nonlinear regression analysis
119 with a sigmoidal dose-response equation using Prism 5.0 (GraphPad software, USA).

120

121 cAMP luminescence assay.

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

124 candidate ligands. cAMP luminescence response was measured for 30 min after injection using
125 a FLEX station 3 (Molecular Devices) at room temperature. Data were analysed using SoftMax
126 Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of
127 10^{-5} M.

128 *Animals and tissue sampling*

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

143 *Reverse transcription quantitative PCR (RT-qPCR)*

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

148 DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random
149 hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200 U MMuLV Reverse Transcriptase
150 (Promega) at 37°C for 1 h in the appropriate buffer. The reaction was stopped by incubation at
151 70°C for 10 min. The GoTaq® qPCR Master Mix (Promega) was used for real time monitoring
152 of amplification (5 ng of cDNA template, 40 cycles: 95°C/15 s, 60°C/15 s) with the following
153 primers: Qs-Cragi-TKR (5'-ATGGCCCACAAGCGGATG-3') and Qa-Cragi-TKR (5'-
154 GGTGGACACAAACGCCGT-3') as sense (Qs) and antisense (Qa) primers for Cragi-TKR
155 cDNA and Qs-Cragi-TK (5'-GCATACCAGAATCATCAA-3') and Qa-Cragi-TK (5'-
156 GTTTATTGTTCCGAACTAAT -3') for Cragi-TK precursor cDNA. Accurate amplification of
157 the target amplicon was checked by performing a melting curve analysis. Using Qs-Cg-EF (5'-
158 ACCACCCTGGTGAGATCAAG-3') and Qa-Cg-EF (5'-ACGACGATCGCATTCTCTT-3')
159 primers, a parallel amplification of oyster Elongation Factor 1α (EF1 α) transcript
160 (BAD15289) was carried out to normalize the expression data of *Cragi-TKR* and *Cragi-TK*
161 transcripts. EF1 α was found as a reliable normalization gene as no significant difference
162 (p<0.05) of Ct values was observed between the different samples compared. Coefficient of
163 variation of EF1 α was less than 5%. Thus, the relative level of each gene expression was
164 calculated for one copy of the EF1 α reference gene by using the following formula: $N = 2^{(Ct_{EF1\alpha} - Ct_{Cg-cDNA})}$
165 The PCR amplification efficiency (E; $E = 10^{(-1/slope)}$) for each primer pair was
166 determined by linear regression analysis of a dilution series to ensure that E ranged from 1.98
167 to 2.02. The specificity of the primer pairs was confirmed by melting curve analysis at the end
168 of each RT-qPCR run.

169 *Statistical analysis*

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

173 Significance was set at $p < 0.05$.

174

175 **Results:**

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

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

190 *Oyster TKs specifically activate Cragi-TKR.*

191 A calcium mobilization assay was used to identify the cognate ligands of Cragi-TKR [33].
192 Transiently transfected HEK293T cells expressing the oyster receptor and the promiscuous G
193 protein $G_{\alpha 16}$ were challenged with the three oyster synthetic TKs (Cragi-TK1: FGFAPMR-
194 amide, Cragi-TK2: ARFFGLR-amide and Cragi-TK3: FRFTALR-amide). These TKs are
195 derived from the Cragi-TK neuropeptide precursor by posttranslational processing (Fig.3A) and
196 have previously been characterized as part of *C. gigas*' repertoire of neuropeptides [25]. Since
197 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 [Arg⁷] and [Phe³] alanine-substituted peptides, a second group comprising
208 [Arg²] and [Phe⁴] alanine-substituted peptides for which the modification only moderately
209 affected the potency and a third group including the [Gly⁵] and [Leu⁶] 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

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

229 **Discussion.**

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

245 In all lophotrochozoan species investigated so far, only one specific TKR has been identified
246 [13,23]. The occurrence of a natalisin type of receptor is unlikely in *C. gigas* since no
247 homologous neuropeptide has been found among the exhaustive neuropeptide repertoires of

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

251 Cragi-TKR behaves as a genuine TKR. Similar to its vertebrate and protostome counterparts, it
252 is specifically and slightly selectively activated by all three oyster TKs encoded by the oyster
253 TK precursor at concentration ranges similar to those required for the activation of TKRs in
254 other species [23,37,42]. Similar to the *Octopus* TKR [13], Cragi-TKR triggers *in vitro* only
255 the phospholipase C β -mediated calcium transduction pathway, a feature distinct to some insect
256 and vertebrate receptors which additionally transduce their signal via an increase in cAMP
257 levels [36,43–45]. Although distinct in sequence, Cragi-TKs exhibit only minor potency
258 differences suggesting that they may be functionally redundant. To determine the essential
259 amino acids of Cragi-TKs, a structure activity relationship analysis was performed using a
260 series of synthetic analogues of Cragi-TK2 (ARFFGLR-amide) in which each amino acid was
261 sequentially replaced by the neutral alanine residue. Considering the C-terminal consensus
262 FX₁GX₂Ramide sequence of protostome TKs, only the replacement of the terminal Arginine or
263 the first Phenylalanine showed drastic negative effects consistent with the high conservation of
264 these two residues possibly due to a strong selective pressure during the evolution of protostome
265 TKs. This also reflects the low activity reported for chordate-type TKs (harbouring a C-terminal
266 methionine instead of an arginine) on protostome receptors [13,23,46]. Change of the N-
267 terminal extension of Cragi-TK2 did not alter significantly the neuropeptide activity. As
268 expected, change of the flexible residue (X₁) of the consensus sequence resulted in only limited
269 effects on the activation of Cragi-TKR. Surprisingly, Cragi-TKR showed higher sensitivity to
270 peptide analogues with an alanine replacing either the conserved Glycine or the penultimate
271 (X₂) residue. The glycine residue does not appear to be crucial since the three naturally
272 occurring oyster TKs display a variability of residues at this position. Interestingly both Cragi-

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

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

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

318 **Conclusion**

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

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

327

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335

336 **Figure legends:**

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

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

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

347

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

365

366

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

373

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

380

381 **Figure 5: Expression of Cragi-TKR and Cragi-TK genes.** (A) Distribution of mRNAs
382 encoding Cragi-TKR in adult tissues, (B) level of expression of Cragi-TKR mRNA in visceral
383 ganglia (VG) and gonads (GO) during gametogenesis, (C) Distribution of mRNAs encoding
384 Cragi-TK precursor in adult tissues, (D) level of expression of Cragi-TK mRNA in visceral
385 ganglia (VG) along an annual reproductive cycle, (E and F) expression levels of Cragi-TKR
386 and Cragi-TK mRNA respectively in VG of four weeks *Isochysis galbana* fed or starved oysters,
387 Each value is the mean + SEM of 5 pools of 6 animals (in adult tissues); 5 pools of 6 animals
388 (VG during gametogenesis) and 19 or 17 independent animals (VG after conditioning with or
389 without food). Expression levels were calculated as the number of copies of Cragi-TKR / Cragi-
390 TK transcripts per 10^3 copies of elongation factor 1α (EF1 α) mRNA. Results were statistically
391 tested with a one-way ANOVA (A, B, D and E) or student's t test (C and F), $p < 0,05$.
392 Significantly different means are indicated by different letters (A, B and D) or *** ($p < 0.001$)
393 (B). No significant statistical difference was observed for (C and E). M: Mantle; G: Gills; LP:
394 Labial Palps; DG: Digestive Gland; Go Gonad; H: heart; AM: Adductor Muscle; VG: Visceral
395 Ganglia; F: Female; M: Male; 0: stage 0 (sexual resting stage); 1: stage 1 (gonial multiplication
396 stage); 2: stage 2 (tubule development and maturation stage); 3: stage 3 (sexual maturity stage)

397

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

401

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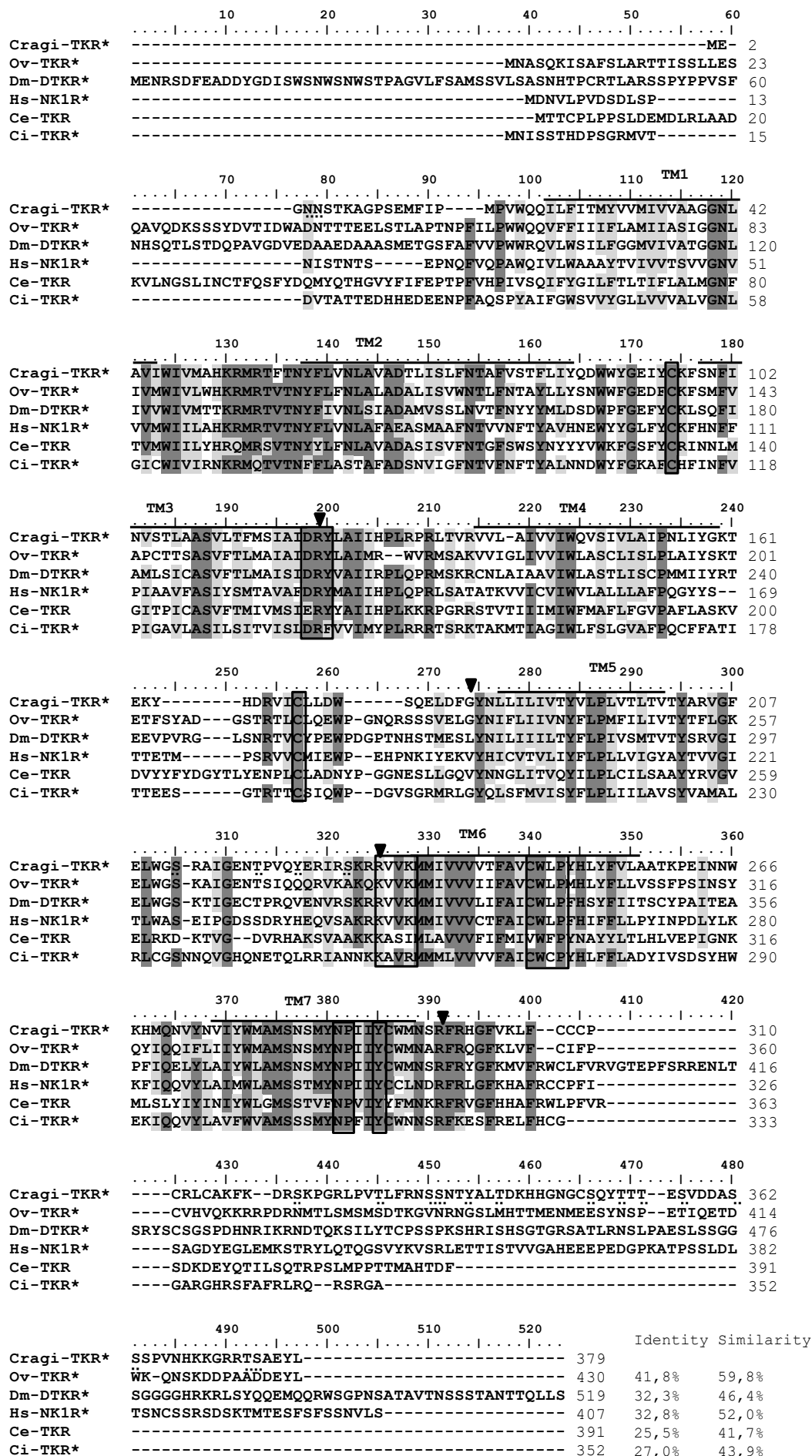


Figure 1

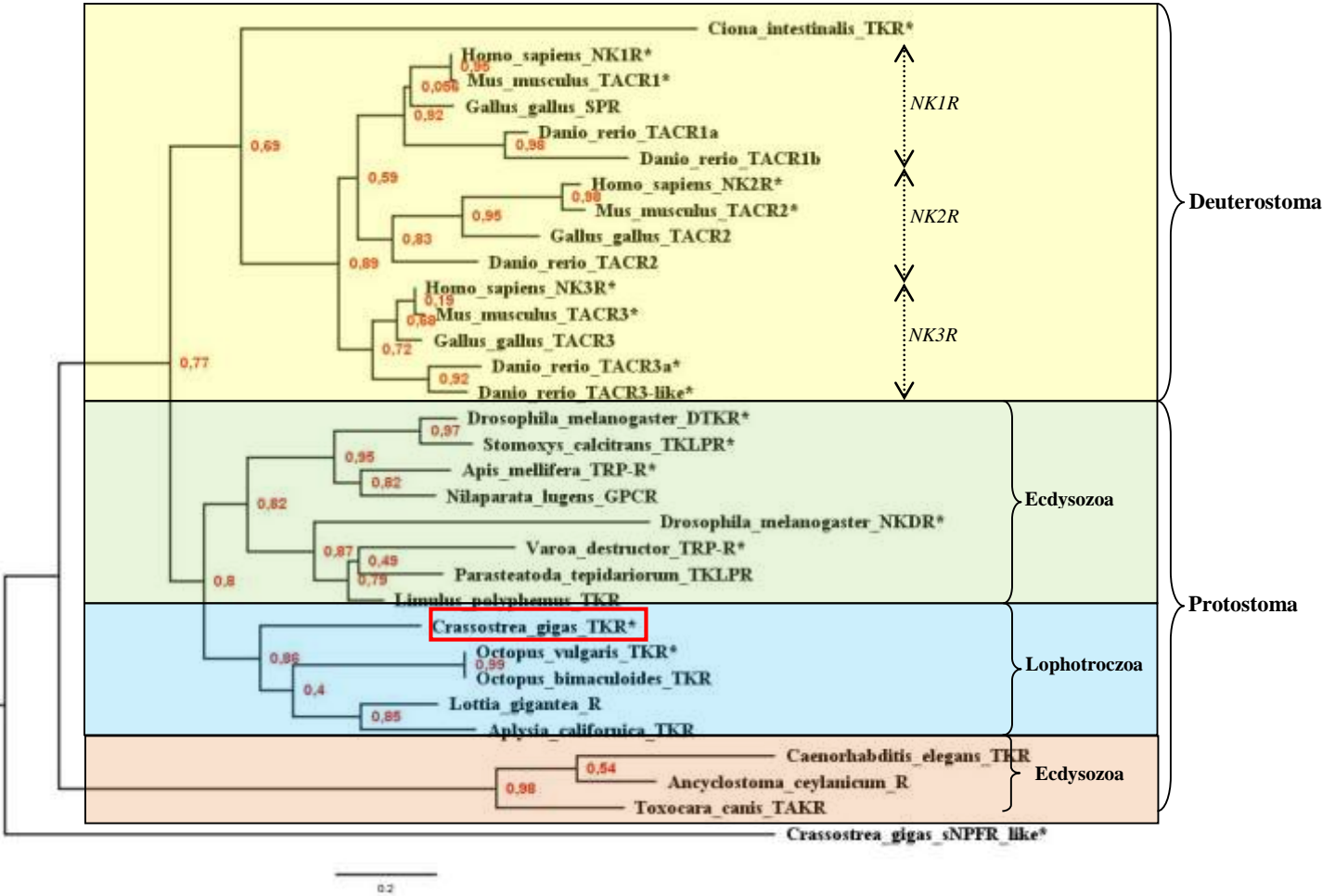


Figure. 2

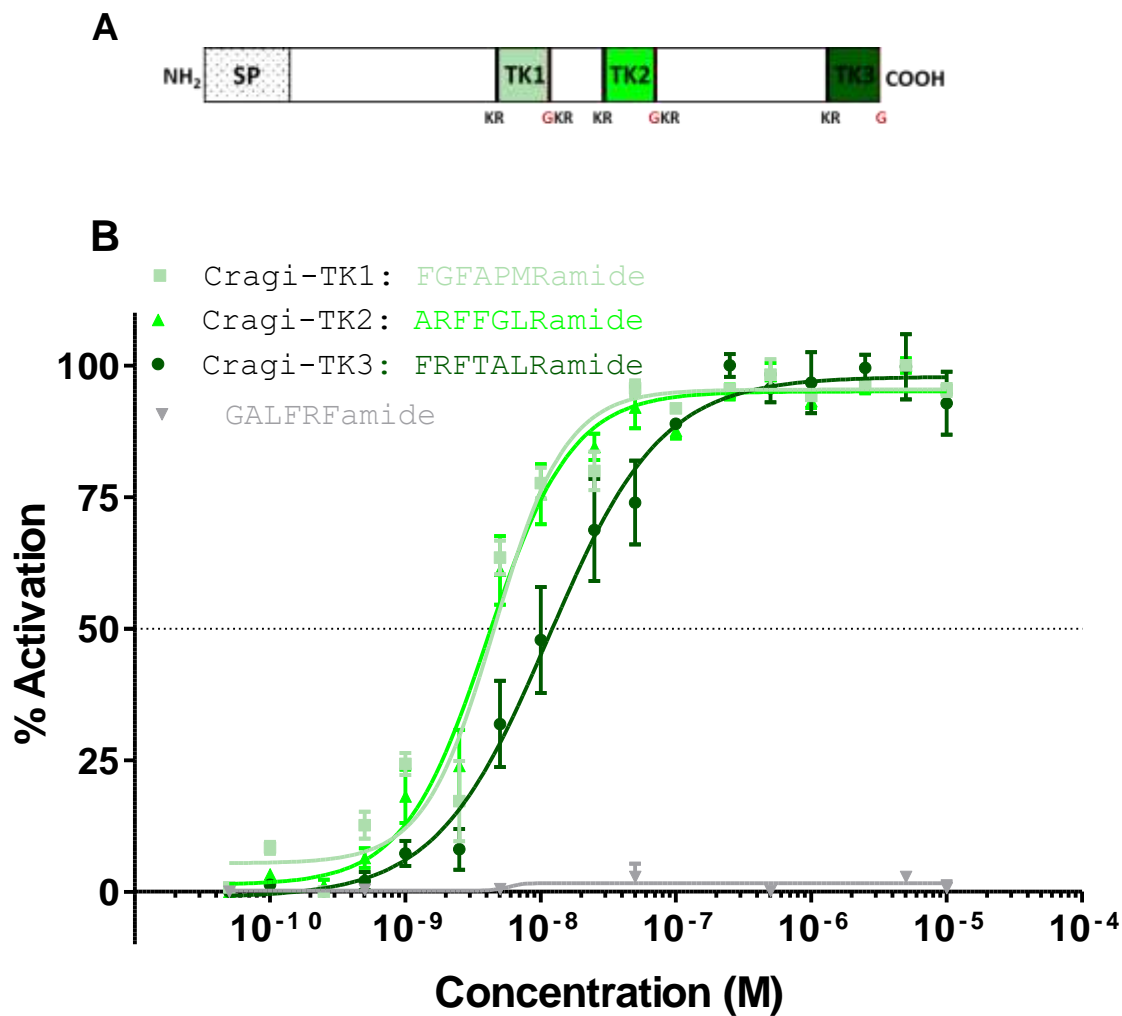


Figure 3

Table 1.

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

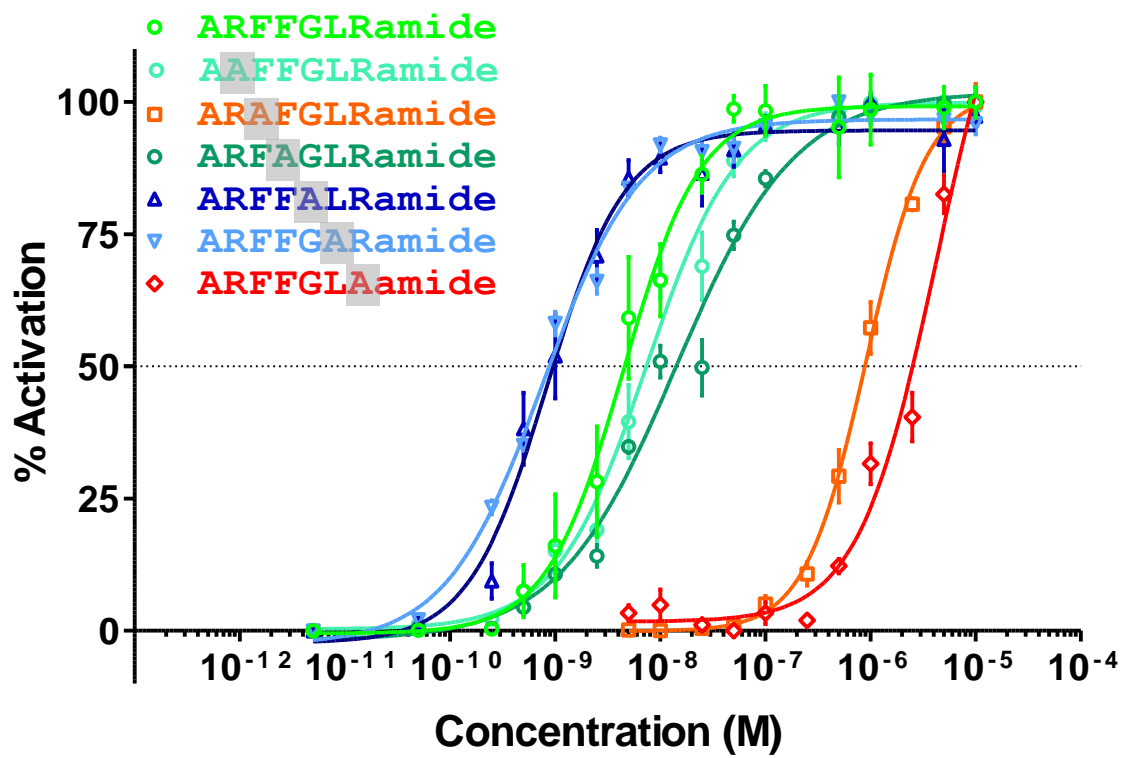
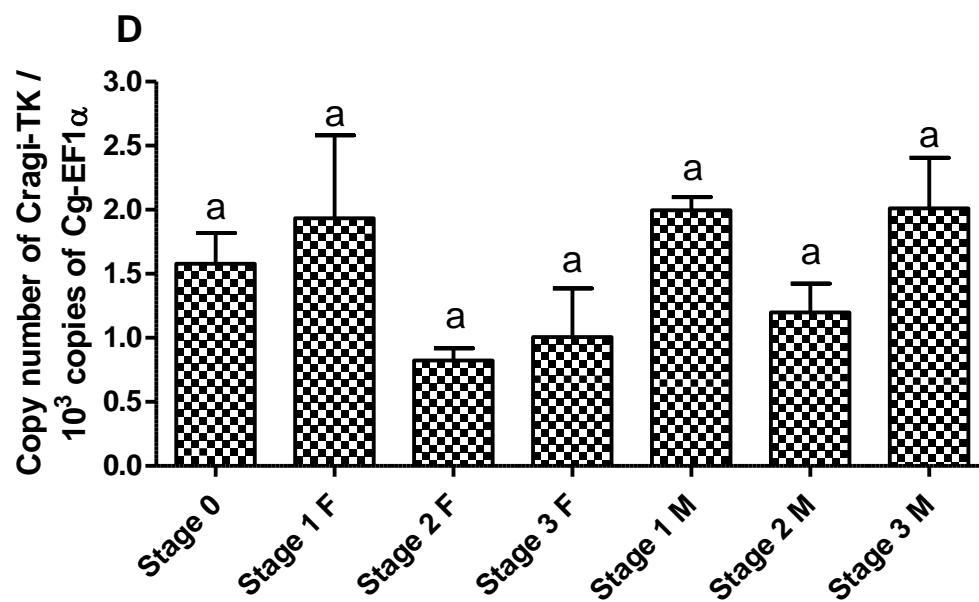
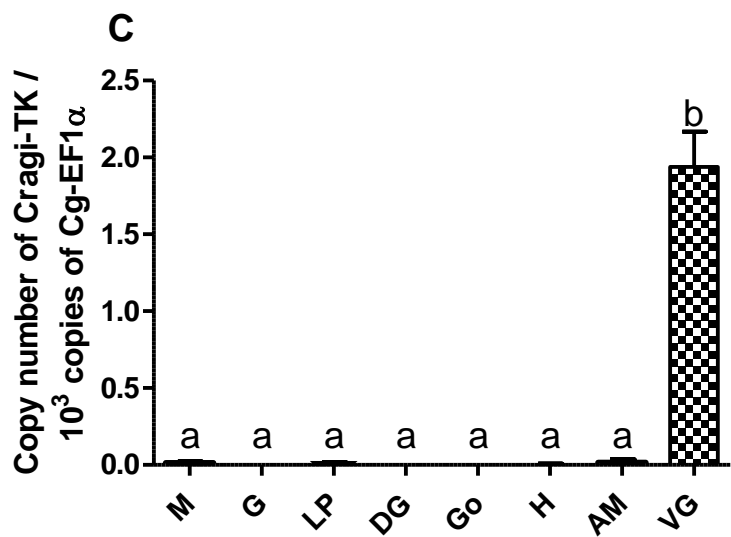
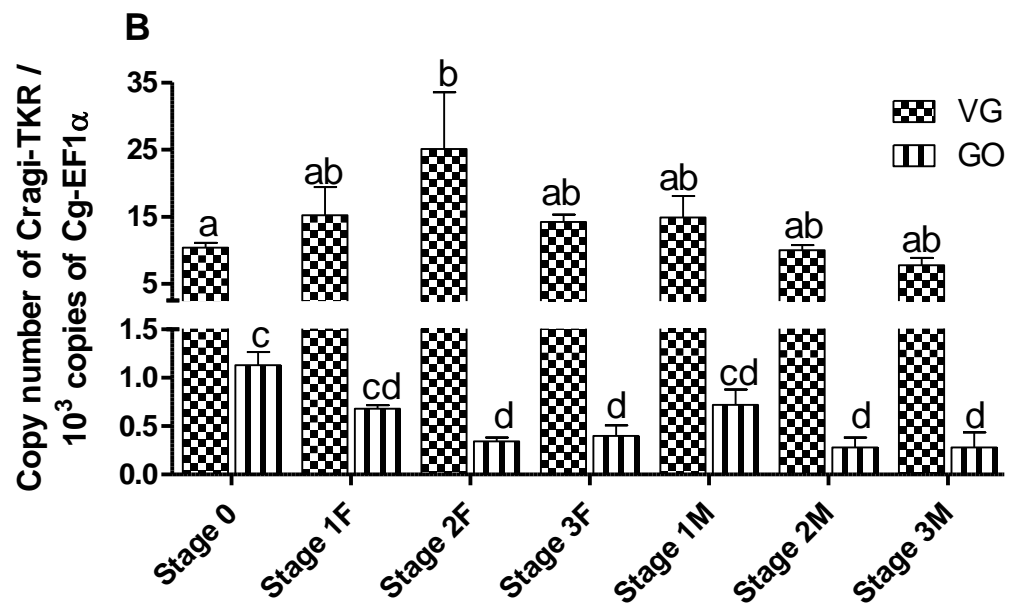
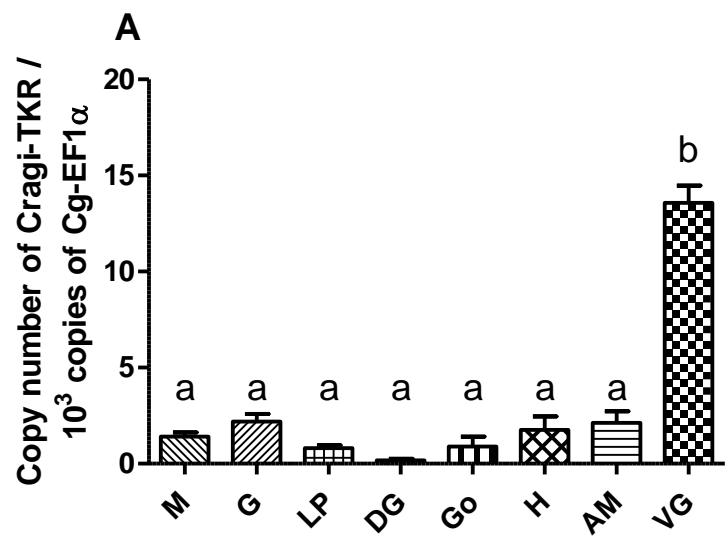


Figure 4.



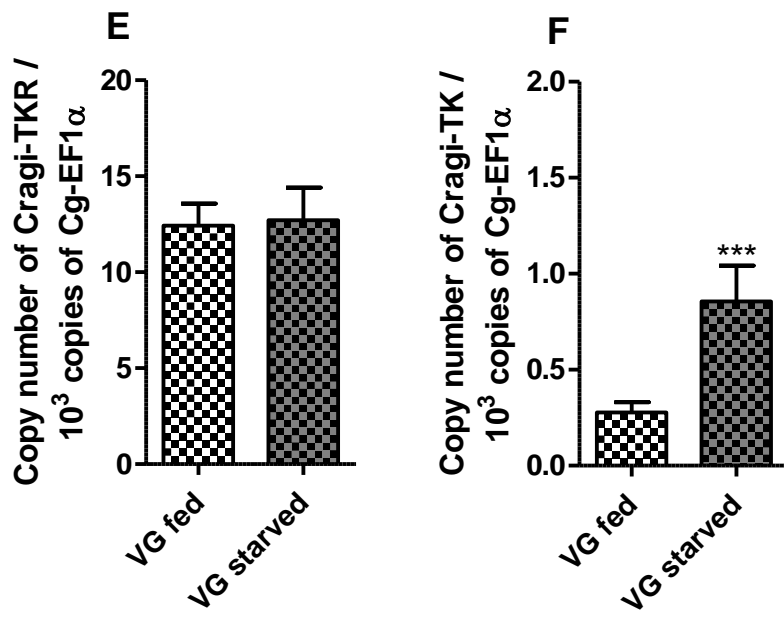


Figure 5

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*	<i>Crassostrea gigas</i>	MF320350	Lophotrochozoa (Mollusc)	Present paper
Ov-TKR*	<i>Octopus vulgaris</i>	Q58A49	Lophotrochozoa (Mollusc)	A. Kanda, K. Takuwa-Kuroda, M. Aoyama, H. Satake, A novel tachykinin-related peptide receptor of <i>Octopus vulgaris</i> - Evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide, FEBS J. 274 (2007) 2229–2239.
Dm-DTKR*	<i>Drosophila melanogaster</i>	P30975	Ecdysozoa (Arthropod)	X.J. Li, W. Wolfgang, Y.N. Wu, R.A. North, M. Forte, Cloning, heterologous expression and developmental regulation of a <i>Drosophila</i> receptor for tachykinin-like peptides., EMBO J. 10 (1991) 3221–9.
Ci-TKR*	<i>Ciona intestinalis</i>	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 , <i>Ciona intestinalis</i> , J. Biol. Chem. 279 (2004) 53798–53805.
Hs-NK1R*	<i>Homo sapiens</i>	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	<i>Caenorhabditis elegans</i>	O44148	Ecdysozoa (Nematode)	No reference

* functionally characterized receptors.

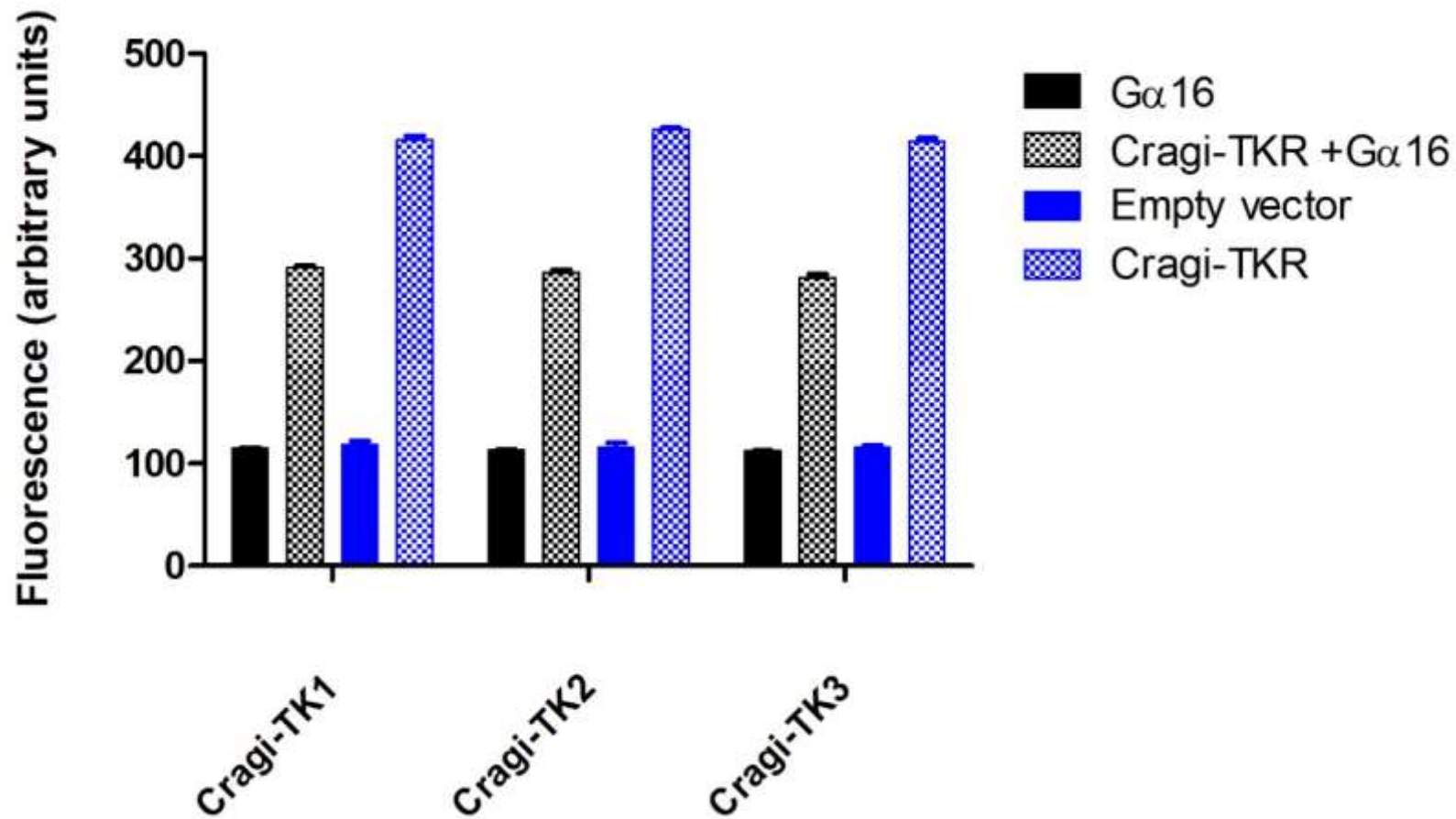
Supplementary table 2: Accession numbers and references of the TKR sequences used for the phylogenetic tree in Figure2

Abbreviation	Species name	Accession number	Phylum	reference
Hs-NK1R*	<i>Homo sapiens</i>	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., <i>Biochemistry</i> . 30 (1991) 10640–6.
Mm-TACR1*	<i>Mus musculus</i>	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., <i>Eur. J. Biochem.</i> 203 (1992) 625–31.
Gg-SPR	<i>Gallus galus</i>	Q9W6I3	Vertebrate	No reference
Dr-TACR1a	<i>Danio rerio</i>	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, <i>J. Mol. Endocrinol.</i> 50 (2013) 115–129.
Dr-TACR1b	<i>Danio rerio</i>	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, <i>J. Mol. Endocrinol.</i> 50 (2013) 115–129.
Hs-NK2R*	<i>Homo sapiens</i>	P21452.3	Vertebrate	S. Arkininstall, I. Emergey, 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., <i>FEBS Lett.</i> 338 (1994) 75–80.
Mm-TACR2*	<i>Mus musculus</i>	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., <i>Eur. J. Biochem.</i> 203 (1992) 625–31.
Gg-TACR2	<i>Gallus galus</i>	E1BRR8	Vertebrate	No reference
Dr-TACR2	<i>Danio rerio</i>	F1QPL8	Vertebrate	No reference
Hs-NK3R*	<i>Homo sapiens</i>	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., <i>Biochem. Biophys. Res. Commun.</i> 179 (1991) 1232–40.

Mm-TACR3*	<i>Mus musculus</i>	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	<i>Gallus galus</i>	F1NJ82	Vertebrate	No reference
Dr-TACR3a	<i>Danio rerio</i>	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	<i>Danio rerio</i>	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 (<i>Danio rerio</i>), Mol. Cell. Endocrinol. 361 (2012) 202–212.
Dm-DTKR*	<i>Drosophila melanogaster</i>	P30975	Ecdysozoa (Arthropod)	X.J. Li, W. Wolfgang, Y.N. Wu, R.A. North, M. Forte, Cloning, heterologous expression and developmental regulation of a <i>Drosophila</i> receptor for tachykinin-like peptides., EMBO J. 10 (1991) 3221–9.
Sc-TKLPR*	<i>Stomoxys calcitrans</i>	AOA1I8PID0	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 <i>Drosophila</i> Schneider 2 cell line, J. Neurochem. 74 (2000) 2182–2189.
Am-TRP-R	<i>Apis mellifera</i>	AOA141CIU0	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 <i>Varroa destructor</i> , Sci. Rep. 6 (2016) 19547.
NI-GPCR	<i>Nilaparvata-lugens</i>	U3U967	Ecdysozoa (Arthropod)	No reference
Dm-NKDR*	<i>Drosophila melanogaster</i>	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 <i>Drosophila</i> tachykinin-related peptide 6, Peptides. 30 (2009) 545–556.

Vd-TRP-R*	<i>Varroa destructor</i>	AOA141CIT9	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 <i>Varroa destructor</i> , Sci. Rep. 6 (2016) 19547.
Pt-TKLPR	<i>Parasteatoda tepidariorum</i>	XP_015910841.1	Ecdysozoa (Arthropod)	No reference
Lp-TKR	<i>Limulus polyphemus</i>	XP_013772923.1	Ecdysozoa (Arthropod)	No reference
Cragi-TKR*	<i>Crassostrea gigas</i>	MF320350	Lophotrochozoa (Mollusc)	Present paper
Ov-TKR*	<i>Octopus vulgaris</i>	Q58A49	Lophotrochozoa (Mollusc)	A. Kanda, K. Takuwa-Kuroda, M. Aoyama, H. Satake, A novel tachykinin-related peptide receptor of <i>Octopus vulgaris</i> - Evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide, FEBS J. 274 (2007) 2229–2239.
Ob-TKR	<i>Octopus bimaculoides</i>	XM_014930159.1	Lophotrochozoa (Mollusc)	No reference
Lg-R	<i>Lottia gigantea</i>	V4BE54	Lophotrochozoa (Mollusc)	No reference
Ac-TKR	<i>Aplysia californica</i>	XP_012936179.1	Lophotrochozoa (Mollusc)	No reference
Ce-TKRF	<i>Caenorhabditis elegans</i>	O44148	Ecdysozoa (Nematode)	No reference
Ac-R	<i>Ancylostoma ceylanicum</i>	AOA016WHR5	Ecdysozoa (Nematode)	No reference
Tc-TAKR	<i>Toxocara canis</i>	AOA0B2V4Q7	Ecdysozoa (Nematode)	No reference
Cg-sNPFR-like	<i>Crassostrea gigas</i>	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 <i>Crassostrea gigas</i> , 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 α ₁₆) of the promiscuous protein G α ₁₆. G α 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.