

CCK-type signalling in an echinoderm

1 Evolutionarily ancient role of cholecystokinin-type neuropeptide signalling as an 2 inhibitory regulator of feeding-related processes revealed in an echinoderm

3

4 Ana B. Tinoco* ¹, Antón Barreiro-Iglesias* ^{1,3}, Luis Alfonso Yañez-Guerra* ^{1,4}, Jérôme
5 Delroisse ^{1,5}, Ya Zhang ¹, Elizabeth F. Gunner ¹, Cleidiane Zampronio ², Alexandra M.
6 Jones², Michaela Egertová ¹, Maurice R. Elphick ¹⁺

7

8 1. Queen Mary University of London, School of Biological & Chemical Sciences, Mile End
9 Road, London, E1 4NS, UK

10 2. School of Life Sciences and Proteomics, Research Technology Platform, University of
11 Warwick, Coventry, UK

12

13 **Present addresses:**

14 3. Department of Functional Biology, CIBUS, Faculty of Biology, Universidade de Santiago
15 de Compostela, 15782, Santiago de Compostela, Spain

16 4. Living Systems Institute, University of Exeter, Exeter EX4 4QD, UK

17 5. University of Mons, Biology of Marine Organisms and Biomimetics Unit, 7000 Mons,
18 Belgium

19

20 * these authors contributed equally

21 + Corresponding author: Prof. M.R. Elphick, Queen Mary University of London, School of
22 Biological & Chemical Sciences, Mile End Road, London, E1 4NS, UK

23 m.r.elphick@qmul.ac.uk

24

25 **E-mail addresses:**

26 Ana. B. Tinoco: a.b.tinoco@qmul.ac.uk

27 Antón Barreiro-Iglesias: anton.barreiro@usc.es

28 Luis Alfonso Yañez-Guerra: L.Yanez-Guerra@exeter.ac.uk

29 Jérôme Delroisse: Jerome.delroisse@umons.ac.be

30 Ya Zhang: yazhang94@gmail.com

31 Elizabeth F. Gunner: elizabethis@sky.com

32 Cleidiane Zampronio: c.g.zampronio@warwick.ac.uk

33 Michaela Egertová: m.egertova@qmul.ac.uk

34 Alexandra M. Jones: alex.jones@warwick.ac.uk

CCK-type signalling in an echinoderm

35 **Abstract**

36 Cholecystokinin (CCK) / sulfakinin (SK)-type neuropeptides regulate feeding and
37 digestion in chordates and protostomes (e.g. insects). Here we characterised CCK/SK-type
38 signalling for the first time in a non-chordate deuterostome - the starfish *Asterias rubens*
39 (phylum Echinodermata). In this species, two neuropeptides (ArCCK1, ArCCK2) derived from
40 the precursor protein ArCCKP act as ligands for a CCK/SK-type receptor (ArCCKR) and are
41 expressed in the nervous system, digestive system, tube feet and body wall. Furthermore,
42 ArCCK1 and ArCCK2 cause dose-dependent contraction of cardiac stomach, tube foot and
43 body wall apical muscle preparations *in vitro* and injection of these neuropeptides *in vivo*
44 triggers cardiac stomach retraction and inhibition of the onset of feeding in *A. rubens*. Thus, an
45 evolutionarily ancient role of CCK/SK-type neuropeptides as inhibitory regulators of feeding-
46 related processes in the Bilateria has been conserved in the unusual and unique context of the
47 extra-oral feeding behaviour and pentaradial body plan of an echinoderm.

CCK-type signalling in an echinoderm

48 **Introduction**

49 The peptide hormones cholecystokinin (CCK) and gastrin were discovered and named
50 on account of their effects as stimulators of gall bladder contraction and gastric secretion of
51 pepsin/acid, respectively, in mammals (Edkins 1906, Ivy 1929). Determination of the
52 structures of CCK and gastrin revealed that they have the same C-terminal structural motif
53 (Trp-Met-Asp-Phe-NH₂), indicative of a common evolutionary origin (Gregory et al. 1964,
54 Mutt and Jorpes 1968). However, CCK and gastrin are derived from different precursor
55 proteins, which are subject to cell/tissue-specific processing to give rise to bioactive peptides
56 of varying length (e.g. CCK-8 and CCK-33; gastrin-17 and gastrin-34) (Boel et al. 1983,
57 Deschenes et al. 1984, Rehfeld et al. 2007). Furthermore, CCK and gastrin have a tyrosine
58 residue at positions seven and six from the C-terminal amide, respectively, which can be
59 sulphated post-translationally (Rehfeld et al. 2007). The effects of CCK and gastrin in
60 mammals are mediated by two G-protein coupled receptors (GPCRs), CCK-A (CCKR1) and
61 CCK-B (CCKR2), with both sulphated and non-sulphated forms of CCK and gastrin acting as
62 ligands for the CCK-B receptor, whilst the CCK-A receptor is selectively activated by
63 sulphated CCK (Deweert et al. 1993, Dufresne et al. 2006, Kopin et al. 1992, Lee et al. 1993,
64 Noble and Roques 1999, Wank et al. 1992). Mediated by these receptors, gastrin and CCK
65 have a variety of physiological/behavioural effects in mammals. Thus, in the gastrointestinal
66 system gastrin stimulates growth of the stomach lining, gastric contractions and gastric
67 emptying (Crean et al. 1969, Dockray et al. 2005, Gregory and Tracy 1964, Vizi et al. 1973),
68 whilst CCK stimulates pancreatic enzyme secretion, contraction of the pyloric sphincter and
69 intestinal motility (Chen et al. 2004, Gutiérrez et al. 1974, Harper and Raper 1943, Rehfeld
70 2017, Shaw and Jones 1978, Vizi et al. 1973). Furthermore, CCK also has behavioural effects
71 that include inhibition of food intake as a mediator of satiety and stimulation of aggression and
72 anxiogenesis (Chandra and Liddle 2007, Gibbs et al. 1973, Singh et al. 1991, Smith et al. 1981).

73 Phylogenomic studies indicate that genome duplication in a common ancestor of the
74 vertebrates gave rise to genes encoding CCK-type and gastrin-type precursor proteins (Dupre
75 and Tostivint 2014). Accordingly, invertebrate chordates that are the closest extant relatives of
76 vertebrates (e.g. the urochordate *Ciona intestinalis*) have a single gene encoding a “hybrid”
77 CCK/gastrin-like peptide (e.g. cionin) with a sulphated tyrosine residue at both positions six
78 and seven from the C-terminal amide (Johnsen and Rehfeld 1990, Monstein et al. 1993,
79 Thorndyke and Dockray 1986). Furthermore, CCK-type peptides stimulate gastric enzyme
80 secretion in the sea-squirt *Styela clava*, providing evidence of evolutionarily ancient roles as

CCK-type signalling in an echinoderm

81 regulators of gastrointestinal physiology in chordates (Bevis and Thorndyke 1981, Thorndyke
82 and Bevis 1984).

83 Evidence that the phylogenetic distribution of CCK/gastrin-type peptides may extend
84 beyond chordates to other phyla was first obtained with the detection of substances
85 immunoreactive with antibodies to CCK and/or gastrin in a variety of invertebrates, including
86 arthropods, annelids, molluscs and cnidarians (Dockray et al. 1981, El-Salhy et al. 1980,
87 Grimmelikhuijzen et al. 1980, Kramer et al. 1977, Larson and Vigna 1983, Rzasa et al. 1982).
88 However, molecular evidence of the evolutionary antiquity of CCK/gastrin-type signalling was
89 obtained with the purification and sequencing of a CCK-like peptide named leucosulfakinin,
90 which was isolated from the insect (cockroach) *Leucophaea maderae* (Nachman et al. 1986a).
91 Subsequently, GPCRs that are homologs of the vertebrate CCKA/CCKB-type receptors have
92 been identified and pharmacologically characterised as receptors for sulfakinin (SK)-type
93 peptides in a variety of insects, including *Drosophila melanogaster* (Bloom et al. 2019, Kubiak
94 et al. 2002, Yu et al. 2013b, Yu and Smaghe 2014b). Furthermore, investigation of the
95 physiological roles of SK-type signalling in insects has revealed similarities with findings from
96 vertebrates. Thus, in several insect species SK-type peptides have myotropic effects on the gut
97 (Al-Alkawi et al. 2017, Marciniak et al. 2011, Nachman et al. 1986a, Nachman et al. 1986b,
98 Nichols 2007, Palmer et al. 2007, Predel et al. 2001, Schoofs et al. 1990) and/or affect digestive
99 enzyme release (Harshini et al. 2002a, b, Nachman et al. 1997, Zels et al. 2015). Furthermore,
100 at a behavioural level there is evidence that SK-type peptides act as satiety factors (Al-Alkawi
101 et al. 2017, Bloom et al. 2019, Downer et al. 2007, Maestro et al. 2001, Meyering-Vos and
102 Muller 2007, Nässel and Zandawala 2019, Nichols et al. 2008, Wei et al. 2000, Yu et al. 2013a,
103 Yu et al. 2013b, Yu and Smaghe 2014b, Zels et al. 2015) and regulate locomotion and
104 aggression in insects (Chen et al. 2012, Nässel and Williams 2014, Nässel and Zandawala 2019,
105 Nichols et al. 2008).

106 The discovery and functional characterisation of SK-type signalling in insects and other
107 arthropods indicated that the evolutionary origin CCK/SK-type signalling can be traced back
108 to the common ancestor of the Bilateria. Consistent with this hypothesis, CCK/SK-type
109 signalling systems have been discovered in a variety of protostome invertebrates, including the
110 nematode *Caenorhabditis elegans*, the mollusc *Crassostrea gigas* and the annelid *Capitella*
111 *teleta* (Janssen et al. 2008, Mirabeau and Joly 2013, Schwartz et al. 2018). Furthermore, some
112 insights into the physiological roles of CCK/SK-type signalling in non-arthropod protostomes
113 have been obtained, including causing a decrease in the frequency of spontaneous contractions
114 of the *C. gigas* hindgut (Schwartz et al. 2018), stimulation of digestive enzyme secretion in *C.*

CCK-type signalling in an echinoderm

115 *elegans* and *Pecten maximus* (Janssen et al. 2008, Nachman et al. 1997) and evidence of a role
116 in regulation of feeding and energy storage in *C. gigas* (Schwartz et al. 2018).

117 Little is known about CCK-type signalling in the Ambulacraria (echinoderms and
118 hemichordates) - deuterostome invertebrates that occupy an ‘intermediate’ phylogenetic
119 position with respect to chordates and protostomes (Furlong and Holland 2002, Telford et al.
120 2015). Prior to the genome sequencing era, use of immunohistochemical methods revealed
121 CCK-like immunoreactive cells in the intestine of sea cucumbers (Phylum Echinodermata) and
122 vertebrate CCK/gastrin-type peptides were found to cause relaxation of sea cucumber intestine
123 (García-Arrarás et al. 1991). More recently, analysis of transcriptome/genome sequence data
124 has enabled identification of transcripts/genes encoding CCK-type peptide precursors and
125 CCK-type receptors in echinoderms and hemichordates (Burke et al. 2006, Chen et al. 2019,
126 Jekely 2013, Mirabeau and Joly 2013, Semmens et al. 2016, Zandawala et al. 2017). However,
127 functional characterisation of native CCK-type peptides and receptors has yet to be reported
128 for an echinoderm or hemichordate species. We have established the common European
129 starfish *Asterias rubens* as an experimental model for molecular and functional characterisation
130 of neuropeptides, obtaining novel insights into the evolution and comparative physiology of
131 several neuropeptide signalling systems (Cai et al. 2018, Elphick et al. 2018, Lin et al. 2017a,
132 Lin et al. 2018, Odekunle et al. 2019, Semmens and Elphick 2017, Tian et al. 2017, Tian et al.
133 2016, Tinoco et al. 2018, Yáñez-Guerra et al. 2018, Yáñez-Guerra et al. 2020, Zhang et al.
134 2020). Accordingly, here we used *A. rubens* to enable the first detailed molecular, anatomical
135 and pharmacological analysis of CCK-type signalling in an echinoderm.

136

CCK-type signalling in an echinoderm

137 **Results**

138

139 **Cloning and sequencing of a cDNA encoding ArCCKP**

140 Analysis of *A. rubens* neural transcriptome sequence data has revealed the presence of
141 a CCK-type precursor in *A. rubens*, which was named ArCCKP (Semmens et al. 2016). Here,
142 cloning and sequencing of a cDNA encoding ArCCKP confirmed the sequence obtained from
143 transcriptome data (**Figure 1 – figure supplement 1**).

144

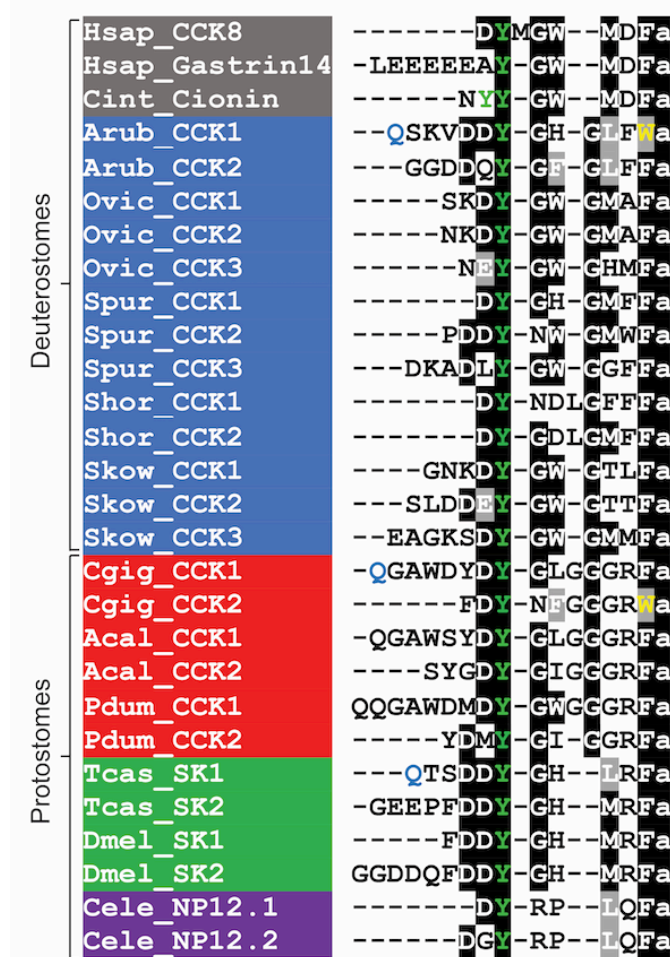
145 **Identification of ArCCKP-derived neuropeptides in extracts of *A. rubens* radial nerve** 146 **cords**

147 ArCCKP comprises two putative CCK-like neuropeptide sequences that are bounded
148 by dibasic or tetrabasic cleavage sites. Both neuropeptide sequences have a C-terminal glycine
149 residue, which is a potential substrate for post-translational amidation, and both neuropeptide
150 sequences contain a tyrosine residue, which could be either sulphated or non-sulphated (ns) in
151 the mature neuropeptides. Furthermore, an N-terminal glutamine residue (Q) in one of the
152 neuropeptide sequences is a potential substrate for N-terminal pyroglutamylation (pQ) (**Figure**
153 **1 – figure supplement 1; Figure 1 - figure supplement 2a**).

154 LC-MS-MS analysis of *A. rubens* radial nerve cord extracts revealed the presence of
155 four CCK-type peptides derived from ArCCKP: pQSKVDDY(SO₃H)GHGLFW-NH₂
156 (ArCCK1; **Figure 1 – figure supplement 2b**), pQSKVDDYGHGLFW-NH₂ [ArCCK1(ns);
157 **Figure 1 – figure supplement 2c**], GGDDQY(SO₃H)GFGLFF-NH₂ (ArCCK2; **Figure 1 –**
158 **figure supplement 2d**) and GGDDQYGFGLFF-NH₂ [ArCCK2(ns); **Figure 1 – figure**
159 **supplement 2e**]. Thus, mass spectrometry confirmed that i). the peptides are C-terminally
160 amidated, ii). the peptides are detected with or without tyrosine sulphation and iii). an N-
161 terminal glutamine is post-translationally converted to pyroglutamate in the mature ArCCK1
162 and ArCCK1(ns) peptides.

163 Having determined the structures of CCK-type neuropeptides derived from ArCCKP,
164 the sequences of ArCCK1 and ArCCK2 were aligned with the sequences of CCK-type peptides
165 that have been identified in other taxa (**Figure 1**). This revealed a number of evolutionarily
166 conserved features, including a tyrosine residue (typically sulphated) and a C-terminal amide
167 group that are separated by five to seven intervening residues. The C-terminal residue in the
168 majority of CCK-type peptides, including ArCCK2, is a phenylalanine residue. However,
169 ArCCK1 is atypical in having a C-terminal tryptophan residue, which is also a feature of a
170 CCK-type peptide in the bivalve mollusc *C. gigas*.

CCK-type signalling in an echinoderm



171
 172 **Figure 1. Comparison of the *A. rubens* CCK-type neuropeptides ArCCK1 and ArCCK2**
 173 **with CCK/SK-type neuropeptides from other taxa.** Conserved residues are highlighted,
 174 with conservation in more than 70% of sequences highlighted in black and conservative
 175 substitutions highlighted in grey. Experimentally verified conversion of an N-terminal
 176 glutamine residue (Q) to pyroglutamate in the mature peptide is indicated by the letter Q being
 177 shown in light blue. Tyrosine (Y) residues that are known or predicted to be subject to post-
 178 translational sulphation are shown in green. The C-terminal tryptophan (W) in ArCCK1 and in
 179 a *C. gigas* CCK-type peptide are shown in yellow to highlight that this feature is atypical of
 180 CCK-type peptides. Predicted or experimentally verified C-terminal amides are shown as the
 181 letter “a” in lowercase. Species names are highlighted in taxon-specific colours: grey
 182 (Chordata), blue (Ambulacraria), red (Lophotrochozoa), green (Arthropoda) and purple
 183 (Nematoda). Abbreviations are as follows: Acal (*Aplysia californica*), Arub (*Asterias rubens*),
 184 Cele (*Caenorhabditis elegans*), Cgig (*Crassostrea gigas*), Cint (*Ciona intestinalis*), Dmel
 185 (*Drosophila melanogaster*), Hsap (*Homo sapiens*), Ovic (*Ophionotus victoriae*), Pdum
 186 (*Platynereis dumerilii*), Shor (*Stichopus horrens*), Skow (*Saccoglossus kowalevskii*), Spur
 187 (*Strongylocentrotus purpuratus*), Tcas (*Tribolium castaneum*). The accession numbers of the
 188 sequences are listed in **Figure 1 – source data 1**. The nucleotide sequence of a cloned cDNA
 189 encoding the *A. rubens* cholecystokinin-type precursor is shown in **Figure 1 – figure**
 190 **supplement 1**. Mass spectroscopic analysis of the structures of the peptides derived from the
 191 *A. rubens* cholecystokinin-type precursor is presented in **Figure 1 – figure supplement 2**. The
 192 raw data for the results shown in **Figure 1 – figure supplement 2** can be found in **Figure 1 –**
 193 **source data 2**.

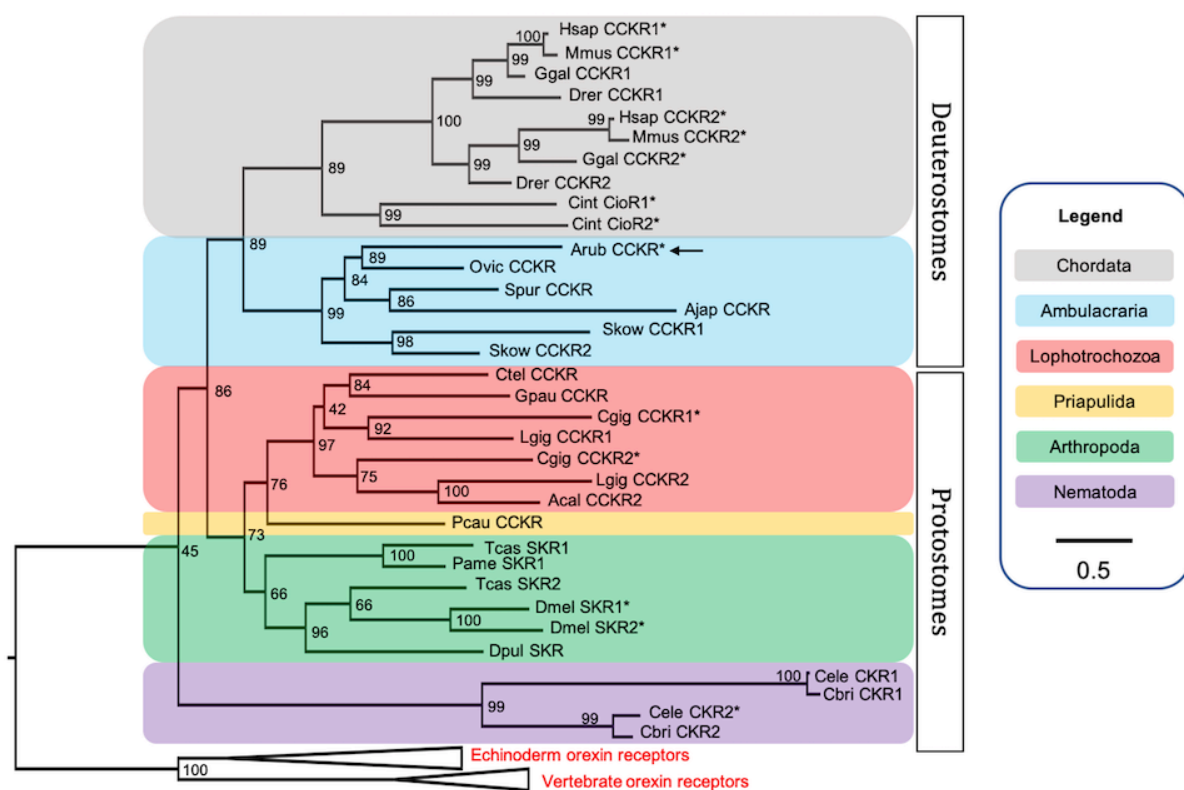
194

CCK-type signalling in an echinoderm

195 Identification of a CCK-type receptor in *A. rubens*

196 BLAST analysis of *A. rubens* neural transcriptome sequence data identified a transcript
 197 that encodes a 434-residue protein (ArCCKR) that shares high sequence similarity with CCK-
 198 type receptors from other taxa (**Figure 2 – figure supplement 1**). Phylogenetic analysis
 199 revealed that ArCCKR groups within a clade including CCK-type receptors that have been
 200 pharmacologically characterised in other taxa, including the human and mouse CCK/gastrin
 201 receptors CCKR1 and CCKR2, the *C. intestinalis* cionin receptors CioR1 and CioR2, the
 202 *Drosophila melanogaster* sulfakinin receptors SKR1 and SKR2, and the recently characterised
 203 *C. gigas* receptors CCKR1 and CCKR2 (**Figure 2**). Thus, this demonstrates that ArCCKR is
 204 an ortholog of CCK/SK-type receptors that have been characterised in other taxa. Furthermore,
 205 reflecting known animal phylogenetic relationships, ArCCKR is positioned within a branch of
 206 the tree that comprises CCK-type receptors from deuterostomes, and more specifically it is
 207 positioned within an ambulacrarian clade that comprises CCK-type receptors from other
 208 echinoderms and from the hemichordate *Saccoglossus kowalevskii*.

209 Analysis of the amino acid sequence of ArCCKR using the Protter tool (Omasits et al.
 210 2014) revealed seven predicted transmembrane domains, as expected for a GPCR, and three
 211 potential N-glycosylation sites in the predicted extracellular N-terminal region of the receptor
 212 (**Figure 2 – figure supplement 2**).



CCK-type signalling in an echinoderm

214 **Figure 2. Phylogenetic tree showing that the predicted *A. rubens* CCK-type receptor**
215 **(ArCCKR; arrow) is an ortholog of CCK-type receptors in other taxa, which include**
216 **receptors (*) for which the peptide ligands have been identified experimentally.** The tree
217 was generated using the maximum likelihood method (Guindon et al. 2009), with the
218 percentage of replicate trees in which two or more sequences form a clade in a bootstrap test
219 (1000 replicates) shown at the node of each clade (Zhaxybayeva and Gogarten 2002). Orexin
220 receptors were included as an outgroup. The tree is drawn to scale, with branch lengths in the
221 same units as those of the evolutionary distances used to infer the phylogenetic tree.
222 Evolutionary analyses were conducted using the IQ-tree server (Trifinopoulos et al. 2016).
223 Taxa are colour-coded as explained in the key. Abbreviations of species names are as follows:
224 Acal (*Aplysia californica*), Ajap (*Apostichopus japonicus*), Arub (*Asterias rubens*), Cbri
225 (*Caenorhabditis briggsae*), Cele (*Caenorhabditis elegans*), Cgig (*Crassostrea gigas*), Cint
226 (*Ciona intestinalis*), Ctel (*Capitella teleta*), Dmel (*Drosophila melanogaster*), Dpul (*Daphnia*
227 *pulex*), Drer (*Danio rerio*), Ggal (*Gallus gallus*), Gpau (*Glossoscolex paulistus*), Hsap (*Homo*
228 *sapiens*), Lgig (*Lottia gigantea*), Mmus (*Mus musculus*), Ovic (*Ophionotus victoriae*), Pame
229 (*Periplaneta americana*), Pcau (*Priapululus caudatus*), Skow (*Saccoglossus kowalevskii*),
230 Spur (*Strongylocentrotus purpuratus*), Tcas (*Tribolium castaneum*). The accession numbers of
231 the sequences used for this phylogenetic tree are listed in **Figure 2 – source data 1**. The
232 nucleotide sequence and the predicted topology of ArCCKR are shown in **Figure 2 – figure**
233 **supplement 1** and **Figure 2 – figure supplement 2**, respectively.
234

235 **ArCCK1 and ArCCK2 are ligands for ArCCKR**

236 Previous studies on other species have revealed that sulphation of the tyrosine residue
237 in CCK-type peptides is often important for receptor activation and bioactivity (Dufresne et al.
238 2006, Kubiak et al. 2002, Schwartz et al. 2018, Sekiguchi et al. 2012, Yu et al. 2015).
239 Accordingly, neuropeptides derived from ArCCKP were detected in *A. rubens* radial nerve
240 cord extracts with sulphated tyrosines (ArCCK1 and ArCCK2; **Figure 1 – figure supplement**
241 **2b, d**). Therefore, the sulphated peptides ArCCK1 and ArCCK2 were synthesized and tested
242 as ligands for ArCCKR. However, because non-sulphated forms of ArCCK1 (ArCCK1(ns))
243 and ArCCK2 (ArCCK2(ns)) were also detected *A. rubens* radial nerve extracts (**Figure 1 –**
244 **figure supplement 2c, e**), we also synthesized and tested ArCCK2(ns) to investigate if absence
245 of tyrosine sulphation affects receptor activation. Using CHO-K1 cells expressing aequorin as
246 an assay system, ArCCK1, ArCCK2 and ArCCK2(ns) did not elicit luminescence responses
247 when tested on cells transfected with an empty vector (**Figure 3**). However, all three peptides
248 caused concentration-dependent stimulation of luminescence in CHO-K1 cells transfected with
249 ArCCKR (**Figure 3**). The EC₅₀ values for ArCCK1, ArCCK2 and ArCCK2(ns) were 0.25 nM
250 (**Figure 3a**), 0.12 nM (**Figure 3b**) and 48 μM (**Figure 3c**), respectively. Thus, although
251 activation of ArCCKR was observed *in vitro* with ArCCK2(ns) (**Figure 3c**), this peptide is five
252 to six orders of magnitude less potent than ArCCK1 and ArCCK2 as a ligand for ArCCKR.
253 This indicates that the non-sulphated peptides ArCCK1(ns) and ArCCK2(ns) that were

CCK-type signalling in an echinoderm

254 detected in *A. rubens* radial nerve extracts are unlikely to have physiological effects *in vivo*.
255 Furthermore, two other *A. rubens* neuropeptides that share modest C-terminal sequence
256 similarity with the *A. rubens* CCK-type peptides - the SALMFamide neuropeptide S2
257 (SGPYSFNSGLTF-NH₂) and the tachykinin-like peptide ArTK2 (GGGVPHVFQSGGIF-
258 NH₂) - were found to be inactive when tested as ligands for ArCCKR at concentrations ranging
259 from 10⁻¹² to 10⁻⁴ M) (**Figure 3 – figure supplement 1**), demonstrating the specificity of
260 ArCCK1 and ArCCK2 as ligands for ArCCKR.

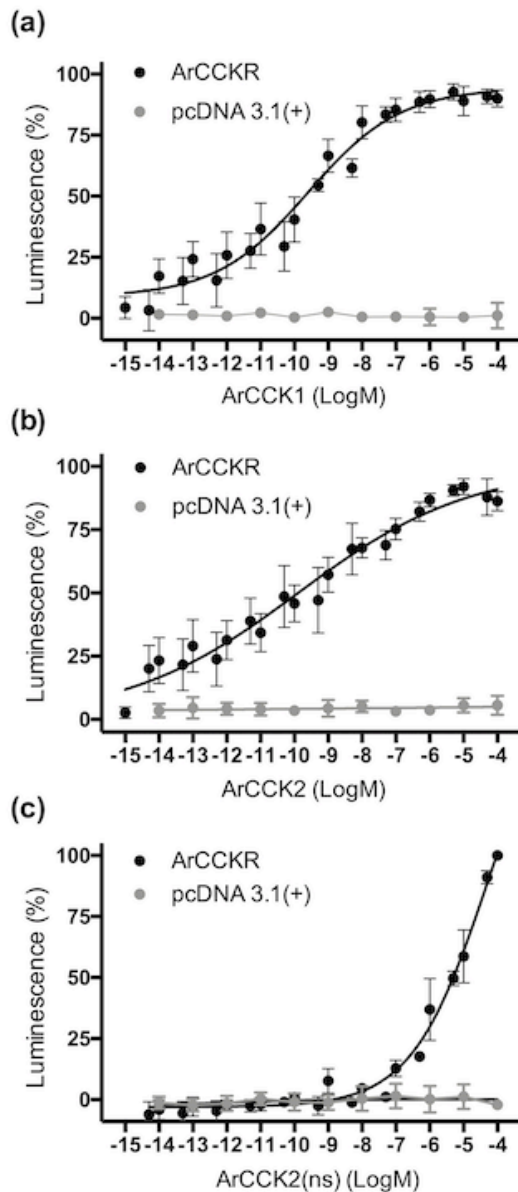


Figure 3. Experimental demonstration that the *A. rubens* CCK-type peptides ArCCK1 and ArCCK2 act as ligands for the *A. rubens* CCK-type receptor ArCCKR. The sulphated peptides ArCCK1 (a), ArCCK2 (b) and the non-sulphated peptide ArCCK2(ns) (c) trigger dose-dependent luminescence in CHO-K1 cells stably expressing mitochondrial targeted apoaequorin (G5A) that were co-transfected with plasmids encoding the promiscuous human G-protein G α 16 and ArCCKR (black). Control experiments where cells were transfected with an empty pcDNA 3.1(+) vector are shown in grey. Each point represents mean values (\pm s.e.m) from at least four independent experiments performed in triplicate. Luminescence is expressed as a percentage of the maximal response observed in each experiment. The EC₅₀ values for ArCCK1 (a) and ArCCK2 (b) are 0.25 nM and 0.12 nM, respectively. In comparison, the absence of tyrosine (Y) sulphation in ArCCK2(ns) (c) causes a massive loss of potency (EC₅₀ = 48 μ M), indicating that the sulphated peptides act as ligands for ArCCKR physiologically. A graph showing the selectivity of ArCCKR as a receptor for CCK-type peptides is presented in **Figure 3 – figure supplement 1**

293

294 Localisation of ArCCKP expression in *A. rubens* using mRNA *in situ* hybridization

295 To gain anatomical insights into the physiological roles of CCK-type neuropeptides in
296 starfish, mRNA *in situ* hybridisation methods were employed to enable analysis of the

CCK-type signalling in an echinoderm

297 distribution of the ArCCKP transcript in *A. rubens*. As described below and illustrated in
298 **Figure 4**, expression of ArCCKP was observed in the central nervous system, digestive system,
299 body wall and tube feet.

300 The central nervous system of *A. rubens* comprises radial nerve cords that extend along the
301 oral side of each arm, with two rows of tube feet (locomotory organs) on either side. The five
302 radial nerve cords are linked by a circumoral nerve ring in the central disk (Pentreath and
303 Cobb 1972). Analysis of ArCCKP mRNA expression revealed stained cells in both the
304 ectoneural and hyponeural regions of the radial nerve cords (**Figure 4a**) and circumoral nerve
305 ring (**Figure 4b, c**). Furthermore, the specificity of staining observed with anti-sense probes
306 was confirmed by an absence of staining in tests with sense probes (**Figure 4a**). Stained cells
307 were also revealed in the ectoneural segmental branches of the radial nerve cords (**Figure 4d**)
308 and in the marginal nerves (**Figure 4e**), which run parallel with the radial nerve cords lateral
309 to the outer row of tube feet. ArCCKP-expressing cells were also revealed in tube feet, with
310 stained cells located in the podium proximal to its junction with the radial and marginal
311 nerves (**Figure 4e**) and in the tube foot disk (**Figure 4f**). In the digestive system, ArCCKP-
312 expressing cells were revealed in the mucosa of the oesophagus (**Figure 4g**), cardiac stomach
313 (**Figure 4i, h**), pyloric stomach (**Figure 4i**), pyloric ducts (**Figure 4j**), pyloric caeca (**Figure**
314 **4j**) and intestine (**Figure 4k, l**). ArCCKP expressing cells were also revealed in the external
315 epithelium of the body wall (**Figure 4m, n**).

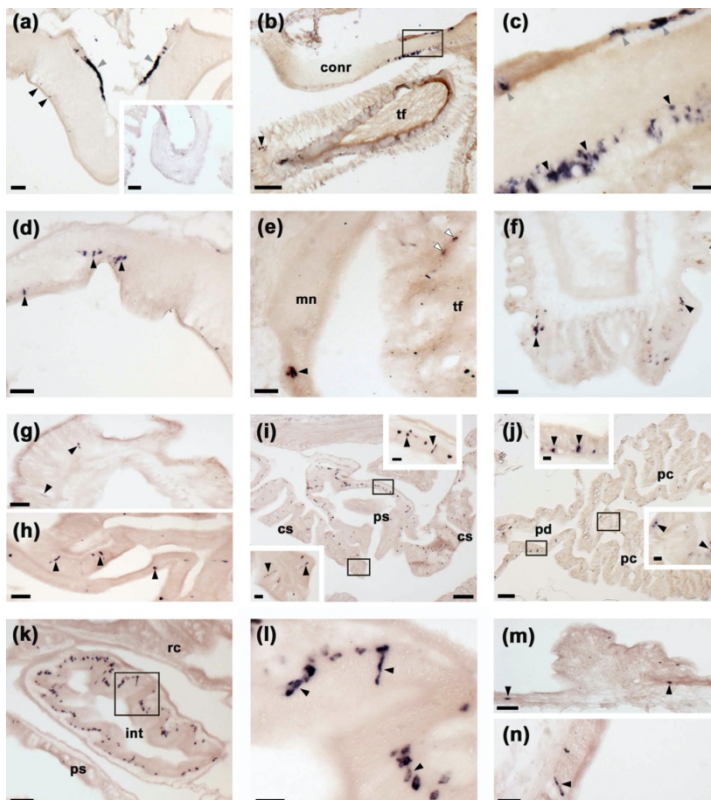


Figure 4. Localisation of ArCCKP expression in *A. rubens* using mRNA *in situ* hybridization. (a) Using antisense probes, ArCCKP-expressing cells are revealed in the ectoneural (black arrowheads) and hyponeural (grey arrowheads) regions of a radial nerve cord. The specificity of staining with antisense probes is demonstrated by the absence of staining in a radial nerve cord section incubated with sense probes (see inset). (b) ArCCKP-expressing cells in the circumoral nerve ring (see boxed area) and in the disk region of a peri-oral tube foot (black arrowhead). (c) High magnification image of the boxed area in (b), showing stained cells in the ectoneural (black arrowheads) and hyponeural (grey arrowheads) regions of the circumoral nerve ring. (d) ArCCKP-expressing cells in a lateral branch of the radial nerve cord (black arrowheads). (e)

CCK-type signalling in an echinoderm

338 ArCCKP-expressing cells adjacent to the marginal nerve (black arrowhead) and in the stem of
339 a tube foot (white arrowheads). **(f)** ArCCKP-expressing cells (black arrowheads) adjacent to
340 the basal nerve ring in the disk region of a tube foot. **(g)** ArCCKP-expressing cells (black
341 arrowheads) in the mucosal layer of the oesophagus. **(h)** ArCCKP-expressing cells (black
342 arrowheads) in the mucosal layer of the cardiac stomach. **(i)** ArCCKP-expressing cells (black
343 arrowheads) in the cardiac stomach and pyloric stomach, with the boxed regions shown at
344 higher magnification in the insets. **(j)** ArCCKP-expressing cells (black arrowheads) in the
345 pyloric duct and pyloric caeca, with the boxed regions shown at higher magnification in the
346 insets. **(k, l)** ArCCKP-expressing cells (black arrowheads) in an oblique section of the intestine;
347 the boxed region in **(k)** is shown at higher magnification in **(l)**. **(m, n)** ArCCKP-expressing cells
348 (black arrowheads) in the external epithelium of the body wall. Abbreviations: conr, circumoral
349 nerve ring; int, intestine; mn, marginal nerve; pc, pyloric caecum; pd, pyloric duct; ps, pyloric
350 stomach; rc, rectal caeca; tf, tube foot. Scale bars: [b, i, j] = 120 μm ; [a, a-inset, k] = 60
351 μm ; [d, e, f, g, h, m] = 32 μm ; [c, i-insets, j-insets, l, n] = 16 μm .
352

353 Immunohistochemical localisation of ArCCK1 in *A. rubens*

354 Use of mRNA *in situ* hybridisation (see above) revealed the location of cells expressing
355 ArCCKP in *A. rubens*. However, a limitation of this technique is that it does not reveal the
356 axonal processes of neuropeptidergic neurons. Therefore, to enable this using
357 immunohistochemistry, we generated and affinity-purified rabbit antibodies to ArCCK1.
358 ELISA analysis of antiserum revealed the presence of antibodies to the ArCCK1 peptide
359 antigen (**Figure 5 - figure supplement 1a**) and ELISA analysis of affinity-purified antibodies
360 to the ArCCK1 antigen peptide revealed the specificity of these antibodies for ArCCK1
361 because they do not cross-react with ArCCK2, ArCCK2(ns) or the starfish luqin-type
362 neuropeptide ArLQ (**Figure 5 - figure supplement 1b**). Immunohistochemical tests with
363 affinity-purified ArCCK1 antibodies revealed extensive immunostaining in sections of *A.*
364 *rubens*, as described in detail below and illustrated in **Figure 5**.

365 ArCCK1-immunoreactive (ir) cells were revealed in the ectoneural and hyponeural
366 regions of the radial nerve cords (**Figure 5a, b**). Furthermore, dense networks of
367 immunostained fibres were revealed in the ectoneural neuropile, with bilaterally symmetrical
368 regional variation in the density of immunostaining (**Figure 5b**). Likewise, ArCCK1-ir cells
369 were revealed in the ectoneural and hyponeural regions of the circumoral nerve ring, also with
370 regional variation in the density of immunostained fibres in the ectoneural neuropile (**Figure**
371 **5c**). Immunostained cells and/or processes were also revealed in the segmental lateral branches
372 of the radial nerve cords (**Figure 5d**) and in the marginal nerve cords (**Figure 5e**). Consistent
373 with the expression of ArCCKP/ArCCK1 in the hyponeural region of radial nerve cords,
374 ArCCK1-ir fibres were revealed in the lateral motor nerves (**Figure 5e**). In tube feet, ArCCK1-

CCK-type signalling in an echinoderm

375 ir fibres were revealed in the sub-epithelial nerve plexus of the podium and in the basal nerve
376 ring of the disk region (**Figure 5c,f**).

377 ArCCK1-ir cells and/or fibres were revealed in the mucosa and basiepithelial nerve
378 plexus, respectively, of many regions of the digestive system, including the peristomial
379 membrane (**Figure 5g**), oesophagus (**Figure 5g**), cardiac stomach (**Figure 5h, i, j**), pyloric
380 stomach (**Figure 5k**), pyloric ducts (**Figure 5l**), pyloric caeca (**Figure 5l**) and intestine (**Figure**
381 **5m**). Consistent with patterns of ArCCKP transcript expression (see above), regional
382 differences in the abundance of stained cells and fibres were observed. Regions of the digestive
383 system containing denser populations of CCK1-ir cells and/or fibres include the lateral pouches
384 of the cardiac stomach (**Figure 5h**), the roof of the pyloric stomach (**Figure 5k**) and the
385 intestine (**Figure 5m**).

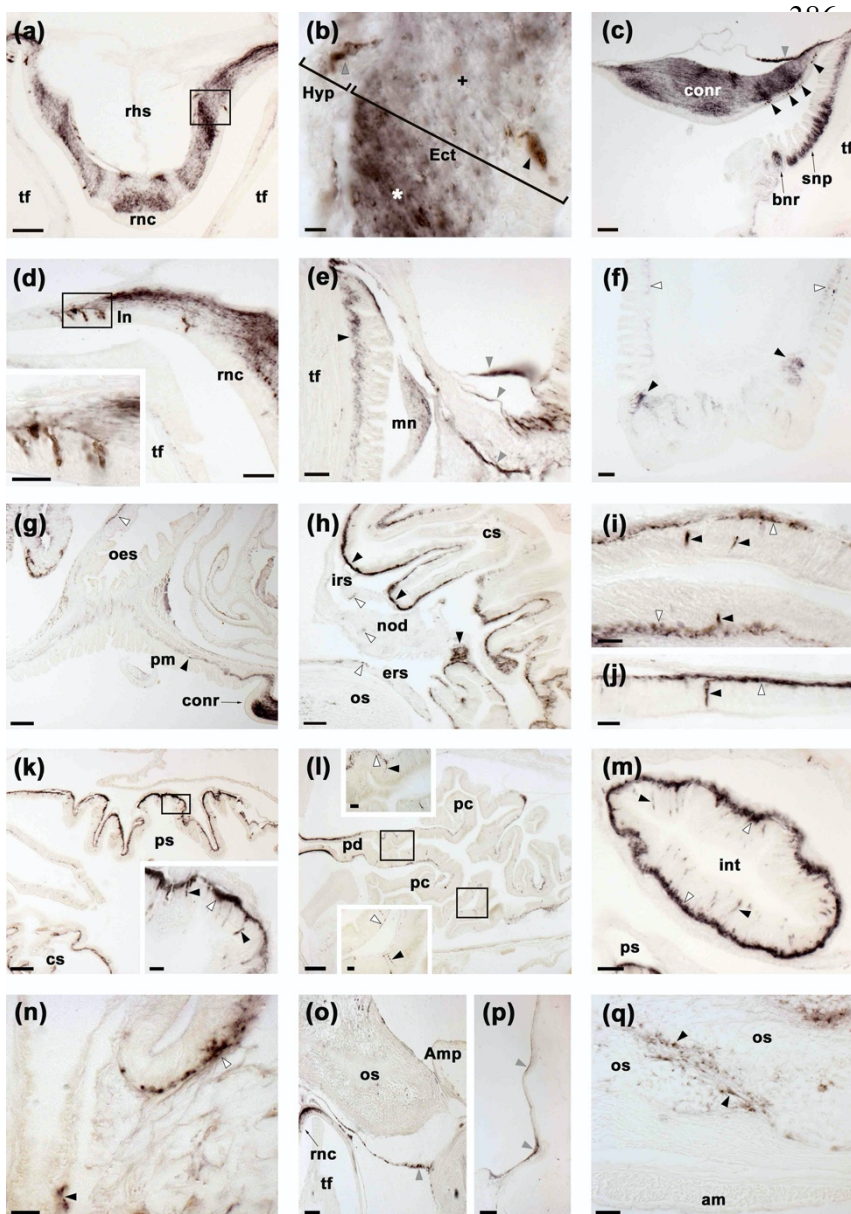


Figure 5. Localisation of ArCCK1 expression in *A. rubens* using immunohistochemistry.

(a) ArCCK1-immunoreactivity (ArCCK1-ir) in a transverse section of the V-shaped radial nerve cord, with bilaterally symmetrical regional variation in the density of immunostaining in the ectoneural neuropile. (b) High magnification image of the boxed region in (a), showing stained cell bodies in the hyponeural (grey arrowhead) and ectoneural (black arrowhead) regions of the radial nerve cord. Regions of the ectoneural neuropile containing a higher (*) and lower (+) densities of immunostained fibres can be seen here. (c) ArCCK1-ir in the circumoral nerve ring, with stained cells present in the hyponeural region (grey arrowheads) and in the ectoneural epithelium (black arrowheads); in the ectoneural neuropile there

CCK-type signalling in an echinoderm

420 is regional variation in the density of immunostained fibres. Immunostaining can also be seen
421 here in the sub-epithelial nerve plexus and basal nerve ring of an adjacent peri-oral tube foot.
422 **(d)** ArCCK1-ir in cells and fibres in a lateral branch of the radial nerve cord; the inset shows
423 immunostained cells in the boxed region at higher magnification. **(e)** ArCCK1-ir in the
424 marginal nerve, the sub-epithelial nerve plexus of an adjacent tube foot (black arrowhead) and
425 in branches of the lateral motor nerve (grey arrowheads). **(f)** ArCCK1-ir in the sub-epithelial
426 nerve plexus (white arrowheads) and basal nerve ring (black arrowheads) of a tube foot. **(g)**
427 ArCCK1-ir in the basi-epithelial nerve plexus of the peristomial membrane (black arrowhead)
428 and the oesophagus (white arrowhead); immunostaining in the circumoral nerve can also be
429 seen here. **(h)** ArCCK1-ir in the lateral pouches of the cardiac stomach; note that the density
430 of immunostained fibres is highest (black arrowheads) in regions of the mucosa adjacent to the
431 intrinsic retractor strand; immunostaining in the intrinsic retractor strand, nodule and extrinsic
432 retractor strand can also be seen here (white arrowheads). **(i, j)** High magnification images of
433 cardiac stomach tissue showing ArCCK1-ir in cell bodies (black arrowheads) and their
434 processes in the basiepithelial nerve plexus (white arrowheads); note that in **(j)** a process
435 emanating from an immunostained cell body can be seen projecting into the plexus. **(k)**
436 ArCCK1-ir in the cardiac stomach and pyloric stomach; the boxed region is shown at higher
437 magnification in the inset, showing immunostaining in cells (black arrowheads) and the basi-
438 epithelial nerve plexus (white arrowhead). **(l)** ArCCK1-ir in the pyloric duct and pyloric caeca;
439 the boxed regions are shown at higher magnification in the insets, where immunostained cells
440 (black arrowheads) and fibres (white arrowheads) can be seen. **(m)** ArCCK1-ir in an oblique
441 section of the intestine, with immunostained cells in the mucosa (black arrowheads) and intense
442 immunostaining in the basi-epithelial nerve plexus (white arrowheads). **(n)** ArCCK1-ir in the
443 basi-epithelial nerve plexus of the body wall external epithelium (white arrowhead) and in the
444 lining of a papula (black arrowhead). **(o)** ArCCK1-ir in nerve fibres projecting around the base
445 of a tube foot at its junction with the neck of its ampulla. **(p)** ArCCK1-ir in nerve fibres located
446 in the coelomic lining of the lateral region of the body wall. **(q)** ArCCK1-ir in inter-ossicular
447 tissue of the body wall. Abbreviations: am, apical muscle; Amp, ampulla; bnr, basal nerve ring;
448 conr, circumoral nerve ring; cs, cardiac stomach; Ect, ectoneural; ers, extrinsic retractor strand;
449 Hyp, hyponeural; int, intestine; irs, intrinsic retractor strand; ln, lateral nerve; mn, marginal
450 nerve; nod, nodule; oes, oesophagus; os, ossicle; pm, peristomial membrane; pc, pyloric
451 caecum; pd, pyloric duct; ps, pyloric stomach; rhs, radial hemal strand; rnc, radial nerve cord;
452 snp, sub-epithelial nerve plexus; tf, tube foot. Scale bars: [g, k, l] = 120 μm ; [a, c, f, h),
453 o, p)] = 60 μm ; [d, e, m, q)] = 32 μm ; [d-inset, i, j), k-inset, l-insets, n)] = 16 μm ; [b)] =
454 6 μm . Graphs showing ELISA-based characterisation of the antibodies to ArCCK1 used here
455 for immunohistochemistry are presented in **Figure 5 – figure supplement 1**.

456

457 Immunostaining was observed in the sub-epithelial nerve plexus of the external
458 epithelium of the body wall (**Figure 5n**) and in papulae (**Figure 5n**), which are protractable
459 appendages that penetrate through the body wall to enable gas exchange between external
460 seawater and the coelomic fluid (Cobb 1978). Immunostained fibres were also observed in
461 branches of the lateral motor nerves located in the coelomic lining of the body wall (**Figure**
462 **5o,p**). However, no staining was observed in the apical muscle - a thickening of longitudinally
463 orientated muscle that is located under the coelomic epithelial layer of the body wall along the
464 aboral midline of each arm (**Figure 5q**). The bulk of the body wall in *A. rubens* is comprised

CCK-type signalling in an echinoderm

465 of calcite ossicles that are interconnected by muscles and collagenous tissue and ArCCK1-ir
466 fibres were revealed in the inter-ossicular tissue (**Figure 5q**).

467

468 **ArCCK1 and ArCCK2 cause concentration-dependent contraction of *in vitro* cardiac**
469 **stomach, tube foot and apical muscle preparations from *A. rubens***

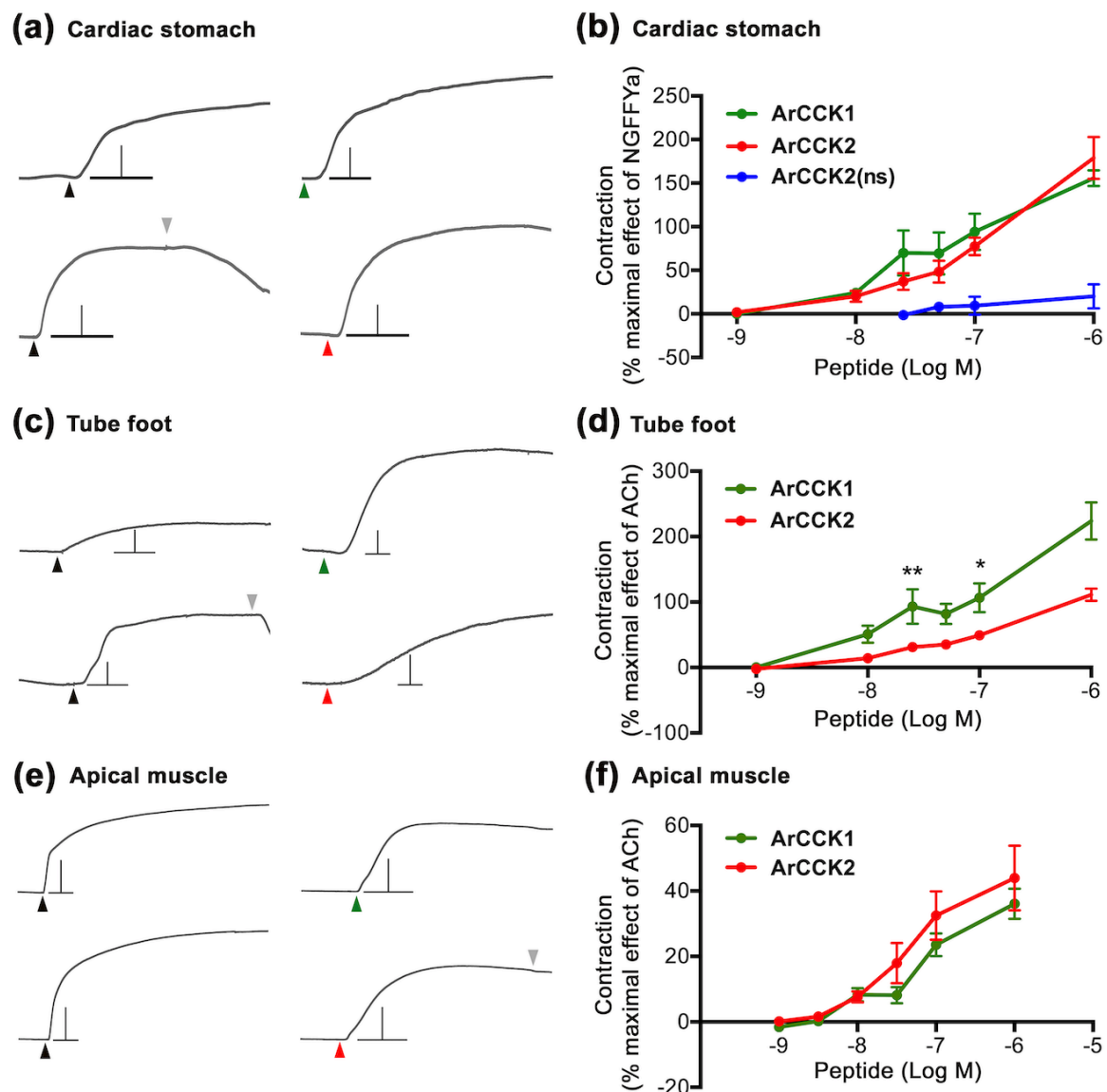
470 Informed by the localisation of ArCCKP/ArCCK1 expression in the cardiac stomach
471 and tube feet of *A. rubens*, we tested the effects of ArCCKP-derived neuropeptides on *in vitro*
472 preparations of these organs. Both ArCCK1 and ArCCK2 caused concentration-dependent
473 contraction of cardiac stomach preparations when tested at concentrations ranging from 1 nM
474 to 1 μ M (**Figure 6a, b**). ArCCK2(ns) caused modest contraction of cardiac stomach
475 preparations by comparison with ArCCK2 (**Figure 6b**), and comparison of the ArCCK2 and
476 ArCCK2(ns) data using a 2-way ANOVA revealed a significant difference in the effects of the
477 peptides on cardiac stomach preparations, irrespective of concentration ($P < 0.0001$). However,
478 2-way ANOVA analysis revealed no significant difference in the effects of ArCCK1 and
479 ArCCK2 on cardiac stomach preparations. To enable normalisation of the effects of the
480 ArCCKP-derived peptides between experiments, the neuropeptide NGFFYamide was also
481 tested on each preparation at a concentration of 100 nM (**Figure 6a**), and at this concentration
482 the effects of ArCCK1 and ArCCK2 (1 μ M) were not significantly different (Student t-test; P
483 > 0.05) to the effect of NGFFYamide (data not shown).

484 Consistent with the effects of ArCCKP-derived neuropeptides on cardiac stomach
485 preparations, ArCCK1 and ArCCK2 (1 nM to 1 μ M) also caused concentration-dependent
486 contraction of tube foot preparations (**Figure 6c, d**). Furthermore, comparison of the effects of
487 ArCCK1 and ArCCK2 on tube feet using a 2-way ANOVA revealed significant differences,
488 irrespective of concentration ($P < 0.0001$). In addition, Bonferroni's multiple comparison test
489 showed that ArCCK1 is significantly more effective than ArCCK2 when tested at
490 concentrations of 25 nM and 100 nM ($P < 0.01$ and 0.05 respectively). Furthermore,
491 ArCCK2(ns) peptide did not cause contraction of tube foot preparations *in vitro* (data not
492 shown).

493 Although ArCCKP/ArCCK1 expression was not detected in the apical muscle (see
494 above), we nevertheless tested ArCCK1 and ArCCK2 on this preparation because previous
495 studies have revealed that other neuropeptides cause contraction (Tian et al. 2017) or relaxation
496 (Cai et al. 2018, Lin et al. 2017a, Tinoco et al. 2018) of the apical muscle. Interestingly,
497 consistent with the effects of ArCCKP-derived neuropeptides on cardiac stomach and tube foot
498 preparations, both ArCCK1 and ArCCK2 caused contraction of apical muscle preparations

CCK-type signalling in an echinoderm

499 (Figure 6e, f). However, by comparison with the effect of acetylcholine, which was tested at
 500 concentration of 10 μ M to normalise effects of the peptides on different preparations, the
 501 contracting actions of ArCCK1 and ArCCK2 were only ~40% of the effect of 10 μ M ACh at
 502 the highest concentration tested (1 μ M; Figure 6f). In contrast, the mean effects of ArCCK1
 503 and ArCCK2 on tube foot preparations at 1 μ M were ~220% and ~110% of the effect of 10
 504 μ M ACh (Figure 6d). No significant differences in the effects of ArCCK1 and ArCCK2 on
 505 apical muscle preparations were observed (2-way ANOVA, $P > 0.05$) and, as was observed
 506 with tube feet, ArCCK2(ns) had no effect on apical muscle preparations (data not shown).



507

508 **Figure 6. ArCCK1 and ArCCK2 cause concentration-dependent contraction of *in vitro***
 509 **preparations of cardiac stomach, tube foot and apical muscle from *A. rubens*.** (a)
 510 Representative recordings of the effects ArCCK1 (1 μ M; green arrowhead) and ArCCK2 (1
 511 μ M; red arrowhead) in causing contraction of cardiac stomach preparations and compared with
 512 the effect of NGFFYamide (100 nM; black arrowhead) on the same preparation. The downward

CCK-type signalling in an echinoderm

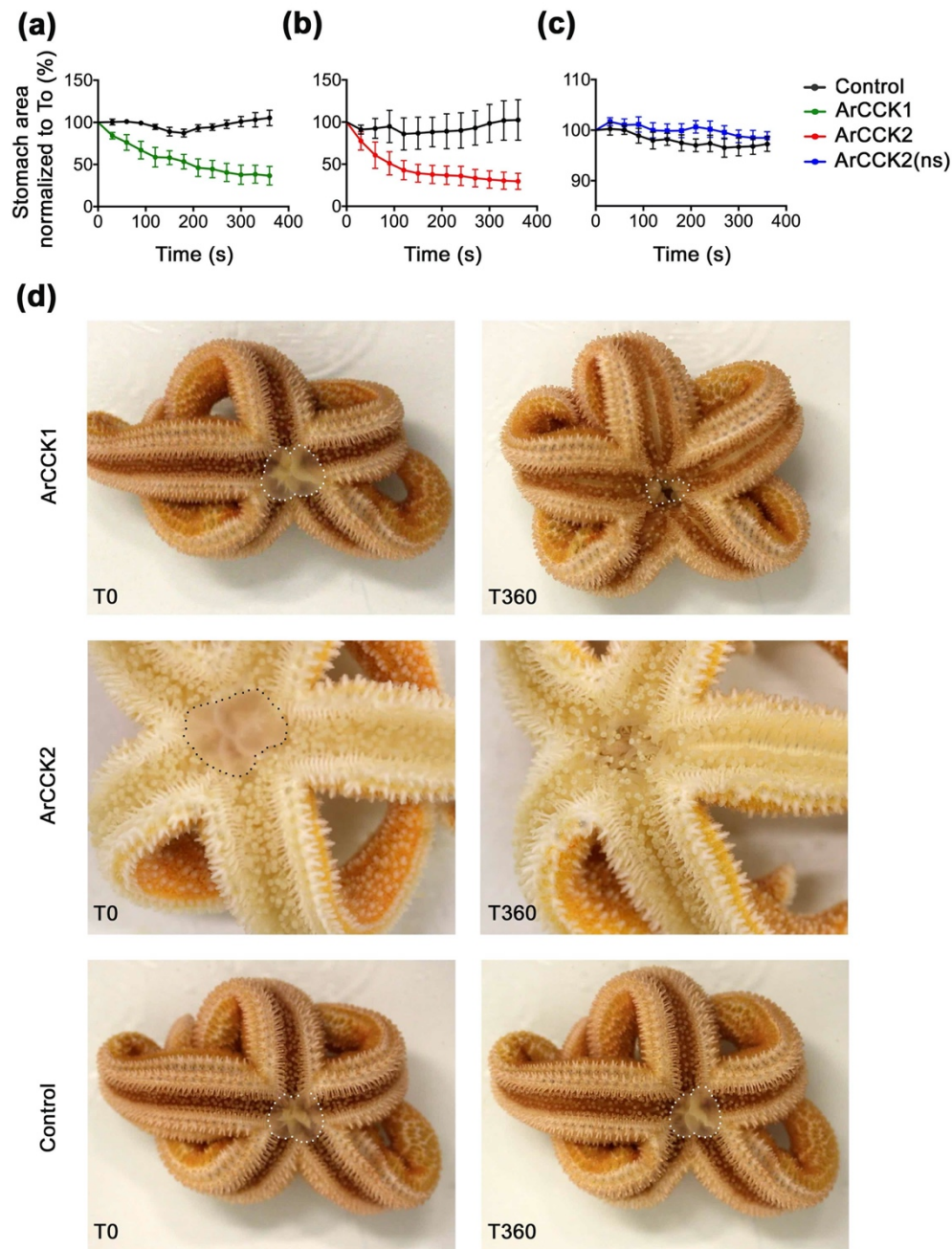
513 pointing grey arrowhead shows when a preparation was washed with seawater. Scale bar:
514 vertical 0.5 mV; horizontal 1 min. **(b)** Concentration-response curves comparing the effects of
515 ArCCK1, ArCCK2 and ArCCK2(ns) on cardiac stomach preparations. The effects of peptides
516 (means \pm s.e.m; n = 5 – 9) were normalized to the effect of 100 nM NGFFYamide (NGFFYa).
517 **(c)** Representative recordings of the effects ArCCK1 (1 μ M; green arrowhead) and ArCCK2
518 (1 μ M; red arrowhead) in causing contraction of tube foot preparations and compared with the
519 effect of acetylcholine (10 μ M; black arrowhead) on the same preparation. The downward
520 pointing grey arrowhead shows when a preparation was washed with seawater. Scale bar:
521 vertical 0.08 mV; horizontal 1 min. **(d)** Concentration-response curves comparing the effects
522 of ArCCK1 and ArCCK2 on tube foot preparations. The effects of peptides (means \pm SEM; n
523 = 8 - 10) were normalized to the effect of 10 μ M acetylcholine (ACh). * indicates statistically
524 significant differences between ArCCK1 and ArCCK2 when tested at concentrations of 25 nM
525 and 100 nM ($P < 0.01$ and $P < 0.05$ respectively) as determined by 2-way ANOVA and
526 Bonferroni's multiple comparison test. **(e)** Representative recordings of the effects ArCCK1 (1
527 μ M; green arrowhead) and ArCCK2 (1 μ M; red arrowhead) in causing contraction of apical
528 muscle preparations and compared to the effect of acetylcholine (10 μ M; black arrowhead) on
529 the same preparation. The downward pointing grey arrowhead shows when a preparation was
530 washed with seawater. Scale bar: vertical 0.4 mV; horizontal 1 min. **(f)** Concentration-response
531 curves comparing the effects of ArCCK1 and ArCCK2 on apical muscle preparations. The
532 effects of peptides (means \pm s.e.m; n = 20 - 23) were normalized to the effect of 10 μ M
533 acetylcholine (ACh).
534

535 **ArCCK1 and ArCCK2 trigger cardiac stomach retraction *in vivo***

536 Previous studies have revealed that the starfish neuropeptide NGFFYamide causes
537 contraction of cardiac stomach preparations *in vitro* and triggers retraction of the everted
538 cardiac stomach *in vivo* (Semmens et al. 2013). Because both ArCCK1 and ArCCK2 also cause
539 contraction of cardiac stomach preparations *in vitro*, it was of interest to investigate if these
540 neuropeptides also trigger retraction of the everted cardiac stomach *in vivo*. As reported
541 previously (Semmens et al. 2013), cardiac stomach eversion was induced by immersing starfish
542 in seawater containing 2% added MgCl₂. In control experiments where starfish were injected
543 with water, no retraction of the cardiac stomach was observed (**Figure 7**). However, injection
544 of ArCCK1 or ArCCK2 (10 μ l of 1 mM) triggered cardiac stomach retraction ($P < 0.0001$ for
545 both peptides; 2-way ANOVA) (**Figure 7a, b, d; Videos 1, 2**), consistent with the contracting
546 action of these peptides *in vitro*. ArCCK1 and ArCCK2 triggered cardiac stomach retraction in
547 all animals tested but with some variability in the rate and extent of retraction. Consistent with
548 the modest effect of ArCCK2(ns) on cardiac stomach preparations *in vitro* (**Figure 6b**),
549 injection of ArCCK2(ns) (10 μ l of 1 mM) did not trigger cardiac stomach retraction *in vivo*
550 (**Figure 7c**).

551

CCK-type signalling in an echinoderm



552

553

554 **Figure 7. ArCCK1 and ArCCK2 trigger cardiac stomach retraction in *A. rubens*.** The

555 graphs compare experiments where starfish were first injected with vehicle (black line; 10 μ l

556 of distilled water) and then injected with (a) ArCCK1 (green line; 10 μ l 1 mM), or (b)

557 ArCCK2 (red line; 10 μ l 1 mM), or (c) ArCCK2(ns) (blue line; 10 μ l 1 mM). Stomach

558 eversion was induced by placing starfish in seawater containing 2% MgCl₂ and then the area

559 of cardiac stomach everted (in 2D) at 30 s intervals (0-360 s) following injection of water

560 (control) or peptide was measured, normalizing to the area of cardiac stomach everted at the

561 time of injection (T0). Data (means \pm s.e.m) were obtained from 6 (ArCCK1), 7 (ArCCK2)

562 or 8 (ArCCK2(ns)) experiments. Both ArCCK1 and ArCCK2 cause retraction of the cardiac

563 stomach, with >50% reduction in the area of cardiac stomach everted within 360 s (see

564 videos 1 and 2), whereas ArCCK2(ns) has no effect. (d) Photographs from representative

565 experiments showing that injection of ArCCK1 (10 μ l 1 mM at T0) or ArCCK2 (10 μ l 1 mM

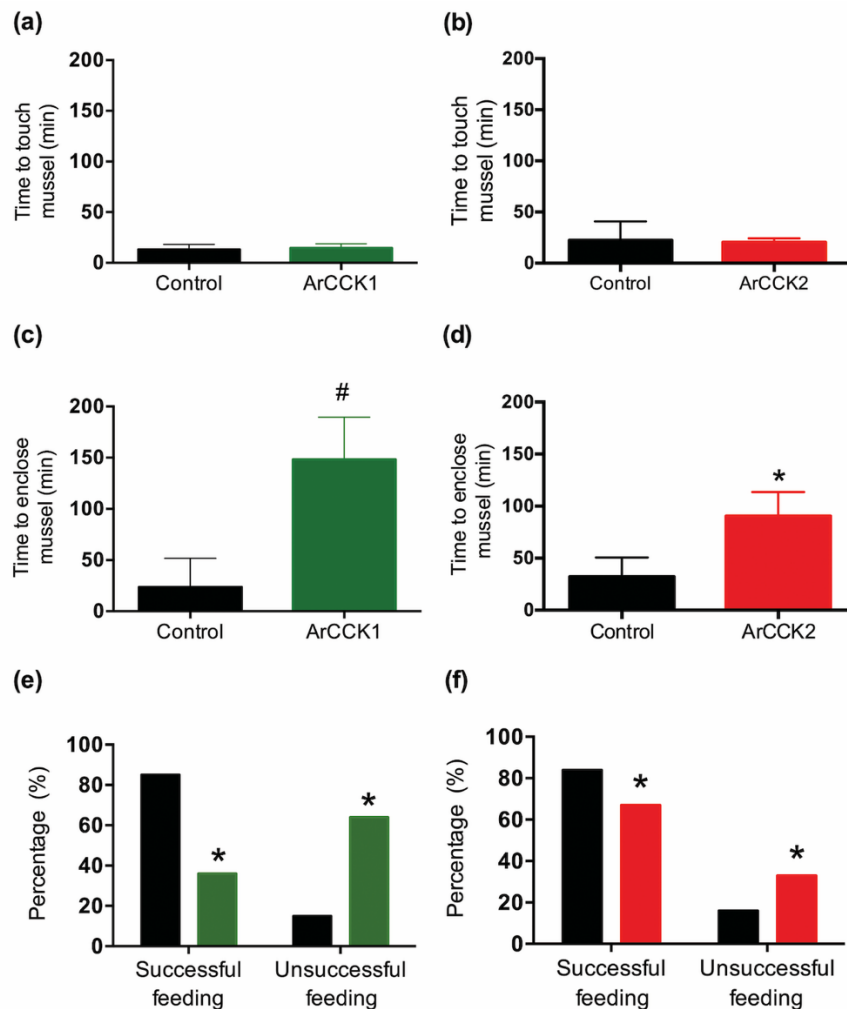
CCK-type signalling in an echinoderm

566 at T0) causes retraction of the everted cardiac stomach (marked with white or black dots),
567 which is reflected in a reduction in the area everted after 360 s (T360). By way of
568 comparison, in a control experiment injection with vehicle (10 µl of distilled water at T0)
569 does not trigger cardiac stomach retraction.
570

571 **ArCCK1 and ArCCK2 inhibit feeding behaviour in *A. rubens***

572 The effects of ArCCK1 and ArCCK2 in triggering cardiac stomach retraction in *A.*
573 *rubens* (see above) suggested that CCK-type signalling may have a physiological role in
574 inhibition and/or termination of feeding behaviour in starfish, which would be consistent with
575 the physiological roles of CCK-type neuropeptides in other taxa (Al-Alkawi et al. 2017,
576 Downer et al. 2007, Kang et al. 2011, Maestro et al. 2001, Meyering-Vos and Muller 2007,
577 Nachman et al. 1986b, Nässel et al. 2019, Rehfeld 2017, Roman et al. 2017, Wei et al. 2000,
578 Yu et al. 2013a, Zels et al. 2015, Zhang et al. 2017). Therefore, we performed experiments to
579 specifically investigate if ArCCK1 and ArCCK2 have inhibitory effects on starfish feeding
580 behaviour on prey (mussels). Injection of ArCCK1 or ArCCK2 (10 µl of 1 mM) did not affect
581 the time taken for starfish to make first contact with a mussel (time to touch; **Figure 8a, b**).
582 However, compared to water-injected controls the mean time taken for starfish to adopt a
583 feeding posture (time to enclose) was higher in neuropeptide-injected animals, reaching
584 statistical significance ($P < 0.05$) with ArCCK2 treatment ($P < 0.05$; **Figure 8d**) but showing
585 only a tendency to an increased time to enclose with ArCCK1 treatment ($P = 0.0523$; **Figure**
586 **9c**). This increased time to enclose was also reflected in an increased number of advances to
587 touch the mussel in the starfish treated with ArCCK1 or ArCCK2 (data not shown). Moreover,
588 by comparison with control starfish (water-injected), fewer starfish injected with ArCCK1 or
589 ArCCK2 proceeded to initiation of a feeding posture after the first touch ($P < 0.0001$ and $P <$
590 0.01 for ArCCK1 and ArCCK2 respectively; **Figure 8e, f**). Another observation indicative of
591 an inhibitory effect on feeding behaviour was that four and two of the starfish from ArCCK1-
592 and ArCCK2-treated groups, respectively, did not initiate feeding on a mussel within the 5 h
593 (300 min) observation period of the experiment, although feeding was commenced later and
594 within 24 h of initiating the experiment.

CCK-type signalling in an echinoderm



595

596 **Figure 8. Effects of ArCCK1 and ArCCK2 on feeding behaviour in *A. rubens*.** To
597 investigate if injection of ArCCK1 and/or ArCCK2 affects feeding behaviour, starved animals
598 were presented with a mussel as prey and then behaviour was observed. By comparison with
599 vehicle-injected animals (control; 10 μ l of distilled water; shown in black), injection of
600 ArCCK1 (a; 10 μ l of 1 mM; shown in green) or ArCCK2 (b; 10 μ l of 1 mM; shown in red)
601 had no effect on the time taken for starfish to make first contact with the mussel. However, by
602 comparison with vehicle-injected animals (control; 10 μ l of distilled water; shown in black)
603 injection of ArCCK1 (c; 10 μ l of 1 mM; shown in green) or ArCCK2 (d; 10 μ l of 1 mM; shown
604 in red) causes an increase in the time elapsed before starfish enclose a mussel. Data are
605 expressed as means \pm s.e.m (n = 13 for control- and 11 for ArCCK1-treated groups; n = 19 for
606 control- and 19 for ArCCK2-treated groups). # indicates a nearly statistically significant
607 difference (P = 0.0523) between vehicle-injected and ArCCK1-injected groups, as determined
608 by two-tailed Mann-Whitney U-test. * indicates statistically significant differences (P < 0.05)
609 between vehicle-injected and ArCCK2-injected groups, as determined by two-tailed Welch's
610 unequal variances t-test. Furthermore, injection of ArCCK1 (e; 10 μ l of 1 mM; shown in green)
611 or ArCCK2 (f; 10 μ l of 1 mM; shown in red) causes a significant decrease in the percentage of
612 starfish that initiate feeding after the mussel is touched for the first time, by comparison with
613 vehicle-injected animals (control; 10 μ l of distilled water; shown in black). * indicates
614 statistically significant differences (P < 0.0001 and P < 0.01 for ArCCK1- and ArCCK2-treated
615 groups respectively) between vehicle-injected and ArCCK1- or ArCCK2-injected groups, as
616 determined by Fisher's exact test.
617

CCK-type signalling in an echinoderm

618 **Discussion**

619 The evolutionary origin of CCK/SK-type neuropeptide signalling has been traced to the
620 common ancestor of the Bilateria, informed by the molecular characterisation of the
621 orthologous CCK-type and SK-type neuropeptide signalling systems in chordates and
622 protostomes, respectively (Bloom et al. 2019, Janssen et al. 2008, Johnsen and Rehfeld 1990,
623 Kubiak et al. 2002, Mirabeau and Joly 2013, Monstein et al. 1993, Nachman et al. 1986a,
624 Schwartz et al. 2018, Yu et al. 2013b, Yu and Smagghe 2014b). Here we report the first
625 molecular and functional characterisation of a CCK/SK-type neuropeptide signalling system
626 in a non-chordate deuterostome - the starfish *A. rubens* (phylum Echinodermata). This provides
627 a key ‘missing link’ in our knowledge of the evolution and comparative physiology of
628 CCK/SK-type signalling, complementing previously reported investigations of CCK-type
629 signalling in chordates and SK-type signalling in protostome invertebrates (e.g. insects).

630

631 **Molecular characterisation of CCK-type signalling system in an echinoderm - the starfish**

632 *A. rubens*

633 Two CCK-type neuropeptides (ArCCK1 and ArCCK2) derived from the precursor
634 protein ArCCKP were detected by mass spectrometry in *A. rubens* radial nerve cord extracts.
635 An evolutionarily conserved feature of CCK-type neuropeptides is a tyrosine (Y) residue that
636 is post-translationally modified by the addition of a sulphate group (Dufresne et al. 2006,
637 Schwartz et al. 2018, Yu and Smagghe 2014a) and accordingly both ArCCK1 and ArCCK2
638 have a sulphated tyrosine. However, non-sulphated forms of these two peptides, ArCCK1(ns)
639 and ArCCK2(ns), were also detected in *A. rubens* nerve cord extracts. Analysis of *A. rubens*
640 neural transcriptome sequence data identified a GPCR (ArCCKR) that is an ortholog of CCK-
641 type receptors that have been characterised in other taxa. Furthermore, heterologous expression
642 of ArCCKR in CHO-K1 cells revealed that the sulphated forms of ArCCK1 and ArCCK2 are
643 potent agonists for ArCCKR, whereas ArCCK2(ns) exhibited little or no agonist activity on
644 this receptor. Therefore, we conclude that the sulphated neuropeptides ArCCK1 and ArCCK2
645 and the GPCR ArCCKR comprise the CCK-type neuropeptide signalling system in the starfish
646 *A. rubens*. The requirement for the tyrosine residues in ArCCK1 and ArCCK2 to be sulphated
647 in order that these peptides can act as potent ligands for ArCCKR is consistent with the
648 properties of many CCK-type peptides in other taxa, including peptides that act as ligands for
649 CCKR1 in mammals and SK-type peptides that act as ligands for the *Drosophila* receptor DSK-
650 R1 (Dufresne et al. 2006, Kubiak et al. 2002). However, this is not a universal feature of CCK-
651 type signalling; for example, non-sulphated CCK-type peptides act as potent ligands for

CCK-type signalling in an echinoderm

652 CCK2R in mammals (Dufresne et al. 2006) and for CKR2a and CKR2b in the nematode *C.*
653 *elegans* (Janssen et al. 2008).

654 Comparison of the sequences of ArCCK1 and ArCCK2 with the sequences of CCK-
655 type peptides that have been identified in other taxa reveal a number of evolutionarily
656 conserved features, including C-terminal amidation and the aforementioned tyrosine (Y)
657 residue. The position of the tyrosine (Y) residue with respect to the C-terminal amide is variable
658 amongst CCK-type peptides, with between five and seven intervening residues. Accordingly,
659 in ArCCK1, ArCCK2 and CCK-type peptides from other echinoderms and hemichordates
660 (collectively Ambulacraria) there are six intervening residues. In ArCCK1 the tyrosine (Y)
661 residue is preceded by an aspartate (D) residue and in both ArCCK1 and ArCCK2 the tyrosine
662 (Y) residue is followed by a glycine (G) residue; accordingly, a DYG motif is a feature of
663 CCK-type peptides in many other taxa. ArCCK1, but not ArCCK2, has an N-terminal
664 pyroglutamate in its mature form and this post-translational modification is also predicted or
665 known to occur in some CCK-type peptides in other taxa – e.g. the SK1 and SK2 peptides from
666 the cockroach *L. maderae* (Predel et al. 1999) and the CCK/SK-type peptides in the molluscs
667 *Pinctata fucata* and *C. gigas* (Schwartz et al. 2018, Stewart et al. 2014). The C-terminal residue
668 of ArCCK2 is a phenylalanine residue (F) and in this respect ArCCK2 is like the majority of
669 CCK-type peptides that have been identified in other taxa. It is noteworthy, therefore, that the
670 C-terminal residue of ArCCK1 is a tryptophan (W) residue, which is also a feature of an
671 ArCCK1-like peptide sequence in another starfish species – the crown-of-thorns starfish
672 *Acanthaster planci* (Smith et al. 2017). Furthermore, this unusual feature of one of the two
673 CCK-type peptides that occur in starfish species appears to be unique to this class of
674 echinoderms (the Asteroidea) because CCK-type peptides that have been identified in other
675 echinoderms all have the more typical C-terminal phenylalanine (F) residue (Chen et al. 2019,
676 Zandawala et al. 2017). Interestingly, however, it is not completely unique to starfish because
677 in the bivalve mollusc *C. gigas* there are two CCK-type peptides, one of which has a C-terminal
678 phenylalanine (F) and another that has a tryptophan (W) residue (Schwartz et al. 2018). Thus,
679 it appears that CCK-type neuropeptides with a C-terminal tryptophan (W) residue may have
680 evolved independently in starfish and in the bivalve mollusc *C. gigas*.

681

682 **Functional characterization of CCK-type neuropeptides in an echinoderm - the starfish** 683 ***A. rubens***

684 Having identified the molecular components of a CCK-type neuropeptide signalling
685 system in *A. rubens*, comprising the sulphated neuropeptides ArCCK1 and ArCCK2 and their

CCK-type signalling in an echinoderm

686 receptor ArCCKR, our next objective was to gain insights into the physiological roles of CCK-
687 type neuropeptides in starfish. Using mRNA *in situ* hybridisation and immunohistochemistry,
688 we examined the anatomical expression patterns of ArCCKP transcripts and one of the
689 neuropeptides derived from ArCCKP (ArCCK1), respectively. This revealed a widespread
690 pattern of expression, including the central nervous system (CNS), tube feet and other body
691 wall associated structures and the digestive system, as discussed below. This pattern of
692 expression can be interpreted with reference to the anatomy of the starfish nervous system
693 (Cobb 1970, Mashanov et al. 2016, Smith 1937), digestive system (Anderson 1953, 1954) and
694 body wall (Blowes et al. 2017) and in comparison with the distribution of other neuropeptides
695 in *A. rubens* (Cai et al. 2018, Elphick et al. 1995, Lin et al. 2017a, Lin et al. 2018, Moore and
696 Thorndyke 1993, Odekunle et al. 2019, Tian et al. 2017, Tinoco et al. 2018, Yáñez-Guerra et
697 al. 2018, Zhang et al. 2020).

698 The starfish CNS consists of radial nerve cords and a circumoral nerve ring, where cells
699 expressing ArCCKP/ArCCK1 were revealed in both the ectoneural and hyponeural regions in
700 common with other neuropeptides such as the calcitonin-type neuropeptide ArCT (Cai et al.
701 2018), pedal peptide/orcokinin-type peptides (Lin et al. 2017a, Lin et al. 2018); the
702 gonadotropin-releasing hormone-type peptide ArGnRH (Tian et al. 2017); and the
703 somatostatin-type peptide ArSS2 (Zhang et al. 2020). The presence of an extensive network of
704 immunostained fibres in the neuropile of the ectoneural region is consistent with neuronal
705 expression of ArCCKP/ArCCK1. Furthermore, regional variation in the density of ArCCK1-ir
706 fibres in the ectoneural neuropile was observed. The ectoneural region is thought to contain
707 sensory neurons, interneurons and motor neurons (Brusca 2017, Cobb 1970, Smith 1937) but
708 the functional identity of neuronal cell types and the neural circuitry are not known. Therefore,
709 it is not possible at present to determine the functional properties of ArCCK-ir neurons in the
710 ectoneural region of the CNS. However, the hyponeural region of the starfish CNS only
711 comprises motoneurons and the projection pathways of the axons of these motoneurons have
712 been reported (Lin et al. 2017a, Smith 1950). Thus, the axons of hyponeural motor neurons
713 project around the tube feet and coalesce as a fibre bundle known as the lateral motor nerve
714 (Lin et al. 2017a, Smith 1937). Consistent, with the expression of ArCCKP in hyponeural cells,
715 ArCCK1-ir fibres can be seen in the lateral motor nerves, as has been previously reported for
716 other *A. rubens* neuropeptides (Cai et al. 2018, Lin et al. 2017a, Lin et al. 2018, Zhang et al.
717 2020). Branches of the lateral motor nerves project into the coelomic lining of the body wall
718 in starfish (Smith 1937, 1950) and accordingly ArCCK1-ir fibres were observed in coelomic
719 lining of the body wall in *A. rubens*. Furthermore, the presence of ArCCK1-ir fibres in inter-

CCK-type signalling in an echinoderm

720 ossicular tissue of the body wall in *A. rubens* suggests that ArCCKP-expressing hyponeural
721 cells may include motoneurons that innervate inter-ossicular muscles and/or inter-ossicular
722 mutable collagenous tissue (Blowes et al. 2017).

723 ArCCKP/ArCCK1 is widely expressed in the digestive system of *A. rubens*, including
724 the oesophagus, peristomial membrane, cardiac stomach, pyloric stomach, pyloric duct, pyloric
725 caeca, intestine and rectal caeca. Consistent with the expression of ArCCKP/ArCCK1 in the
726 cardiac stomach, ArCCK1 and ArCCK2 cause concentration-dependent contraction of cardiac
727 stomach preparations *in vitro*. ArCCKP/ArCCK1-expressing cells are present in the mucosal
728 wall of the cardiac stomach and a dense network of ArCCK1-ir fibres is present in the
729 basiepithelial nerve plexus of the cardiac stomach, particularly in the highly folded lateral
730 pouches of the cardiac stomach. Therefore, CCK-type neuropeptides released by fibres in the
731 basiepithelial nerve plexus of the cardiac stomach may diffuse across an intervening thin layer
732 of collagenous tissue to cause contraction of visceral muscle. Other peptides that cause cardiac
733 stomach contraction *in vitro* have been identified in *A. rubens*, which include NGFFYamide
734 (Semmens et al. 2013), the GnRH-type peptide ArGnRH and the corazonin-type neuropeptide
735 ArCRZ (Tian et al. 2017). In comparison with effects of NGFFYamide on cardiac stomach
736 preparations *in vitro* (Semmens et al. 2013), ArCCK1 and ArCCK2 exhibit lower potency but
737 higher efficacy. In comparison with effects of ArGnRH and ArCRZ on cardiac stomach
738 preparations *in vitro* (Tian et al. 2017), ArCCK1 and ArCCK2 exhibit higher potency and
739 efficacy. These observations on the *in vitro* effects ArCCK1 and ArCCK2 on cardiac stomach
740 preparations provided a basis for examining the *in vivo* effects of these peptides on feeding
741 related processes in *A. rubens*.

742 Starfish exhibit one of the most remarkable feeding behaviours in the animal kingdom
743 – they evert their stomach out of their mouth and digest large prey externally, and once the
744 prey has been digested, the stomach is withdrawn. Extra-oral feeding in starfish requires
745 relaxation of muscle in the wall of the cardiac stomach and in intrinsic and extrinsic retractor
746 strands to enable stomach eversion. Then when external digestion and ingestion of prey tissue
747 is completed, contraction of the musculature enables cardiac stomach retraction (Anderson
748 1954). Previous studies have identified neuropeptides that cause relaxation of the cardiac
749 stomach *in vitro* and trigger cardiac stomach eversion when injected *in vivo*; for example, the
750 SALMFamide neuropeptide S2 (Melarange et al. 1999), the vasopressin/oxytocin-type
751 neuropeptide asterotocin (Odekunle et al. 2019) and the somatostatin-type neuropeptide ArSS2
752 (Zhang et al. 2020). Conversely, NGFFYamide has been identified as a neuropeptide in *A.*
753 *rubens* that triggers contraction of the cardiac stomach *in vitro* and retraction of the everted

CCK-type signalling in an echinoderm

754 stomach when injected *in vivo* (Semmens et al. 2013). The *in vitro* effects of ArCCK1 and
755 ArCCK2 in causing contraction of the cardiac stomach and the presence of ArCCK1-ir fibres
756 in the basiepithelial nerve plexus of the cardiac stomach and in retractor strands suggest that
757 CCK-type peptides may participate in mechanisms of cardiac stomach retraction
758 physiologically. Accordingly, injection of 10 µl of 1 mM ArCCK1 or 1 mM ArCCK2 triggered
759 partial or complete retraction of the cardiac stomach within a test period of six minutes. These
760 experiments indicate that CCK-type peptides may be involved in physiological mechanisms of
761 cardiac stomach retraction in starfish. Furthermore, to investigate the importance of tyrosine
762 (Y) sulphation for the bioactivity of CCK-type peptides in *A. rubens*, we also tested the non-
763 sulphated peptide ArCCK2(ns) on cardiac stomach preparations. By comparison with ArCCK1
764 and ArCCK2, ArCCK2(ns) had a very modest contracting effect on cardiac stomach
765 preparations *in vitro* and did not trigger cardiac stomach retraction *in vivo*. This is consistent
766 with low potency of ArCCK2(ns) as an agonist on ArCCKR expressed in CHO cells and
767 indicative that non-sulphated CCK-type peptides are not bioactive physiologically in starfish.

768 The *in vivo* effect of CCK-type neuropeptides in triggering cardiac stomach retraction
769 is indicative of a role in physiological mechanisms that control termination of feeding
770 behaviour in starfish. By way of comparison, the neuropeptide NGFFYamide that triggers
771 retraction of the everted stomach of *A. rubens* also causes a significant delay in the onset of
772 feeding on prey (mussels) when injected *in vivo* (Tinoco et al. 2018). Accordingly, here we
773 observed that starfish injected with CCK-type neuropeptides took longer to enclose prey
774 compared to control animals injected with water. Furthermore, in animals injected with CCK-
775 type neuropeptides the proportion of starfish that successfully consumed prey was fewer than
776 in control animals that were injected with water. Thus, collectively these findings indicate that
777 CCK-type signalling acts as a physiological regulator that inhibits and/or terminates feeding
778 behaviour in starfish.

779 Investigation of the *in vitro* pharmacological effects of neuropeptides in starfish has
780 revealed that some peptides that act as contractants or relaxants of the cardiac stomach also
781 cause contraction or relaxation, respectively, of two other muscular tissues/organs – tube feet
782 and the body wall associated apical muscle. For example, the GnRH-type peptide ArGnRH
783 and the corazonin-type peptide ArCRZ cause contraction of all three preparations (Tian et al.
784 2017) and the SALMFamide-type neuropeptides S1 and S2 and the pedal peptide/orcokinin-
785 type peptide ArPPLN1b (starfish myorelaxant peptide) cause relaxation of all three
786 preparations (Lin et al. 2017a, Melarange and Elphick 2003).

CCK-type signalling in an echinoderm

787 Informed by detection of ArCCKP/ArCCK1 expression in the tube feet of *A. rubens*,
788 we tested ArCCK1 and ArCCK2 on *in vitro* preparations of these locomotory organs and found
789 that both peptides cause dose-dependent contraction. ArCCKP-expressing cells are present at
790 the base of tube foot podium proximal to the junction with the radial nerve cord or marginal
791 nerve and also in the disk region proximal to the basal nerve ring. Consistent with this pattern
792 of precursor protein expression, ArCCK1-ir was revealed in the basiepithelial nerve plexus and
793 in the basal nerve ring. The basiepithelial nerve plexus is separated from the tube foot muscle
794 layer by a thin layer of collagenous tissue and therefore, consistent with mechanisms proposed
795 for control tube foot myoactivity by the neurotransmitter acetylcholine (Florey and Cahill
796 1980, Florey et al. 1975), CCK-type peptides released by nerve processes in the basiepithelial
797 nerve plexus may diffuse across the collagenous tissue layer to cause contraction of the
798 longitudinally orientated tube foot muscle *in vivo*. Furthermore, from a behavioural
799 perspective, CCK-type neuropeptides may participate in neural mechanisms controlling tube
800 foot retraction during locomotor activity and/or for generation of force when the collective
801 pulling power of tube feet is used by starfish to prise apart the valves of prey such as mussels
802 (Lavoie 1956).

803 Although expression of ArCCKP/ArCCK1 was not detected in the apical muscle,
804 ArCCK1-ir fibres were revealed proximally in the coelomic lining of the body wall. Therefore,
805 we also tested ArCCK1 and ArCCK2 on *in vitro* preparations of the apical muscle from *A.*
806 *rubens* and found that both peptides caused dose-dependent contraction. However, by
807 comparison with their effects on tube foot preparations, the effects of ArCCK1 and ArCCK2
808 on apical muscle preparations were quite modest. Thus, the effects of 1 μ M ArCCK1 or
809 ArCCK2 on apical muscle preparations were ~40% of the effect of 10 μ M ACh, whereas the
810 effects of 1 μ M ArCCK1 or ArCCK2 on tube foot preparations were, respectively, 220% and
811 110% of the effect of 10 μ M ACh. Finally, it is noteworthy that ArCCK1-ir fibres were
812 revealed in the inter-ossicular tissue that contains muscles and mutable collagenous tissue that
813 interlink the calcite ossicles of the body wall endoskeleton (Blowes et al. 2017). Therefore,
814 CCK-types neuropeptides in *A. rubens* may also have physiological roles in regulating the
815 contractile state of inter-ossicular muscles and/or the stiffness of inter-ossicular mutable
816 collagenous tissue.

817

818 **Comparative and evolutionary physiology of CCK/SK-type neuropeptide signalling in**
819 **the Bilateria**

CCK-type signalling in an echinoderm

820 Our *in vitro* pharmacological analysis of the effects of ArCCK-type neuropeptides in
821 *A. rubens* revealed a myoexcitatory action, consistent with findings from previous studies on
822 vertebrates and protostome invertebrates. For example, CCK causes pyloric and gallbladder
823 contraction in mammals (Gutiérrez et al. 1974, Rehfeld 2017, Shaw and Jones 1978, Vizi et al.
824 1973) and gut contraction in other vertebrates (Tinoco et al. 2015). Accordingly, SK-type
825 peptides also have myoexcitatory effects in insects (Al-Alkawi et al. 2017, Maestro et al. 2001,
826 Marciniak et al. 2011, Nachman et al. 1986b, Nichols 2007, Palmer et al. 2007, Predel et al.
827 2001). However, CCK-type peptides are not exclusively myoexcitatory because, for example,
828 CCK causes relaxation of the proximal stomach in mammals; however, this myoinhibitory
829 effect of CCK is indirect and mediated by vagal and splanchnic afferents (Takahashi and
830 Owyang 1999). Accordingly, both inhibitory and excitatory effects of CCK-8 on the stomach
831 of a non-mammalian vertebrate, the rainbow trout *Oncorhynchus mykiss*, have been reported
832 (Olsson et al. 1999) and a CCK/SK-type peptide causes a decrease in the frequency of hindgut
833 contraction in the mollusc *C. gigas* (Schwartz et al. 2018). Furthermore, and directly relevant
834 to this study, it has been reported that mammalian CCK-8 causes *in vitro* relaxation of
835 intestine preparations from the sea cucumber of *Holothuria glaberrima* (García-Arrarás et al.
836 1991). With the determination of the amino-acid sequences of native CCK-type neuropeptides
837 in sea cucumbers (Chen et al. 2019, Zandawala et al. 2017), it will now be possible to
838 specifically investigate their pharmacological effects in these animals to make direct
839 comparisons with the findings reported here for starfish.

840 As discussed below, CCK/SK-type neuropeptides are perhaps best known for their roles
841 as inhibitory regulators of feeding. However, in common with other neuropeptides, they are
842 pleiotropic in their physiological roles. Thus, linked to regulation feeding, CCK/SK-type
843 neuropeptides stimulate secretion of gastric acid and/or digestive enzymes in mammals,
844 insects, nematodes, ascidians and molluscs (Bevis and Thorndyke 1981, Chen et al. 2004,
845 Harper and Raper 1943, Harshini et al. 2002b, Janssen et al. 2008, Nachman et al. 1997, Shaw
846 and Jones 1978, Thorndyke and Bevis 1984, Zels et al. 2015). Accordingly, expression of
847 CCK-type peptides by cells in several regions of the digestive system in *A. rubens* may be
848 indicative of a similar role in starfish. Furthermore, CCK precursor transcripts are detected in
849 rat spinal motoneurons (Cortés et al. 1990) and SK-type neuropeptides act as positive growth
850 regulators for neuromuscular junction formation and promote locomotion in larval *Drosophila*
851 (Chen and Ganetzky 2012). In this context, it is noteworthy that CCK-type neuropeptides cause
852 contraction of body wall associated muscles (apical muscle) and organs (tube feet) in starfish.
853 Accordingly, the expression of CCK-type peptides in hyponeural motoneurons, the lateral

CCK-type signalling in an echinoderm

854 motor nerve and inter-ossicular tissue of the body wall of *A. rubens* may reflect evolutionarily
855 ancient and conserved roles of CCK-type neuropeptides as regulators of skeletal muscle
856 function. It is also noteworthy that CCK is one of the most abundantly expressed neuropeptides
857 in the cortex of the mammalian brain, where it is expressed by sub-populations of GABAergic
858 interneurons and acts as a multi-functional molecular switch to regulate the output of cortical
859 neuronal circuits (Lee and Soltesz 2011). Furthermore, evidence that expression of CCK in
860 GABAergic neurons is an evolutionarily ancient association was provided by a recent study
861 reporting co-localisation of CCK-8 and GABA in several different neuronal populations in the
862 brain of the sea lamprey *Petromyzon marinus* (Sobrido-Cameán et al. 2020). GABA-
863 immunoreactive neurons have been revealed in the ectoneural region of the radial nerve cord
864 in *A. rubens* (Newman and Thorndyke 1994) and sub-populations of these neurons may
865 correspond with cells expressing CCK-type neuropeptides reported in this study. However, the
866 inaccessibility and small size of these neurons may preclude investigation of their
867 electrophysiological properties.

868 The key functional insights from this study are our observations that in the starfish *A.*
869 *rubens* CCK-type neuropeptides trigger cardiac stomach contraction and retraction and induce
870 a delay in the onset of feeding and a reduction in predation. These findings are of general
871 interest because of the previously reported evidence that CCK/SK-type neuropeptides mediate
872 physiological mechanisms of satiety and/or regulate feeding behaviour in vertebrates, insects
873 and the mollusc *C. gigas* (Al-Alkawi et al. 2017, Downer et al. 2007, Himick and Peter 1994,
874 Kang et al. 2011, Maestro et al. 2001, Meyering-Vos and Muller 2007, Nachman et al. 1986a,
875 Nachman et al. 1986b, Nässel and Zandawala 2019, Rehfeld 2017, Roman et al. 2017,
876 Schwartz et al. 2018, Wei et al. 2000, Yu et al. 2013a, Yu and Smagghe 2014b, Zels et al. 2015,
877 Zhang et al. 2017). Furthermore, insights into the mechanisms by which CCK/SK-type
878 neuropeptides regulate feeding behaviour in mammals and insects have been obtained. In
879 mammals CCK released by intestinal endocrine cells acts on vagal afferents, which is thought
880 to then lead to activation of calcitonin-gene related peptide (CGRP)-expressing neurons in the
881 parabrachial nucleus that suppress feeding and inhibition of Agouti-related peptide (AgRP)-
882 expressing hypothalamic neurons that promote feeding (Beutler et al. 2017, Essner et al. 2017).
883 In *Drosophila*, SK-type neuropeptides are expressed by a sub-population median
884 neurosecretory cells in the brain that also produce insulin-like peptides and results from a
885 variety of experimental studies indicate that release of SK-type neuropeptides by these neurons
886 induces satiety in both larval and adult flies (Nässel and Williams 2014). Thus, an
887 evolutionarily conserved physiological role of CCK/SK-type neuropeptides as inhibitory

CCK-type signalling in an echinoderm

888 regulators of feeding are mediated by different mechanisms in mammals and insects, which
889 may reflect evolutionary divergence in the anatomy of these taxa. It is interesting, therefore,
890 that in the unique context of the evolutionary and developmental replacement of bilateral
891 symmetry with pentaradial symmetry in adult echinoderms, an ancient role of CCK/SK-type
892 neuropeptides as inhibitory regulators of feeding-related processes has been retained in starfish.
893 Furthermore, because feeding in starfish is accomplished by stomach eversion, there is a direct
894 link between the action of CCK-type neuropeptides on the gastro-intestinal system and
895 inhibition/termination of feeding behaviour. Thus, our findings from starfish reported here for
896 CCK/SK-type neuropeptides and previously for other neuropeptides (Cai et al. 2018, Odekunle
897 et al. 2019, Tian et al. 2017, Tinoco et al. 2018, Zhang et al. 2020) reveal how ancient roles of
898 neuropeptide signalling systems have been preserved in spite of unique and radical
899 evolutionary and developmental changes in the anatomy of echinoderms amongst bilaterian
900 animals.
901

CCK-type signalling in an echinoderm

902 **Material and methods**

903 **Animals**

904 Adult starfish (*Asterias rubens*, Linnaeus, 1758) were collected at low tide near
905 Margate (Kent, UK) or obtained from a fisherman based at Whitstable (Kent, UK). The starfish
906 were maintained in a circulating seawater aquarium under a 12 h -12 h of light - dark cycle
907 (lights on at 8 a.m.) at a temperature of ~12°C and salinity of 32 ‰, located in the School of
908 Biological & Chemical Sciences at Queen Mary University of London. Animals were fed on
909 mussels (*Mytilus edulis*) that were collected at low tide near Margate (Kent, UK). Additionally,
910 juvenile specimens of *A. rubens* (diameter 0.5 - 1.5 cm) used for anatomical studies were
911 collected at the University of Gothenburg Sven Lovén Centre for Marine Infrastructure
912 (Kristineberg, Sweden).

913

914 **Cloning of a cDNA encoding the *Asterias rubens* CCK-type precursor ArCCKP**

915 A cDNA encoding the ArCCK precursor (ArCCKP), including 5' and 3' untranslated
916 regions (UTR) and the complete open reading frame (ORF), was amplified by PCR (Phusion
917 High-Fidelity PCR Master Mix, NEB, Hitchin, Hertfordshire, UK) using specific
918 oligonucleotide primers (5'-TCGCTACTGTTTCTCTCGCA-3' and 5'-
919 AAAGGCGTCAACAACACTGCTT-3'), which were designed using Primer3 software
920 (<http://bioinfo.ut.ee/primer3-0.4.0/>) with reference to the ArCCKP transcript sequence (contig
921 1124413; GenBank accession number KT601716) obtained from *A. rubens* radial nerve cord
922 transcriptome data (Semmens et al. 2016). The PCR product was gel-extracted and purified
923 (QIAquick Gel Extraction Kit, Qiagen, Manchester, UK) before being blunt-end cloned into
924 pBluescript SKII (C) (Agilent Technologies, Stockport, Cheshire, UK) or Zero Blunt® Topo
925 PCR (ThermoFisher Scientific; Waltham, MA, USA) vectors. The clones were sequenced
926 (Eurofins Genomics GmbH, Ebersberg, Germany) using the T7 and T3 sequencing primer
927 sites.

928

929 **Localisation of ArCCKP expression in *A. rubens* using mRNA *in situ* hybridization**

930 To enable visualisation of ArCCKP transcripts in *A. rubens* using mRNA *in situ*
931 hybridization, digoxigenin-labelled RNA probes were synthesised. Zero Blunt® Topo or
932 pBluescript SKII (+) vectors containing the ArCCKP cDNA were purified (Qiagen Maxiprep,
933 Qiagen, Manchester, UK) and 5 µg of the vector was linearized using restriction enzymes
934 (NEB, Hitchin, Hertfordshire, UK). Linearized vector containing the ArCCKP cDNA were
935 cleaned using phenol-chloroform (Sigma-Aldrich, Gillingham, UK) and chloroform-

CCK-type signalling in an echinoderm

936 isomylalcohol (Sigma-Aldrich, Gillingham, UK) extractions and then precipitated using 1/10
937 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol (Honeywell™, Fisher
938 Scientific UK Ltd, Loughborough, UK) at -80°C. The pellet was washed with 70% ice-cold
939 ethanol before air drying and re-suspending in autoclaved water. Sense and antisense RNA
940 probes were synthesized using digoxigenin nucleotide triphosphate (DIG-NTP) mix (Roche,
941 Mannheim, Germany), 5x transcription buffer (NEB, Hitchin, Hertfordshire, UK), 0.2 M
942 dithiothreitol (DTT) (Promega, Madison, USA), placental ribonuclease inhibitor (10 U/μl)
943 (Promega, Madison, USA) and T7 polymerase (50 U/μl) or T3 polymerase (50 U/μl) (NEB,
944 Hitchin, Hertfordshire, UK) with 1 μg of linearised vector containing the ArCCKP cDNA.
945 Template DNA was then digested with RNase free DNase (NEB, Hitchin, Hertfordshire, UK).
946 RNA probes were stored in 25% formamide/2x saline-sodium citrate (25% FA/2x SSC; VWR
947 Chemicals, Leicestershire, UK) at -20°C for long-term storage.

948 To prepare specimens of *A. rubens* for mRNA *in situ* hybridisation, animals were fixed
949 by immersion in 4% paraformaldehyde (PFA; Sigma-Aldrich, Gillingham, UK) in phosphate-
950 buffered saline (PBS) overnight at 4°C. Specimens were washed in PBS, dissected and placed
951 in Morse's solution (10% sodium citrate; 20% formic acid in autoclaved water) to enable
952 decalcification of ossicles in the body wall of starfish. Decalcified specimens were then washed
953 in autoclaved water, dehydrated through a graded ethanol series and then immersed in xylene
954 (Honeywell, Fisher Scientific UK Ltd, Loughborough, UK) before being embedded in paraffin
955 wax. 14 μm sections of *A. rubens* arms and central disk were prepared using a RM 2145
956 microtome (Leica Microsystems [UK], Milton Keynes, UK). Sections were collected on poly-
957 L-lysine coated slides (VWR Chemicals, Lutterworth, Leicestershire, UK) that had been placed
958 on a hot plate and covered with autoclaved water. Slides were left to dry before proceeding
959 with probe hybridization and immunodetection.

960 Slides were kept at 60°C for 1 hour to allow excess wax to melt before leaving to cool
961 at room temperature. Sections were then deparaffinised in xylene and hydrated through a
962 graded ethanol series before being washed in PBS. Sections were then post-fixed in 4%
963 PFA/PBS before washing with buffer containing Proteinase K (PK; Qiagen UK Ltd,
964 Manchester, UK) (1 μg/ml PK, 50 mM Tris-HCl [pH 7.5]; 6.25 mM EDTA in autoclaved
965 water; Thermo Fisher Scientific, Oxford, UK) at 37°C for 12 minutes. Sections were then post-
966 fixed in 4% PFA/PBS before washing with PBS/Tween 0.1% and then acetylated (1.325%
967 triethanolamine [pH 7-8]; 0.25% acetic anhydride; 0.175% HCl in autoclaved water; VWR
968 Chemicals, Lutterworth, UK) for 10 minutes. Sections were washed in PBS/0.1% Tween-20
969 and in 5x SSC. Then sections were dried, placed in a humidified chamber and covered with

CCK-type signalling in an echinoderm

970 hybridisation buffer (50% formamide; 5x SSC; 500 µg/ml yeast total RNA; 50 µg/ml heparin;
971 0.1% Tween-20 in autoclaved water) at room temperature for 2 hours. ArCCK precursor sense
972 and anti-sense probes (500-1000 ng/ml) were denatured in hybridisation buffer at 80°C and
973 placed on ice before adding remaining hybridisation buffer and applying 100 µl probe solution
974 per slide. Slides were covered with a piece of Parafilm (Bemis, Terre Haute, IN, USA) and
975 then placed in a humidified chamber at 65°C overnight. Sections were then washed in 0.2x
976 SSC at 65°C, in 0.2x SSC at room temperature and equilibrated in buffer B1 (10 mM Tris-HCl
977 [pH 7.5]; 150 mM NaCl in autoclaved water). Sections were covered in buffer B1/5% goat
978 serum and placed in a humidified chamber at room temperature for 2 hours. Sections were then
979 dried and covered in an alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:3000;
980 Roche, Mannheim, Germany) in buffer B1/2.5% goat serum at 4°C overnight. Slides were
981 washed in buffer B1 and then equilibrated in buffer B3 (100 mM Tris-HCl [pH 9.5]; 100 mM
982 NaCl; 50 mM MgCl₂ in autoclaved water). Sections were then covered in buffer B3/0.1%
983 Tween-20 with nitro-blue tetrazolium chloride (NBT; Sigma-Aldrich, Gillingham, UK) (75
984 mg/ml in 70% dimethylformamide) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
985 (BCIP; Sigma-Aldrich, Gillingham, UK) substrate solution (50 mg/ml BCIP in autoclaved
986 water) until strong staining was observed. The slides were washed in distilled water to stop the
987 staining reaction and then were dried on a hot plate before rinsing in 100% ethanol and Histo-
988 Clear (National Diagnostics, Fisher Scientific UK Ltd, Loughborough, UK). Sections were
989 mounted with a coverslip on HistoMount solution (National Diagnostics, Fisher Scientific UK
990 Ltd, Loughborough, UK) for long-term storage.

991

992 **Mass spectrometry**

993 Extracts of *A. rubens* radial nerve cords were prepared and analysed using mass
994 spectrometry (NanoLC-ESI-MS/MS), as described in detail previously for the *A. rubens*
995 relaxin-like gonad stimulating peptide, which contains disulphide bridges (Lin et al. 2017b).
996 Aliquots of radial nerve cord extract were not treated with trypsin but were subjected to
997 reduction to break disulphide bridges (using 100 mM dithiothreitol; Sigma Aldrich,
998 Gillingham, UK) followed by alkylation of cysteine residues (using 200 mM iodoacetamide;
999 Sigma Aldrich, Gillingham, UK). Raw data were converted to Mascot generic format using
1000 MSConvert in ProteoWizard Toolkit (v. 3.0.5759) (Kessner et al. 2008). MS spectra were
1001 searched with Mascot engine (Matrix Science, v. 2.4.1) (Nesvizhskii et al. 2003) against a
1002 database comprising 40 *A. rubens* neuropeptide precursor proteins, including ArCCKP
1003 (Semmens et al. 2016), all proteins in GenBank from species belonging to the family Asteroidea

CCK-type signalling in an echinoderm

1004 and the common Repository of Adventitious Proteins Database
1005 (<http://www.thegpm.org/cRAP/index.html>). A no-enzyme search was performed with up to
1006 two missed cleavages and carbamidomethyl as a fixed modification. Post-translational
1007 amidation of C-terminal glycine residues, pyroglutamylation of N-terminal glutamine residues,
1008 sulphation of tyrosine residues and oxidation were included as variable modifications.
1009 Precursor mass tolerance was 10 ppm and product ions were searched at 0.8 Da tolerances.

1010 Scaffold (version Scaffold_4.6.1, Proteome Software Inc.) was used to validate MS/MS
1011 based peptide and protein identifications. Peptide identifications were accepted if they could
1012 be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein
1013 identifications were accepted if they could be established at greater than 95.0% probability and
1014 contained at least two identified peptides.

1015

1016 **Alignment of the *A. rubens* CCK-type neuropeptides ArCCK1 and ArCCK2 with CCK-** 1017 **type peptides from other taxa**

1018 Having used mass spectrometry to confirm the structures of the mature peptides
1019 ArCCK1 and ArCCK2 that are derived from ArCCKP, the sequences of ArCCK1 and ArCCK2
1020 were aligned with CCK-type peptides from other taxa to investigate the occurrence of
1021 evolutionarily conserved residues. The alignment was generated using MAFFT (version 7)
1022 with the following parameters (BLOSUM62, 200 PAM/K=2) and highlighted using
1023 BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) using 70% conservation
1024 as a minimum for highlighting. The accession numbers for the sequences used for this analysis
1025 are shown in **Figure 1 – source data 1**.

1026

1027 **Identification a transcript encoding an *A. rubens* CCK-type receptor**

1028 A transcript encoding an *A. rubens* CCK-type receptor (ArCCKR) was identified by
1029 tBLASTn analysis of the *A. rubens* radial nerve cord transcriptome data (Semmens et al. 2016),
1030 using SequenceServer [<https://www.sequenceserver.com>; (Priyam et al. 2015)] and a
1031 *Strongylocentrotus purpuratus* CCK-type receptor (Accession number XP_782630.3) as the
1032 query sequence. To investigate in more detail the relationship of ArCCKR with CCK-type
1033 receptors that have been identified in other taxa, phylogenetic analyses were performed using
1034 the maximum-likelihood method. The sequences of ArCCKR and CCK-type receptors from a
1035 variety of taxa were aligned using MUSCLE (iterative, 10 iterations, UPGMB as clustering
1036 method) (Edgar 2004). The maximum-likelihood tree was generated using IQ-tree web server
1037 [1000 bootstrap replicates, LG+F+I+G4 substitution model; (Trifinopoulos et al. 2016)]. The

CCK-type signalling in an echinoderm

1038 accession numbers of the protein sequences that were used for this analysis are listed in **Figure**
1039 **2 – source data 1**.

1040

1041 **Pharmacological characterization of ArCCKR**

1042 To enable testing of ArCCK1 and ArCCK2 as candidate ligands for ArCCKR, a full-
1043 length cDNA encoding ArCCKR was synthesized by GenScript (Piscataway, NJ, USA) and
1044 cloned into pcDNA 3.1+ vector (Invitrogen™, ThermoFisher Scientific; Waltham, MA, USA).
1045 A partial Kozak translation initiation sequence (CACC) was introduced upstream to the start
1046 codon (ATG). Chinese hamster ovary (CHO)-K1 cells stably expressing the mitochondrial
1047 targeted calcium-sensitive bioluminescent reporter GFP-aequorin fusion protein (G5A) were
1048 used as a heterologous expression system for ArCCKR. Cells were cultured, co-transfected
1049 with the pcDNA 3.1+ vector containing the ArCCKR cDNA sequence and plasmids encoding
1050 the promiscuous human G-protein G_α16. Then bioluminescence-based receptor assays were
1051 performed, as described previously for *A. rubens* luqin-type receptors (Yáñez-Guerra et al.
1052 2018).

1053 CCK-type peptides in other taxa have a sulphated tyrosine residue that is important for
1054 bioactivity and therefore the ArCCK1 and ArCCK2 peptides were synthesized (Peptide Protein
1055 Research Ltd, Fareham, UK) with sulphated tyrosine residues:
1056 pQSKVDDY(SO₃H)GHGLFW-NH₂ (ArCCK1), and GGDDQY(SO₃H)GFGLFF-NH₂
1057 (ArCCK2). Furthermore, to assess the requirement of tyrosine sulphation for receptor
1058 activation and bioactivity, a non-sulphated form of ArCCK2 was also synthesized:
1059 GGDDQYGFGLFF-NH₂ [ArCCK2(ns)]. The peptides were diluted in distilled water and
1060 tested as candidate ligands for ArCCKR at concentrations ranging from 3 x 10⁻¹⁷ M to 10⁻⁴ M.
1061 Concentration-response data were determined as a percentage of the highest response for each
1062 peptide (100% activation). EC₅₀ values were calculated from concentration-response curves
1063 based on 4 to 6 independent transfections and averaging 2 - 3 replicates in each transfection
1064 using Prism 6.0 (GraphPad software, La Jolla, CA). Cells transfected with an empty vector
1065 were used for control experiments. Other *A. rubens* neuropeptides [Luqin:
1066 EEKTRFPKFMRW-NH₂ (ArLQ); tachykinin-like peptide 2: GGGVPHVFQSGGIFG-NH₂
1067 (ArTK2); (Semmens et al. 2016; Yáñez-Guerra et al. 2018)] were tested at a concentration of
1068 10 μM to assess the specificity of receptor activation.

1069

1070 **Generation and characterisation of antibodies to ArCCK1**

CCK-type signalling in an echinoderm

1071 To facilitate immunohistochemical analysis of the expression of a ArCCKP-derived
1072 neuropeptide in *A. rubens*, we generated antibodies to ArCCK1. To accomplish this an N-
1073 terminally truncated peptide analog of ArCCK1 with the addition of a reactive N-terminal
1074 lysine residue was synthesized (KY(SO₃H)GHGLFW-NH₂, Peptide Protein Research Ltd,
1075 Fareham, UK). This peptide was conjugated to porcine thyroglobulin (Sigma-Aldrich,
1076 Gillingham, UK) as a carrier protein using 5% glutaraldehyde (Sigma-Aldrich, Gillingham,
1077 UK) in phosphate buffer (0.1 M; pH 7.2) and the conjugate was used for immunisation of a
1078 rabbit (70-day protocol; Charles River Biologics, Romans, France). The antigen was emulsified
1079 in Freund's complete adjuvant for primary immunisations (~100 nmol antigen peptide) and in
1080 Freund's incomplete adjuvant for three booster immunisations (~50 nmol antigen peptide). The
1081 presence of antibodies to the antigen peptide in post-immunisation serum samples was assessed
1082 using an enzyme-linked immunosorbent assay (ELISA), in comparison with pre-immune
1083 serum (**Figure 5 – figure supplement 1a**). Antibodies to the antigen peptide were purified
1084 from the final bleed antiserum by affinity-purification using the AminoLink Plus
1085 Immobilization Kit (ThermoFisher Scientific, Waltham, MA, USA), with bound antibodies
1086 eluted using glycine elution buffer [6.3 ml of 100 mM glycine (VWR Chemicals,
1087 Leicestershire, UK) and 0.7 ml of Tris (1M, pH = 7.0)] and trimethylamine (TEA) elution
1088 buffer [6.3 ml of TEA (Sigma-Aldrich, Gillingham, UK) and 0.7 ml of Tris (1M, pH = 7.0)].
1089 Eluates were dialyzed and sodium azide (0.1%) was added for long-term storage of the affinity-
1090 purified antibodies at 4°C. The specificity of antibodies eluted with TEA, which were
1091 subsequently used for immunohistochemistry (see below), was assessed by ELISA by testing
1092 them at a concentration of 1:10 with the following synthetic peptides [100 µl at a concentration
1093 of 1 µM dissolved in carbonate/bicarbonate buffer (25 mM sodium carbonate, 25 mM sodium
1094 bicarbonate, pH = 9.8)]: ArCCK1, ArCCK2, ArCCK2(ns) and ArLQ (**Figure 5 – figure
1095 supplement 1b**). The rabbit antiserum to ArCCK1 has been assigned the RRID:AB_2877176.

1096

1097 **Immunohistochemical localisation of ArCCK1 in *A. rubens***

1098 Small specimens of *A. rubens* (< 6 cm diameter) were fixed by immersion in seawater
1099 Bouin's fluid [75% saturated picric acid (Sigma-Aldrich, Gillingham, UK) in seawater, 25%
1100 formaldehyde, 5% acetic acid] for 3 to 4 days at 4°C and then were decalcified for a week
1101 using a 2% ascorbic acid/0.3 M sodium chloride solution, dehydrated and embedded in paraffin
1102 wax. Sections of the arms and the central disk region (8 µm; transverse or horizontal) were cut
1103 using a microtome (RM 2145, Leica Microsystems [UK], Milton Keynes, UK) and mounted
1104 on chrome alum/gelatin coated microscope slides. Paraffin wax was removed by immersion of

CCK-type signalling in an echinoderm

1105 slides in xylene, and then slides were immersed in 100% ethanol. Endogenous peroxidase
1106 activity was quenched using a 0.3% hydrogen peroxide (VWT Chemicals, Leicestershire,
1107 UK)/methanol solution for 30 min. Subsequently, the slides were rehydrated through a graded
1108 ethanol series (90, 70 and 50%) and distilled water, blocked in 5% goat serum (NGS; Sigma-
1109 Aldrich, Gillingham, UK) made up in PBS containing 0.1% Tween (PBST). Then, the slides
1110 were incubated overnight with affinity-purified rabbit antibodies to ArCCK1 (TEA fraction
1111 diluted 1:10 in 5% NGS/PBST). Following a series of washes in PBST, indirect
1112 immunohistochemical detection was carried out using Peroxidase-AffiniPure Goat Anti-Rabbit
1113 IgG (H+L) conjugated to Horseradish Peroxidase (Jackson ImmunoResearch, West Grove,
1114 PA) diluted 1:1000 in 2% NGS/PBST. Bound antibodies were revealed using a solution
1115 containing 0.015% hydrogen peroxide, 0.05% diaminobenzidine (VWR Chemicals,
1116 Leicestershire, UK) and 0.05% nickel chloride (Sigma-Aldrich, Gillingham, UK) in PBS.
1117 When strong staining was observed, sections were washed in distilled water, dehydrated
1118 through a graded ethanol series (50, 70, 90 and 100%) and washed in xylene before being
1119 mounted with coverslips on DPX mounting medium (ThermoFisher Scientific, Waltham, MA,
1120 USA). Immunostaining was not observed in negative control tests without the primary
1121 antibodies or with primary antibodies that had been pre-adsorbed with the antigen peptide at a
1122 concentration of 20 μ M (data not shown).

1123

1124 **Imaging**

1125 Photographs of sections processed for mRNA *in situ* hybridization or
1126 immunohistochemistry were captured using a QIClick™ CCD Color Camera (Qimagin, British
1127 Columbia, CA) linked to a DMRA2 light microscope (Leica), utilising Volocity® v.6.3.1
1128 image analysis software (PerkinElmer, Seer Green, UK) running on iMac computer (27-inch,
1129 Late 2013 model with OS X Yosemite, version 10.10). Montages of photographs were prepared
1130 using Adobe Photoshop CC (version 19.1.4, x64) running on a MacBook Pro computer (13-
1131 inch, early 2015 model with OS Mojave version 10.14.3).

1132

1133 ***In vitro* pharmacology**

1134 Informed by analysis of the expression of ArCCKP transcripts and ArCCK1 in *A.*
1135 *rubens*, both ArCCK1 and ArCCK2 were tested for myoactivity on cardiac stomach, tube foot,
1136 and apical muscle preparations dissected from specimens of *A. rubens* (n = 5 - 9, 8 - 10 and 20
1137 - 23 respectively) and set up in a 20 ml organ bath, as described previously (Elphick et al.
1138 1995, Melarange and Elphick 2003, Tian et al. 2017). Effects of peptides on preparations were

CCK-type signalling in an echinoderm

1139 assessed and recorded using an isotonic transducer (MLT0015, ADInstruments Pty Ltd)
1140 connected to a bridge amplifier (FE221 Bridge Amp, ADInstruments Pty Ltd) linked to
1141 PowerLab data acquisition hardware (2/36, ADInstruments Pty Ltd). Data was collected and
1142 analysed using LabChart (v8.0.7) software installed on a laptop computer (Lenovo E540,
1143 Windows 7 Professional). Stock solutions of synthetic peptides were prepared in distilled water
1144 and added to the organ bath to achieve final concentrations ranging from 0.1 nM to 1 μ M. To
1145 assess the viability of preparations and to enable normalization of responses to ArCCK1 or
1146 ArCCK2, the starfish neuropeptide NGFFYamide (100 nM) was tested on cardiac stomach
1147 preparations and acetylcholine (ACh; 10 μ M) was tested on tube foot and apical muscle
1148 preparations. To assess the importance of tyrosine sulphation for peptide bioactivity, a non-
1149 sulphated analog of ArCCK2 [ArCCK2(ns)] was also tested on cardiac stomach (n = 5), tube
1150 foot and apical muscle preparations (data not shown).

1151

1152 ***In vivo* pharmacology: testing ArCCK1 and ArCCK2 as cardiac stomach retractants**

1153 *In vitro* pharmacological experiments revealed that both ArCCK1 and ArCCK2 cause
1154 contraction of cardiac stomach preparations. Previous studies have revealed that the
1155 neuropeptide NGFFYamide causes cardiac stomach contraction *in vitro* and also triggers
1156 retraction of the everted cardiac stomach when injected into *A. rubens in vivo* (Semmens et al.
1157 2013). Therefore, experiments were performed to investigate if ArCCK1 and ArCCK2 also
1158 trigger cardiac stomach retraction in *A. rubens*. Twenty specimens of *A. rubens*, which had
1159 been withheld from a food supply for one week, were placed in a glass tank containing 2%
1160 magnesium chloride (MgCl₂; Sigma-Aldrich, Gillingham, UK) dissolved in seawater, which
1161 acts as a muscle relaxant in marine invertebrates (Mayer, 1909). This treatment conveniently
1162 and reproducibly causes eversion of the cardiac stomach in *A. rubens*, typically within a period
1163 of ~30 min (Semmens et al. 2013). Hamilton 75N 10 μ l syringes (Sigma-Aldrich, Gillingham,
1164 UK) were used to inject test compounds into the perivisceral coelom of animals, inserting the
1165 needle through the aboral body wall of the arms proximal to the junctions with the central disk
1166 region. Care was taken to inject neuropeptides [ArCCK1, ArCCK2 and ArCCK2(ns)] or
1167 distilled water (control) into the perivisceral coelom and not into the cardiac stomach. All
1168 animals were first injected with 10 μ l of distilled water (control) and video recorded for 6 min.
1169 The same animals were then injected with 10 μ l of 1 mM peptide and video recorded for 6 min.
1170 Static images from video recordings were captured at 30 s intervals from the time of injection.
1171 Then the two-dimensional area of everted cardiac stomach was measured from the images
1172 using the ImageJ software (version 1.0; <http://rsb.info.nih.gov/ij>) and normalized as a

CCK-type signalling in an echinoderm

1173 percentage of the area of cardiac stomach everted at the time of injection (T₀).

1174

1175 ***In vivo* pharmacology: testing effects of ArCCK1 and ArCCK2 on feeding behaviour**

1176 Previous studies have revealed that the starfish neuropeptide NGFFYamide inhibits the
1177 onset of feeding behaviour of *A. rubens* on a prey species – the mussel *Mytilus edulis* (Tinoco
1178 et al. 2018). Here the same methods employed by Tinoco et al. (2018) were used to investigate
1179 if ArCCK1 and/or ArCCK2 affect feeding behaviour in starfish. Sixty-two adult starfish (n =
1180 24 for ArCCK1; n = 38 for ArCCK2) that met the following criteria were used: (i) all 5 arms
1181 were intact, (ii) exhibited a normal righting response (Lawrence and Cowell 1996) and (iii)
1182 after twenty-four days of starvation, exhibited normal feeding behaviour on a mussel. Then,
1183 starfish were fasted for twenty-four days and transferred to and kept individually in Plexiglas
1184 aquaria, as described previously (Tinoco et al. 2018). After three days of acclimation (twenty-
1185 seven days of starvation at this point) and at 10 a.m., these animals were then divided into a
1186 control group (to be injected with distilled water), with 13 animals used for the ArCCK1
1187 experiment (mean diameter of 12.4 ± 0.3 cm) and 19 animals used for the ArCCK2 experiment
1188 (mean diameter of 12.9 ± 0.4 cm), and a test group (to be injected with ArCCK1 or ArCCK2),
1189 with 11 animals used for the ArCCK1 experiment (mean diameter of 12.6 ± 0.3 cm) and 19
1190 animals used for the ArCCK2 experiment (mean diameter of 12.9 ± 0.5 cm). The starfish were
1191 then injected with 10 µl of distilled water (control group) or 10 µl of 1 mM ArCCK1 or
1192 ArCCK2 peptides (test group) to achieve an estimated final concentration in the perivisceral
1193 coelom of ~1 µM, which is the concentration at which ArCCK peptides were found to have a
1194 maximal effect when tested on *in vitro* preparations of the cardiac stomach. The time taken for
1195 starfish to make first contact with a mussel (tube feet touching the mussel or time to touch the
1196 mussel), the number of attempts to touch as well as the time to enclose the mussel (indicated
1197 by a feeding posture) was recorded. Starfish that were feeding after 24 h were included in data
1198 analysis and any starfish in the control or test group that had not fed on a mussel after 24 hours
1199 were discarded from data analysis.

1200

1201 **Statistical analyses**

1202 Data were presented as means ± standard error of the mean (s.e.m). The *in vitro* or *in*
1203 *vivo* pharmacological effects of starfish CCK-type peptides on cardiac stomach, apical muscle
1204 and tube foot preparations were analysed by 2-way ANOVA, using type of substance tested
1205 and concentration/time as independent factors and Bonferroni's multiple comparison test.
1206 Apical muscle and tube foot data were transformed to logarithms to obtain a normal distribution

CCK-type signalling in an echinoderm

1207 and homogeneity of variances. The *in vitro* effects of ArCCK1 and ArCCK2 (1 μ M) on cardiac
1208 stomach preparations were compared with the *in vitro* effect of NGFFYamide (100 nM) using
1209 a two-tailed Student's t-test. The effect of ArCCK1 on feeding behaviour was analysed by two-
1210 tailed Mann-Whitney U-test (time to touch and time to enclose) because these data did not
1211 follow a normal distribution when analysed using the D'Agostino & Pearson omnibus
1212 normality test. The effect of ArCCK2 on feeding behaviour was analysed by two-tailed
1213 Student's t-test (time to touch) or Welch's unequal variances t-test (time to enclose). Fisher's
1214 exact test was used to analyse the percentage of successful feeding after the first touch for
1215 control and treated starfish. Statistical analyses were carried out using Prism 6 (GraphPad
1216 software, La Jolla, CA, USA) and differences were considered statistically significant at $p <$
1217 0.05.
1218

CCK-type signalling in an echinoderm

1219 **Acknowledgments**

1220 This work was supported by grants awarded to MRE by BBSRC (BB/M001644/1) and the
1221 Leverhulme Trust (RPG-2018-200) and to AMJ by BBSRC (BB/M001032/1). LAYG was
1222 supported by a PhD studentship awarded by the Mexican Council of Science and Technology
1223 (CONACyT studentship no. 518612) and Queen Mary University of London. YZ was
1224 supported by a PhD studentship awarded by the China Scholarship Council and Queen Mary
1225 University of London. JD is currently a postdoctoral researcher supported by Fund for
1226 Scientific Research of Belgium (F.R.S.-FNRS).

CCK-type signalling in an echinoderm

1227

1228 **References**

- 1229 Al-Alkawi H, Lange AB, and Orchard I. 2017. "Cloning, localization, and physiological
1230 effects of sulfakinin in the kissing bug, *Rhodnius prolixus*." *Peptides* **98**:15-22. doi:
1231 <https://doi.org/10.1016/j.peptides.2016.12.017>.
- 1232 Anderson JM. 1953. "Structure and function in the pyloric caeca of *Asterias Forbesi*." *The*
1233 *Biological Bulletin* **105** (1):47-61. doi: <https://doi.org/10.2307/1538555>.
- 1234 Anderson JM. 1954. "Studies on the Cardiac Stomach of the Starfish, *Asterias forbesi*."
1235 *Biological Bulletin* **107** (2):157-173. doi: <https://doi.org/10.2307/1538604>.
- 1236 Beutler LR, Chen Y, Ahn JS, Lin YC, Essner RA, and Knight ZA. 2017. "Dynamics of Gut-
1237 Brain Communication Underlying Hunger." *Neuron* **96** (2):461-475.e5. doi:
1238 <https://doi.org/10.1016/j.neuron.2017.09.043>.
- 1239 Bevis PJR, and Thorndyke MC. 1981. "Stimulation of gastric enzyme secretion by porcine
1240 cholecystokinin in the ascidian *Styela clava*." *General and Comparative*
1241 *Endocrinology* **45** (4):458-464. doi: [https://doi.org/10.1016/0016-6480\(81\)90049-6](https://doi.org/10.1016/0016-6480(81)90049-6).
- 1242 Bloom M, Lange AB, and Orchard I. 2019. "Identification, Functional Characterization, and
1243 Pharmacological Analysis of Two Sulfakinin Receptors in the Medically-Important
1244 Insect *Rhodnius prolixus*." *Scientific Reports* **9** (1):13437-13437. doi:
1245 <https://doi.org/10.1038/s41598-019-49790-x>.
- 1246 Blowes LM, Egertová M, Liu Y, Davis GR, Terrill NJ, Gupta HS, and Elphick MR. 2017.
1247 "Body wall structure in the starfish *Asterias rubens*." *Journal of Anatomy* **231**
1248 (3):325-341. doi: <https://doi.org/10.1111/joa.12646>.
- 1249 Boel E, Vuust J, Norris F, Norris K, Wind A, Rehfeld JF, and Marcker KA. 1983. "Molecular
1250 cloning of human gastrin cDNA: evidence for evolution of gastrin by gene
1251 duplication." *Proceedings of the National Academy of Sciences of the United States*
1252 *of America* **80** (10):2866-2869. doi: <https://doi.org/10.1073/pnas.80.10.2866>.
- 1253 Brusca RC. 2017. "Structure and Evolution of Invertebrate Nervous Systems edited by
1254 Andreas Schmidt-Rhaesa, Steffen Harzsch, and Günter Purschke." *The Quarterly*
1255 *Review of Biology* **92** (1):102-103. doi: <https://doi.org/10.1086/690877>.
- 1256 Burke RD, Angerer LM, Elphick MR, Humphrey GW, Yaguchi S, Kiyama T, Liang S, Mu
1257 X, Agca C, Klein WH, Brandhorst BP, Rowe M, Wilson K, Churcher AM, Taylor JS,
1258 Chen N, Murray G, Wang D, Mellott D, Olinski R, Hallböök F, and Thorndyke MC.
1259 2006. "A genomic view of the sea urchin nervous system." *Developmental Biology*
1260 **300** (1):434-60. doi: <https://doi.org/10.1016/j.ydbio.2006.08.007>.
- 1261 Cai W, Kim C-H, Go H-J, Egertová M, Zampronio CG, Jones AM, Park NG, and Elphick
1262 MR. 2018. "Biochemical, Anatomical, and Pharmacological Characterization of
1263 Calcitonin-Type Neuropeptides in Starfish: Discovery of an Ancient Role as Muscle
1264 Relaxants." *Frontiers in Neuroscience* **12** (382). doi:
1265 <https://doi.org/10.3389/fnins.2018.00382>.
- 1266 Chandra R, and Liddle RA. 2007. "Cholecystokinin." *Current Opinion in Endocrinology,*
1267 *Diabetes, and Obesity* **14** (1):63-7. doi:
1268 <https://doi.org/10.1097/MED.0b013e3280122850>.
- 1269 Chen D, Zhao CM, Håkanson R, Samuelson LC, Rehfeld JF, and Friis-Hansen L. 2004.
1270 "Altered control of gastric acid secretion in gastrin-cholecystokinin double mutant
1271 mice." *Gastroenterology* **126** (2):476-87. doi:
1272 <https://doi.org/10.1053/j.gastro.2003.11.012>.
- 1273 Chen M, Talarovicova A, Zheng Y, Storey KB, and Elphick MR. 2019. "Neuropeptide
1274 precursors and neuropeptides in the sea cucumber *Apostichopus japonicus*: a

CCK-type signalling in an echinoderm

- 1275 genomic, transcriptomic and proteomic analysis." *Scientific Reports* **9** (1):8829. doi:
1276 <https://doi.org/10.1038/s41598-019-45271-3>.
- 1277 Chen X, and Ganetzky B. 2012. "A neuropeptide signaling pathway regulates synaptic
1278 growth in *Drosophila*." *The Journal of Cell Biology* **196** (4):529-43. doi:
1279 <https://doi.org/10.1083/jcb.201109044>.
- 1280 Chen X, Peterson J, Nachman RJ, and Ganetzky B. 2012. "Drosulfakinin activates CCKLR-
1281 17D1 and promotes larval locomotion and escape response in *Drosophila*." *Fly*
1282 (*Austin*) **6** (4):290-7. doi: <https://doi.org/10.4161/fly.21534>.
- 1283 Cobb JL. 1970. "The significance of the radial nerve cords in asteroids and echinoids."
1284 *Zeitschrift für Zellforschung und Mikroskopische Anatomie (Vienna, Austria : 1948)*
1285 **108** (4):457-74. doi: <https://doi.org/10.1007/BF00339653>.
- 1286 Cobb JL. 1978. "An ultrastructural study of the dermal papulae of the starfish, *Asterias*
1287 *rubens*, with special reference to innervation of the muscles." *Cell and Tissue*
1288 *Research* **187** (3):515-23. doi: <https://doi.org/10.1007/BF00229616>.
- 1289 Cortés R, Arvidsson U, Schalling M, Ceccatelli S, and Hökfelt T. 1990. "In situ hybridization
1290 studies on mRNAs for cholecystokinin, calcitonin gene-related peptide and choline
1291 acetyltransferase in the lower brain stem, spinal cord and dorsal root ganglia of rat
1292 and guinea pig with special reference to motoneurons." *Journal of Chemical*
1293 *Neuroanatomy* **3** (6):467-85.
- 1294 Crean GP, Marshall MW, and Rumsey RD. 1969. "Parietal cell hyperplasia induced by the
1295 administration of pentagastrin (ICI 50,123) to rats." *Gastroenterology* **57** (2):147-55.
1296 doi: [https://doi.org/10.1016/S0016-5085\(19\)33930-7](https://doi.org/10.1016/S0016-5085(19)33930-7).
- 1297 Deschenes RJ, Lorenz LJ, Haun RS, Roos BA, Collier KJ, and Dixon JE. 1984. "Cloning and
1298 sequence analysis of a cDNA encoding rat preprocholecystokinin." *Proceedings of*
1299 *the National Academy of Sciences* **81** (3):726-730. doi:
1300 <https://doi.org/10.1073/pnas.81.3.726>.
- 1301 Deweerth A, Pisegna JR, Huppi K, and Wank SA. 1993. "Molecular Cloning, Functional
1302 Expression and Chromosomal Localization of the Human Cholecystokinin Type A
1303 Receptor." *Biochemical and Biophysical Research Communications* **194** (2):811-818.
1304 doi: <https://doi.org/10.1006/bbrc.1993.1894>.
- 1305 Dockray G, Dimaline R, and Varro A. 2005. "Gastrin: Old hormone, new functions."
1306 *Pflugers Archiv European Journal of Physiology* **449** (4):344-355. doi:
1307 <https://doi.org/10.1007/s00424-004-1347-5>.
- 1308 Dockray GJ, Duve H, and Thorpe A. 1981. "Immunochemical characterization of
1309 gastrin/cholecystokinin-like peptides in the brain of the blowfly, *Calliphora*
1310 *vomitaria*." *General and Comparative Endocrinology* **45** (4):491-6. doi:
1311 [https://doi.org/10.1016/0016-6480\(81\)90053-8](https://doi.org/10.1016/0016-6480(81)90053-8).
- 1312 Downer KE, Haselton AT, Nachman RJ, and Stoffolano JG, Jr. 2007. "Insect satiety:
1313 sulfakinin localization and the effect of drosulfakinin on protein and carbohydrate
1314 ingestion in the blow fly, *Phormia regina* (Diptera: Calliphoridae)." *Journal of Insect*
1315 *Physiology* **53** (1):106-12. doi: <https://doi.org/10.1016/j.jinsphys.2006.10.013>.
- 1316 Dufresne M, Seva C, and Fourmy D. 2006. "Cholecystokinin and gastrin receptors."
1317 *Physiological Reviews* **86** (3):805-47. doi:
1318 <https://doi.org/10.1152/physrev.00014.2005>.
- 1319 Dupre D, and Tostivint H. 2014. "Evolution of the gastrin-cholecystokinin gene family
1320 revealed by synteny analysis." *General and Comparative Endocrinology* **195**:164-73.
1321 doi: <https://doi.org/10.1016/j.ygcen.2013.10.019>.
- 1322 Edgar RC. 2004. "MUSCLE: multiple sequence alignment with high accuracy and high
1323 throughput." *Nucleic Acids Research* **32** (5):1792-7. doi:
1324 <https://doi.org/10.1093/nar/gkh340>.

CCK-type signalling in an echinoderm

- 1325 Edkins JS. 1906. "The chemical mechanism of gastric secretion." *The Journal of Physiology*
1326 **34** (1-2):133-44. doi: <https://doi.org/10.1113/jphysiol.1906.sp001146>.
- 1327 El-Salhy M, Abou-el-Ela R, Falkmer S, Grimelius L, and Wilander E. 1980.
1328 "Immunohistochemical evidence of gastro-entero-pancreatic neurohormonal peptides
1329 of vertebrate type in the nervous system of the larva of a dipteran insect, the hoverfly,
1330 *Eristalis aeneus*." *Regulatory Peptides* **1** (3):187-204. doi:
1331 [https://doi.org/10.1016/0167-0115\(80\)90271-2](https://doi.org/10.1016/0167-0115(80)90271-2).
- 1332 Elphick MR, Mirabeau O, and Larhammar D. 2018. "Evolution of neuropeptide signalling
1333 systems." *The Journal of Experimental Biology* **221** (3). doi:
1334 <https://doi.org/10.1242/jeb.151092>.
- 1335 Elphick MR, Newman SJ, and Thorndyke MC. 1995. "Distribution and action of
1336 SALMFamide neuropeptides in the starfish *Asterias rubens*." *The Journal of*
1337 *Experimental Biology* **198** (12):2519.
- 1338 Essner RA, Smith AG, Jamnik AA, Ryba AR, Trutner ZD, and Carter ME. 2017. "AgRP
1339 Neurons Can Increase Food Intake during Conditions of Appetite Suppression and
1340 Inhibit Anorexigenic Parabrachial Neurons." *The Journal of Neuroscience* **37**
1341 (36):8678-8687. doi: <https://doi.org/10.1523/jneurosci.0798-17.2017>.
- 1342 Florey E, and Cahill MA. 1980. "Cholinergic motor control of sea urchin tube feet: evidence
1343 for chemical transmission without synapses." *The Journal of Experimental Biology*
1344 **88**:281-92.
- 1345 Florey E, Cahill MA, and Rathmayer M. 1975. "Excitatory actions of GABA and of
1346 acetylcholine in sea urchin tube feet." *Comparative Biochemistry and Physiology*
1347 *Part C: Comparative Pharmacology* **51** (1):5-12. doi: [https://doi.org/10.1016/0306-](https://doi.org/10.1016/0306-4492(75)90031-3)
1348 [4492\(75\)90031-3](https://doi.org/10.1016/0306-4492(75)90031-3).
- 1349 Furlong RF, and Holland PW. 2002. "Bayesian phylogenetic analysis supports monophyly of
1350 ambulacraria and of cyclostomes." *Zoological Science* **19** (5):593-9. doi:
1351 <https://doi.org/10.2108/zsj.19.593>.
- 1352 García-Arrarás JE, Torres-Avillán I, and Ortíz-Miranda S. 1991. "Cells in the intestinal
1353 system of holothurians (Echinodermata) express cholecystokinin-like
1354 immunoreactivity." *General and Comparative Endocrinology* **83** (2):233-42. doi:
1355 [https://doi.org/10.1016/0016-6480\(91\)90026-3](https://doi.org/10.1016/0016-6480(91)90026-3).
- 1356 Gibbs J, Young RC, and Smith GP. 1973. "Cholecystokinin elicits Satiety in Rats with Open
1357 Gastric Fistulas." *Nature* **245**:323. doi: <https://doi.org/10.1038/245323a0>.
- 1358 Gregory H, Hardy PM, Jones DS, Kenner GW, and Sheppard RC. 1964. "The antral
1359 Hormone Gastrin: Structure of Gastrin." *Nature* **204** (4962):931-933. doi:
1360 <https://doi.org/10.1038/204931a0>.
- 1361 Gregory RA, and Tracy HJ. 1964. "The constitution and properties of two gastrins extracted
1362 from hog antral mucosa: Part II The properties of two gastrins isolated from hog
1363 antral mucosa." *Gut* **5** (2):107-117. doi: <https://doi.org/10.1136/gut.5.2.107>.
- 1364 Grimmelikhuijzen CJ, Sundler F, and Rehfeld JF. 1980. "Gastrin/CCK-like immunoreactivity
1365 in the nervous system of coelenterates." *Histochemistry* **69** (1):61-8. doi:
1366 <https://doi.org/10.1007/bf00508367>.
- 1367 Guindon S, Delsuc F, Dufayard JF, and Gascuel O. 2009. "Estimating maximum likelihood
1368 phylogenies with PhyML." *Methods in Molecular Biology* **537**:113-37. doi:
1369 https://doi.org/10.1007/978-1-59745-251-9_6.
- 1370 Gutiérrez JG, Chey WY, and Dinoso VP. 1974. "Actions of Cholecystokinin and Secretin on
1371 the Motor Activity of the Small Intestine in Man." *Gastroenterology* **67** (1):35-41.
1372 doi: [https://doi.org/10.1016/S0016-5085\(19\)32922-1](https://doi.org/10.1016/S0016-5085(19)32922-1).

CCK-type signalling in an echinoderm

- 1373 Harper AA, and Raper HS. 1943. "Pancreozymin, a stimulant of the secretion of pancreatic
1374 enzymes in extracts of the small intestine." *The Journal of physiology* **102** (1):115-
1375 125. doi: <https://doi.org/10.1113/jphysiol.1943.sp004021>.
- 1376 Harshini S, Nachman RJ, and Sreekumar S. 2002a. "In vitro release of digestive enzymes by
1377 FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina*
1378 *arenosella* (Walk.)." *Peptides* **23** (10):1759-63. doi: [https://doi.org/10.1016/s0196-9781\(02\)00152-3](https://doi.org/10.1016/s0196-9781(02)00152-3).
- 1380 Harshini S, Nachman RJ, and Sreekumar S. 2002b. "Inhibition of digestive enzyme release
1381 by neuropeptides in larvae of *Opisina arenosella* (Lepidoptera: Cryptophasidae)." *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular*
1382 *Biology* **132** (2):353-8. doi: [https://doi.org/10.1016/s1096-4959\(02\)00047-7](https://doi.org/10.1016/s1096-4959(02)00047-7).
- 1384 Himick BA, and Peter RE. 1994. "CCK/gastrin-like immunoreactivity in brain and gut, and
1385 CCK suppression of feeding in goldfish." *The American Journal of Physiology* **267**
1386 (3 Pt 2):R841-51. doi: <https://doi.org/10.1152/ajpregu.1994.267.3.R841>.
- 1387 Ivy ACO, E. 1929. "A hormone mechanism for gall-bladder contraction & evacuation." *The*
1388 *American Journal of Surgery* **7** (4):455-459. doi: [https://doi.org/10.1016/S0002-9610\(29\)90551-1](https://doi.org/10.1016/S0002-9610(29)90551-1).
- 1390 Janssen T, Meelkop E, Lindemans M, Verstraelen K, Husson SJ, Temmerman L, Nachman
1391 RJ, and Schoofs L. 2008. "Discovery of a cholecystokinin-gastrin-like signaling
1392 system in nematodes." *Endocrinology* **149** (6):2826-39. doi:
1393 <https://doi.org/10.1210/en.2007-1772>.
- 1394 Jekely G. 2013. "Global view of the evolution and diversity of metazoan neuropeptide
1395 signaling." *Proceedings of the National Academy of Sciences of the United States of*
1396 *America* **110** (21):8702-7. doi: <https://doi.org/10.1073/pnas.1221833110>.
- 1397 Johnsen AH, and Rehfeld JF. 1990. "Cionin: a disulfotyrosyl hybrid of cholecystokinin and
1398 gastrin from the neural ganglion of the protochordate *Ciona intestinalis*." *Journal of*
1399 *Biological Chemistry* **265** (6):3054-8.
- 1400 Kang KS, Yahashi S, and Matsuda K. 2011. "Effect of the N-methyl-d-aspartate receptor
1401 antagonist on locomotor activity and cholecystokinin-induced anorexigenic action in a
1402 goldfish model." *Neuroscience Letters* **488** (3):238-41. doi:
1403 <https://doi.org/10.1016/j.neulet.2010.11.036>.
- 1404 Kessner D, Chambers M, Burke R, Agus D, and Mallick P. 2008. "ProteoWizard: open
1405 source software for rapid proteomics tools development." *Bioinformatics* **24**
1406 (21):2534-6. doi: <https://doi.org/10.1093/bioinformatics/btn323>.
- 1407 Kopin AS, Lee YM, McBride EW, Miller LJ, Lu M, Lin HY, Kolakowski LF, and Beinborn
1408 M. 1992. "Expression cloning and characterization of the canine parietal cell gastrin
1409 receptor." *Proceedings of the National Academy of Sciences* **89** (8):3605-3609. doi:
1410 <https://doi.org/10.1073/pnas.89.8.3605>.
- 1411 Kramer KJ, Speirs RD, and Childs CN. 1977. "Immunochemical evidence for a gastrin-like
1412 peptide in insect neuroendocrine system." *General and Comparative Endocrinology*
1413 **32** (4):423-426. doi: [https://doi.org/10.1016/0016-6480\(77\)90224-6](https://doi.org/10.1016/0016-6480(77)90224-6).
- 1414 Kubiak TM, Larsen MJ, Burton KJ, Bannow CA, Martin RA, Zantello MR, and Lowery DE.
1415 2002. "Cloning and functional expression of the first *Drosophila melanogaster*
1416 sulfakinin receptor DSK-R1." *Biochemical and Biophysical Research*
1417 *Communications* **291** (2):313-20. doi: <https://doi.org/10.1006/bbrc.2002.6459>.
- 1418 Larson BA, and Vigna SR. 1983. "Species and tissue distribution of cholecystokinin/gastrin-
1419 like substances in some invertebrates." *General and Comparative Endocrinology* **50**
1420 (3):469-75.
- 1421 Lavoie ME. 1956. "How Sea Stars Open Bivalves." *Biological Bulletin* **111** (1):114-122. doi:
1422 <https://doi.org/10.2307/1539188>.

CCK-type signalling in an echinoderm

- 1423 Lawrence JM, and Cowell BC. 1996. "The righting response as an indication of stress in
1424 stichaster striatus (Echinodermata, asteroidea)." *Marine and Freshwater Behaviour
1425 and Physiology* **27** (4):239-248. doi: <https://doi.org/10.1080/10236249609378969>.
- 1426 Lee SY, and Soltesz I. 2011. "Cholecystokinin: a multi-functional molecular switch of
1427 neuronal circuits." *Developmental Neurobiology* **71** (1):83-91. doi:
1428 <https://doi.org/10.1002/dneu.20815>.
- 1429 Lee YM, Beinborn M, McBride EW, Lu M, Kolakowski LF, Jr., and Kopin AS. 1993. "The
1430 human brain cholecystokinin-B/gastrin receptor. Cloning and characterization." *The
1431 Journal of Biological Chemistry* **268** (11):8164-9.
- 1432 Lin M, Egertová M, Zampronio CG, Jones AM, and Elphick MR. 2017a. "Pedal
1433 peptide/orcokinin-type neuropeptide signaling in a deuterostome: The anatomy and
1434 pharmacology of starfish myorelaxant peptide in *Asterias rubens*." *The Journal of
1435 Comparative Neurology* **525** (18):3890-3917. doi: <https://doi.org/10.1002/cne.24309>.
- 1436 Lin M, Egertová M, Zampronio CG, Jones AM, and Elphick MR. 2018. "Functional
1437 characterization of a second pedal peptide/orcokinin-type neuropeptide signaling
1438 system in the starfish *Asterias rubens*." *The Journal of Comparative Neurology* **526**
1439 (5):858-876. doi: <https://doi.org/10.1002/cne.24371>.
- 1440 Lin M, Mita M, Egertová M, Zampronio CG, Jones AM, and Elphick MR. 2017b. "Cellular
1441 localization of relaxin-like gonad-stimulating peptide expression in *Asterias rubens*:
1442 New insights into neurohormonal control of spawning in starfish." *The Journal of
1443 Comparative Neurology* **525** (7):1599-1617. doi: <https://doi.org/10.1002/cne.24141>.
- 1444 Maestro JL, Aguilar R, Pascual N, Valero ML, Piulachs MD, Andreu D, Navarro I, and
1445 Belles X. 2001. "Screening of antifeedant activity in brain extracts led to the
1446 identification of sulfakinin as a satiety promoter in the German cockroach. Are
1447 arthropod sulfakinins homologous to vertebrate gastrins-cholecystokinins?"
1448 *European Journal of Biochemistry* **268** (22):5824-30. doi:
1449 <https://doi.org/10.1046/j.0014-2956.2001.02527.x>.
- 1450 Marciniak P, Kuczer M, and Rosinski G. 2011. "New physiological activities of
1451 myosuppressin, sulfakinin and NVP-like peptide in *Zophobas atratus* beetle." *Journal
1452 of Comparative Physiology. B: Biochemical, Systemic, and Environmental Physiology*
1453 **181** (6):721-30. doi: <https://doi.org/10.1007/s00360-011-0563-5>.
- 1454 Mashanov V, Zueva O, Rubilar T, Epherra L, and García-Arrarás JE. 2016. *Echinodermata
1455 in structure and evolution of invertebrate nervous systems*. Vol. **Chapter: 51**. Oxford,
1456 UK: Oxford University Press.
- 1457 Melarange R, and Elphick MR. 2003. "Comparative analysis of nitric oxide and
1458 SALMFamide neuropeptides as general muscle relaxants in starfish." *Journal of
1459 Experimental Biology* **206** (5):893-899. doi: <https://doi.org/10.1242/jeb.00197>.
- 1460 Melarange R, Potton DJ, Thorndyke MC, and Elphick MR. 1999. "SALMFamide
1461 neuropeptides cause relaxation and eversion of the cardiac stomach in starfish."
1462 *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**
1463 (1430):1785. doi: <https://doi.org/10.1098/rspb.1999.0847>.
- 1464 Meyering-Vos M, and Muller A. 2007. "RNA interference suggests sulfakinins as satiety
1465 effectors in the cricket *Gryllus bimaculatus*." *Journal of Insect Physiology* **53**
1466 (8):840-8. doi: <https://doi.org/10.1016/j.jinsphys.2007.04.003>.
- 1467 Mirabeau O, and Joly JS. 2013. "Molecular evolution of peptidergic signaling systems in
1468 bilaterians." *Proceedings of the National Academy of Sciences of the United States of
1469 America* **110** (22):e2028-37. doi: <https://doi.org/10.1073/pnas.1219956110>.
- 1470 Monstein HJ, Thorup JU, Folkesson R, Johnsen AH, and Rehfeld JF. 1993. "cDNA deduced
1471 procionin. Structure and expression in protochordates resemble that of

CCK-type signalling in an echinoderm

- 1472 procholecystokinin in mammals." *FEBS Letters* **331** (1-2):60-4. doi:
1473 [https://doi.org/10.1016/0014-5793\(93\)80297-8](https://doi.org/10.1016/0014-5793(93)80297-8).
- 1474 Moore SJ, and Thorndyke MC. 1993. "Immunocytochemical mapping of the novel
1475 echinoderm neuropeptide SALMFamide 1 (S1) in the starfish *Asterias rubens*." *Cell*
1476 *and Tissue Research* **274** (3):605-18. doi: <https://doi.org/10.1007/BF00314559>.
- 1477 Mutt V, and Jorpes JE. 1968. "Structure of Porcine Cholecystokinin-Pancreozymin."
1478 *European Journal of Biochemistry* **6** (1):156-162. doi: <https://doi.org/10.1111/j.1432-1033.1968.tb00433.x>.
- 1480 Nachman RJ, Giard W, Favrel P, Suresh T, Sreekumar S, and Holman GM. 1997. "Insect
1481 Myosuppressins and Sulfakinins Stimulate Release of the Digestive Enzyme α -
1482 Amylase in Two Invertebrates: The Scallop *Pecten maximus* and Insect
1483 *Rhynchophorus ferrugineus*." *Annals of the New York Academy of Sciences* **814**
1484 (1):335-338. doi: <https://doi.org/10.1111/j.1749-6632.1997.tb46178.x>.
- 1485 Nachman RJ, Holman GM, Cook BJ, Haddon WF, and Ling N. 1986a. "Leucosulfakinin-II, a
1486 blocked sulfated insect neuropeptide with homology to cholecystokinin and gastrin."
1487 *Biochemical and Biophysical Research Communications* **140** (1):357-64. doi:
1488 [https://doi.org/10.1016/0006-291x\(86\)91098-3](https://doi.org/10.1016/0006-291x(86)91098-3).
- 1489 Nachman RJ, Holman GM, Haddon WF, and Ling N. 1986b. "Leucosulfakinin, a sulfated
1490 insect neuropeptide with homology to gastrin and cholecystokinin." *Science* **234**
1491 (4772):71-3. doi: <https://doi.org/10.1126/science.3749893>.
- 1492 Nässel DR, Pauls D, and Huetteroth W. 2019. "Neuropeptides in modulation of *Drosophila*
1493 behavior: how to get a grip on their pleiotropic actions." *Current Opinion in Insect*
1494 *Science* **36**:1-8. doi: <https://doi.org/10.1016/j.cois.2019.03.002>.
- 1495 Nässel DR, and Williams MJ. 2014. "Cholecystokinin-Like Peptide (DSK) in *Drosophila*,
1496 Not Only for Satiety Signaling." *Frontiers in endocrinology* **5**:219-219. doi:
1497 <https://doi.org/10.3389/fendo.2014.00219>.
- 1498 Nässel DR, and Zandawala M. 2019. "Recent advances in neuropeptide signaling in
1499 *Drosophila*, from genes to physiology and behavior." *Progress in Neurobiology*. doi:
1500 <https://doi.org/10.1016/j.pneurobio.2019.02.003>.
- 1501 Nesvizhskii AI, Keller A, Kolker E, and Aebersold R. 2003. "A statistical model for
1502 identifying proteins by tandem mass spectrometry." *Analytical Chemistry* **75**
1503 (17):4646-58. doi: <https://doi.org/10.1021/ac0341261>.
- 1504 Newman SJ, and Thorndyke MC. 1994. "Localisation of gamma aminobutyric acid (GABA)-
1505 like immunoreactivity in the echinoderm *Asterias rubens*." *Cell and Tissue Research*
1506 **278** (1):177-85. doi: <https://doi.org/10.1007/bf00305790>.
- 1507 Nichols R. 2007. "The first nonsulfated sulfakinin activity reported suggests nsDSK acts in
1508 gut biology." *Peptides* **28** (4):767-73. doi:
1509 <https://doi.org/10.1016/j.peptides.2007.01.009>.
- 1510 Nichols R, Egle JP, Langan NR, and Palmer GC. 2008. "The different effects of structurally
1511 related sulfakinins on *Drosophila melanogaster* odor preference and locomotion
1512 suggest involvement of distinct mechanisms." *Peptides* **29** (12):2128-2135. doi:
1513 <https://doi.org/10.1016/j.peptides.2008.08.010>.
- 1514 Noble F, and Roques BP. 1999. "CCK-B receptor: chemistry, molecular biology,
1515 biochemistry and pharmacology." *Progress in Neurobiology* **58** (4):349-379. doi:
1516 [https://doi.org/10.1016/S0301-0082\(98\)00090-2](https://doi.org/10.1016/S0301-0082(98)00090-2).
- 1517 Odekunle EA, Semmens DC, Martynyuk N, Tinoco AB, Garewal AK, Patel RR, Blowes LM,
1518 Zandawala M, Delroisse J, Slade SE, Scrivens JH, Egertová M, and Elphick MR.
1519 2019. "Ancient role of vasopressin/oxytocin-type neuropeptides as regulators of
1520 feeding revealed in an echinoderm." *BMC Biology* **17** (1):60. doi:
1521 <https://doi.org/10.1186/s12915-019-0680-2>.

CCK-type signalling in an echinoderm

- 1522 Olsson C, Aldman G, Larsson A, and Holmgren S. 1999. "Cholecystokinin affects gastric
1523 emptying and stomach motility in the rainbow trout *Oncorhynchus mykiss*." *The*
1524 *Journal of Experimental Biology* **202** (Pt 2):161-70.
- 1525 Omasits U, Ahrens CH, Müller S, and Wollscheid B. 2014. "Protter: interactive protein
1526 feature visualization and integration with experimental proteomic data."
1527 *Bioinformatics* **30** (6):884-6. doi: <https://doi.org/10.1093/bioinformatics/btt607>.
- 1528 Palmer GC, Tran T, Duttlinger A, and Nichols R. 2007. "The drosulfakinin 0 (DSK 0)
1529 peptide encoded in the conserved Dsk gene affects adult *Drosophila melanogaster*
1530 crop contractions." *Journal of Insect Physiology* **53** (11):1125-33. doi:
1531 <https://doi.org/10.1016/j.jinsphys.2007.06.001>.
- 1532 Pentreath VW, and Cobb JL. 1972. "Neurobiology of echinodermata." *Biological Reviews of*
1533 *the Cambridge Philosophical Society* **47** (3):363-92. doi:
1534 <https://doi.org/10.1111/j.1469-185x.1972.tb00977.x>.
- 1535 Predel R, Brandt W, Kellner R, Rapus J, Nachman RJ, and Gäde G. 1999. "Post-translational
1536 modifications of the insect sulfakinins: sulfation, pyroglutamate-formation and O-
1537 methylation of glutamic acid." *European Journal of Biochemistry* **263** (2):552-60.
1538 doi: <https://doi.org/10.1046/j.1432-1327.1999.00532.x>.
- 1539 Predel R, Nachman RJ, and Gade G. 2001. "Myostimulatory neuropeptides in cockroaches:
1540 structures, distribution, pharmacological activities, and mimetic analogs." *Journal of*
1541 *Insect Physiology* **47** (4-5):311-24. doi: [https://doi.org/10.1016/s0022-](https://doi.org/10.1016/s0022-1910(00)00129-3)
1542 [1910\(00\)00129-3](https://doi.org/10.1016/s0022-1910(00)00129-3).
- 1543 Priyam A, Woodcroft BJ, Rai V, Munagala A, Moghul I, Ter F, Gibbins MA, Moon H,
1544 Leonard G, Rumpf W, and Wurm Y. 2015. "Sequenceserver: a modern graphical user
1545 interface for custom BLAST databases." *bioRxiv*:033142. doi:
1546 <https://doi.org/10.1101/033142>.
- 1547 Rehfeld JF. 2017. "Cholecystokinin-From Local Gut Hormone to Ubiquitous Messenger."
1548 *Frontiers in Endocrinology* **8**:47. doi: <https://doi.org/10.3389/fendo.2017.00047>.
- 1549 Rehfeld JF, Friis-Hansen L, Goetze JP, and Hansen TV. 2007. "The biology of
1550 cholecystokinin and gastrin peptides." *Current Topics in Medicinal Chemistry* **7**
1551 (12):1154-65. doi: <https://doi.org/10.2174/156802607780960483>.
- 1552 Roman CW, Sloat SR, and Palmiter RD. 2017. "A tale of two circuits: CCK(NTS) neuron
1553 stimulation controls appetite and induces opposing motivational states by projections
1554 to distinct brain regions." *Neuroscience* **358**:316-324. doi:
1555 <https://doi.org/10.1016/j.neuroscience.2017.06.049>.
- 1556 Rzasa P, Kaloustian KV, and Prokop EK. 1982. "Immunochemical evidence for a gastrin-like
1557 peptide in the intestinal tissues of the earthworm *Lumbricus terrestris*." *Comparative*
1558 *Biochemistry and Physiology Part A: Physiology* **71** (4):631-634. doi:
1559 [https://doi.org/10.1016/0300-9629\(82\)90216-X](https://doi.org/10.1016/0300-9629(82)90216-X).
- 1560 Schoofs L, Holman GM, Hayes TK, Nachman RJ, and De Loof A. 1990. "Locustatachykinin
1561 I and II, two novel insect neuropeptides with homology to peptides of the vertebrate
1562 tachykinin family." *FEBS Letters* **261** (2):397-401. doi: [https://doi.org/10.1016/0014-](https://doi.org/10.1016/0014-5793(90)80601-E)
1563 [5793\(90\)80601-E](https://doi.org/10.1016/0014-5793(90)80601-E).
- 1564 Schwartz J, Dubos M-P, Pasquier J, Zatylny-Gaudin C, and Favrel P. 2018. "Emergence of a
1565 cholecystokinin/sulfakinin signalling system in Lophotrochozoa." *Scientific Reports*
1566 **8** (1):16424. doi: <https://doi.org/10.1038/s41598-018-34700-4>.
- 1567 Sekiguchi T, Ogasawara M, and Satake H. 2012. "Molecular and functional characterization
1568 of cionin receptors in the ascidian, *Ciona intestinalis*: the evolutionary origin of the
1569 vertebrate cholecystokinin/gastrin family." *The Journal of Endocrinology* **213** (1):99-
1570 106. doi: <https://doi.org/10.1530/joe-11-0410>.

CCK-type signalling in an echinoderm

- 1571 Semmens DC, Dane RE, Pancholi MR, Slade SE, Scrivens JH, and Elphick MR. 2013.
1572 "Discovery of a novel neurophysin-associated neuropeptide that triggers cardiac
1573 stomach contraction and retraction in starfish." *The Journal of Experimental Biology*
1574 **216** (Pt 21):4047-53. doi: <https://doi.org/10.1242/jeb.092171>.
- 1575 Semmens DC, and Elphick MR. 2017. "The evolution of neuropeptide signalling: insights
1576 from echinoderms." *Briefings in Functional Genomics* **16** (5):288-298. doi:
1577 <https://doi.org/10.1093/bfpg/elx005>.
- 1578 Semmens DC, Mirabeau O, Moghul I, Pancholi MR, Wurm Y, and Elphick MR. 2016.
1579 "Transcriptomic identification of starfish neuropeptide precursors yields new insights
1580 into neuropeptide evolution." *Open Biology* **6** (2):150224. doi:
1581 <https://doi.org/10.1098/rsob.150224>.
- 1582 Shaw RA, and Jones RS. 1978. "The choleric action of cholecystokinin and cholecystokinin
1583 octapeptide in dogs." *Surgery* **84** (5):622-5.
- 1584 Singh L, Lewis AS, Field MJ, Hughes J, and Woodruff GN. 1991. "Evidence for an
1585 involvement of the brain cholecystokinin B receptor in anxiety." *Proceedings of the*
1586 *National Academy of Sciences of the United States of America* **88** (4):1130-3. doi:
1587 <https://doi.org/10.1073/pnas.88.4.1130>.
- 1588 Smith G, Jerome C, Cushin B, Eterno R, and Simansky K. 1981. "Abdominal vagotomy
1589 blocks the satiety effect of cholecystokinin in the rat." *Science* **213** (4511):1036-
1590 1037. doi: <https://doi.org/10.1126/science.7268408>.
- 1591 Smith JE. 1937. "On the Nervous System of the Starfish *Marthasterias glacialis* (L.)."
1592 *Philosophical Transactions of the Royal Society of London, Series B: Biological*
1593 *Sciences* **227** (542):111-173. doi: <https://doi.org/10.1098/rstb.1937.0002>.
- 1594 Smith JE. 1950. "The motor nervous system of the starfish, *Astropecten irregularis* (Pennant),
1595 with special reference to the innervation of the tube feet and ampullae."
1596 *Philosophical Transactions of the Royal Society of London. Series B, Biological*
1597 *sciences* **234** (618):521-58. doi: <https://doi.org/10.1098/rstb.1950.0010>.
- 1598 Smith MK, Wang T, Suwansa-Ard S, Motti CA, Elizur A, Zhao M, Rowe ML, Hall MR,
1599 Elphick MR, and Cummins SF. 2017. "The neuropeptidome of the Crown-of-Thorns
1600 Starfish, *Acanthaster planci*." *Journal of Proteomics* **165**:61-68. doi:
1601 <https://doi.org/10.1016/j.jprot.2017.05.026>.
- 1602 Sobrido-Cameán D, Yáñez-Guerra LA, Robledo D, López-Varela E, Rodicio MC, Elphick
1603 MR, Anadón R, and Barreiro-Iglesias A. 2020. "Cholecystokinin in the central
1604 nervous system of the sea lamprey *Petromyzon marinus*: precursor identification and
1605 neuroanatomical relationships with other neuronal signalling systems." *Brain*
1606 *Structure and Function* **225** (1):249-284. doi: [https://doi.org/10.1007/s00429-019-](https://doi.org/10.1007/s00429-019-01999-2)
1607 [01999-2](https://doi.org/10.1007/s00429-019-01999-2).
- 1608 Stewart MJ, Favrel P, Rotgans BA, Wang T, Zhao M, Sohail M, O'Connor WA, Elizur A,
1609 Henry J, and Cummins SF. 2014. "Neuropeptides encoded by the genomes of the
1610 Akoya pearl oyster *Pinctata fucata* and Pacific oyster *Crassostrea gigas*: a
1611 bioinformatic and peptidomic survey." *BMC Genomics* **15** (1):840. doi:
1612 <https://doi.org/10.1186/1471-2164-15-840>.
- 1613 Takahashi T, and Owyang C. 1999. "Mechanism of cholecystokinin-induced relaxation of the
1614 rat stomach." *Journal of the Autonomic Nervous System* **75** (2-3):123-30. doi:
1615 [https://doi.org/10.1016/s0165-1838\(98\)00181-7](https://doi.org/10.1016/s0165-1838(98)00181-7).
- 1616 Telford MJ, Budd GE, and Philippe H. 2015. "Phylogenomic Insights into Animal
1617 Evolution." *Current biology* **25** (19):R876-87. doi:
1618 <https://doi.org/10.1016/j.cub.2015.07.060>.
- 1619 Thorndyke M, and Dockray GJ. 1986. "Identification and localization of material with
1620 gastrin-like immunoreactivity in the neural ganglion of a protochordate, *Ciona*

CCK-type signalling in an echinoderm

- 1621 intestinalis." *Regulatory Peptides* **16** (3-4):269-79. doi: <https://doi.org/10.1016/0167->
1622 0115(86)90026-1.
- 1623 Thorndyke MC, and Bevis PJ. 1984. "Comparative studies on the effects of cholecystokinins,
1624 caerulein, bombesin 6-14 nonapeptide, and physalaemin on gastric secretion in the
1625 ascidian *Styela clava*." *General and Comparative Endocrinology* **55** (2):251-9. doi:
1626 [https://doi.org/10.1016/0016-6480\(84\)90109-6](https://doi.org/10.1016/0016-6480(84)90109-6).
- 1627 Tian S, Egertová M, and Elphick MR. 2017. "Functional Characterization of Paralogous
1628 Gonadotropin-Releasing Hormone-Type and Corazonin-Type Neuropeptides in an
1629 Echinoderm." *Frontiers in Endocrinology* **8**:259. doi:
1630 <https://doi.org/10.3389/fendo.2017.00259>.
- 1631 Tian S, Zandawala M, Beets I, Baytemur E, Slade SE, Scrivens JH, and Elphick MR. 2016.
1632 "Urbilaterian origin of paralogous GnRH and corazonin neuropeptide signalling
1633 pathways." *Scientific Reports* **6**:28788. doi: <https://doi.org/10.1038/srep28788>.
- 1634 Tinoco AB, Semmens DC, Patching EC, Gunner EF, Egertová M, and Elphick MR. 2018.
1635 "Characterization of NGFFYamide Signaling in Starfish Reveals Roles in Regulation
1636 of Feeding Behavior and Locomotory Systems." *Frontiers in Endocrinology* **9**:507-
1637 507. doi: <https://doi.org/10.3389/fendo.2018.00507>.
- 1638 Tinoco AB, Valenciano AI, Gómez-Boronat M, Blanco AM, Nisembaum LG, De Pedro N,
1639 and Delgado MJ. 2015. "Two cholecystokinin receptor subtypes are identified in
1640 goldfish, being the CCKAR involved in the regulation of intestinal motility."
1641 *Comparative Biochemistry and Physiology: Part A, Molecular & Integrative*
1642 *Physiology* **187**:193-201. doi: <https://doi.org/10.1016/j.cbpa.2015.05.027>.
- 1643 Trifinopoulos J, Nguyen LT, von Haeseler A, and Minh BQ. 2016. "W-IQ-TREE: a fast
1644 online phylogenetic tool for maximum likelihood analysis." *Nucleic Acids Research*
1645 **44** (W1):W232-5. doi: <https://doi.org/10.1093/nar/gkw256>.
- 1646 Vizi SE, Bertaccini G, Impicciatore M, and Knoll J. 1973. "Evidence that Acetylcholine
1647 Released by Gastrin and Related Polypeptides Contributes to their Effect on
1648 Gastrointestinal Motility." *Gastroenterology* **64** (2):268-277. doi:
1649 [https://doi.org/10.1016/S0016-5085\(73\)80038-1](https://doi.org/10.1016/S0016-5085(73)80038-1).
- 1650 Wank SA, Harkins R, Jensen RT, Shapira H, de Weerth A, and Slattery T. 1992.
1651 "Purification, molecular cloning, and functional expression of the cholecystokinin
1652 receptor from rat pancreas." *Proceedings of the National Academy of Sciences* **89**
1653 (7):3125-3129. doi: <https://doi.org/10.1073/pnas.89.7.3125>.
- 1654 Wei Z, Baggerman G, R JN, Goldsworthy G, Verhaert P, De Loof A, and Schoofs L. 2000.
1655 "Sulfakinins reduce food intake in the desert locust, *Schistocerca gregaria*." *Journal*
1656 *of Insect Physiology* **46** (9):1259-1265. doi: <https://doi.org/10.1016/s0022->
1657 1910(00)00046-9.
- 1658 Yáñez-Guerra LA, Delroisse J, Barreiro-Iglesias A, Slade SE, Scrivens JH, and Elphick MR.
1659 2018. "Discovery and functional characterisation of a luqin-type neuropeptide
1660 signalling system in a deuterostome." *Scientific Reports* **8** (1):7220. doi:
1661 <https://doi.org/10.1038/s41598-018-25606-2>.
- 1662 Yáñez-Guerra LA, Zhong X, Moghul I, Butts T, Zampronio CG, Jones AM, Mirabeau O, and
1663 Elphick MR. 2020. "Echinoderms provide missing link in the evolution of
1664 PrRP/sNPF-type neuropeptide signalling." *Elife* **9**. doi:
1665 <https://doi.org/10.7554/eLife.57640>.
- 1666 Yu N, Benzi V, Zotti MJ, Staljanssens D, Kaczmarek K, Zabrocki J, Nachman RJ, and
1667 Smagghe G. 2013a. "Analogues of sulfakinin-related peptides demonstrate reduction in
1668 food intake in the red flour beetle, *Tribolium castaneum*, while putative antagonists
1669 increase consumption." *Peptides* **41**:107-12. doi:
1670 <https://doi.org/10.1016/j.peptides.2012.12.005>.

CCK-type signalling in an echinoderm

- 1671 Yu N, Nachman RJ, and Smagghe G. 2013b. "Characterization of sulfakinin and sulfakinin
1672 receptor and their roles in food intake in the red flour beetle *Tribolium castaneum*."
1673 *General and Comparative Endocrinology* **188**:196-203. doi:
1674 <https://doi.org/10.1016/j.ygcen.2013.03.006>.
- 1675 Yu N, and Smagghe G. 2014a. "CCK(-like) and receptors: structure and phylogeny in a
1676 comparative perspective." *General and Comparative Endocrinology* **209**:74-81. doi:
1677 <https://doi.org/10.1016/j.ygcen.2014.05.003>.
- 1678 Yu N, and Smagghe G. 2014b. "Characterization of sulfakinin receptor 2 and its role in food
1679 intake in the red flour beetle, *Tribolium castaneum*." *Peptides* **53**:232-7. doi:
1680 <https://doi.org/10.1016/j.peptides.2013.12.011>.
- 1681 Yu N, Zotti MJ, Scheys F, Braz AS, Penna PH, Nachman RJ, and Smagghe G. 2015.
1682 "Flexibility and extracellular opening determine the interaction between ligands and
1683 insect sulfakinin receptors." *Scientific Reports* **5**:12627. doi:
1684 <https://doi.org/10.1038/srep12627>.
- 1685 Zandawala M, Moghul I, Yáñez-Guerra LA, Delroisse J, Abylkassimova N, Hugall AF,
1686 O'Hara TD, and Elphick MR. 2017. "Discovery of novel representatives of bilaterian
1687 neuropeptide families and reconstruction of neuropeptide precursor evolution in
1688 ophiuroid echinoderms." *Open Biology* **7** (9). doi:
1689 <https://doi.org/10.1098/rsob.170129>.
- 1690 Zels S, Dillen S, Crabbe K, Spit J, Nachman RJ, and Vanden Broeck J. 2015. "Sulfakinin is
1691 an important regulator of digestive processes in the migratory locust, *Locusta*
1692 *migratoria*." *Insect Biochemistry and Molecular Biology* **61**:8-16. doi:
1693 <https://doi.org/10.1016/j.ibmb.2015.03.008>.
- 1694 Zhang X, Tang N, Qi J, Wang S, Hao J, Wu Y, Chen H, Tian Z, Wang B, Chen D, and Li Z.
1695 2017. "CCK reduces the food intake mainly through CCK1R in Siberian sturgeon
1696 (*Acipenser baerii* Brandt)." *Scientific Reports* **7** (1):12413. doi:
1697 <https://doi.org/10.1038/s41598-017-12646-3>.
- 1698 Zhang Y, Yáñez Guerra LA, Egertová M, Zampronio CG, Jones AM, and Elphick MR. 2020.
1699 "Molecular and functional characterization of somatostatin-type signalling in a
1700 deuterostome invertebrate." *Open Biology* **10** (9):200172. doi:
1701 <https://doi.org/10.1098/rsob.200172>.
- 1702 Zhaxybayeva O, and Gogarten JP. 2002. "Bootstrap, Bayesian probability and maximum
1703 likelihood mapping: exploring new tools for comparative genome analyses." *BMC*
1704 *Genomics* **3**:4. doi: <https://doi.org/10.1186/1471-2164-3-4>.
1705
1706

CCK-type signalling in an echinoderm

1707 **Supplementary data**

1708

1709 **Figure 1- source data 1.** Accession numbers for precursors of the neuropeptides shown in the
 1710 sequence alignment in **Figure 1.**

1711

Precursor/Peptide name	Species name	Accession number or PubMed reference ID
CCK	<i>Aplysia californica</i>	XP_005096263.1
CCK	<i>Asterias rubens</i>	ALJ99958
NP12	<i>Caenorhabditis elegans</i>	O01970
Cionin	<i>Ciona intestinalis</i>	P16240
CCK	<i>Crassostrea gigas</i>	EKC26412.1
SK	<i>Drosophila melanogaster</i>	P09040
CCK	<i>Homo sapiens</i>	P06307
Gastrin	<i>Homo sapiens</i>	P01350
CCK	<i>Ophionotus victoriae</i>	ASK86241
CCK	<i>Platynereis dumerilii</i>	Contig HAMO01025411.1 (transcriptome prediction)
CCK	<i>Strongylocentrotus purpuratus</i>	PMID: 28878039 (predicted from the genomic scaffold AAGJ06000007.1)
CCK	<i>Stichopus horrens</i>	HAMZ01045944.1 (transcript)
CCK	<i>Saccoglossus kowalevskii</i>	XM_002738068.2
SK	<i>Tribolium castaneum</i>	D6WP08

1712

1713

1714

1715 **Figure 1 – source data 2.** Data for the mass spectra shown in **Figure 1 – figure supplement**
 1716 **2.** (not included here – available as separate file)

CCK-type signalling in an echinoderm

1717 1 cg
1718 3 ggcttcaaacttcaacatctcttgttatgctcgtcagctatcgtggttctaaaatcgcat
1719 63 actcttacaacggtccggctgcttacatctctcccaattccgctcgtcatctaaccaaca
1720 123 aagggacctcctctgttaatttgttctactttttgatcgaattgatctgattttcagttt
1721 183 tgattcccactaggaacacgaacttgacgtgtttaaaccgaactgctgggtattttggttc
1722 243 cttctgtcgtgagctgcaagggggccaca **tcgctactgtttctctcgca**ttgcttgtatttt
1723 303 gactgattgaacttatcaacacctatccgtatataggaagttctgctcagatcataatg
1724 M 1
1725 363 agtagttggcttacagtcgccatagcaactgtgacatgccttttgcctttcgccaatcacg
1726 **S S W L T V A I A T V T C L L L S P I T** 21
1727 423 tgcctgcctcttcatgacgtagccgacggtaaggaaggcgggaactcctgcacagcacg
1728 **C L P L H D V A D G K E R R E L L H S T** 41
1729 483 tggtagaccctccggttcaacaggtcaaggcagggaggaattggccgagacgagcaag
1730 **W L D P S G S T G Q G T E E L A E T S K** 61
1731 543 cgactacttggggataacaacagggactcgggcattattgacctcctttagcactgca
1732 **R L L G D N N R D S G I I D L L V A L R** 81
1733 603 gacacaaacacaaaccgagagatctttatcttcaacggcaacacagagacagctcgtaaa
1734 **D T N T N P R D L Y L H G N T E T A R K** 101
1735 663 cgaagacaatccaaggtggatgactacggccatggctctattctggggcaagagaggatcc
1736 **R R Q S K V D D Y G H G L F W G K R G S** 121
1737 723 aactggctcagaccggggtacgagcaatgacagataaggacaccaagaggggctggat
1738 **N W S D H G V R A M T D K D T K R G G D** 141
1739 783 gatcaatatggcttggcttatttttggcaagcgaatgaagaagactacgaagacttt
1740 **D Q Y G F G L F F G K R N E E D Y E D F** 161
1741 843 acgtttagattgttttagcaataaggataacttaaaagctctaaagaatttggcaaagta
1742 **T L *** 163
1743 903 atgttttgaataaggcatagctctttatgataagttaacgcaatatcaaactaatataac
1744 963 tcgacacttctgtgggggttaagtctcaatagagtgtccaactatcgccaaatacaatt
1745 1023 gtgcaaaaaacgtctcgaaatcatttacaataattcacacaaaccatgggtacttggtta
1746 1083 attgtgtaatgctgttgaaacgtgcatgtagtgaattagactgataaatacggata
1747 1143 aatcatgtaaagccagtagcagctcacactgcaattacaacattagcttttagtttaagt
1748 1203 taaagattgacttgtcatgttccgagcaaaaggttgccatcatttgatttttttaatttg
1749 1263 ataggaatcattgttcaaattgacgtttaccgcaagtggtattacacctctctttctgaac
1750 1323 tacatgacaggatcaaaatgacagtttagcagaagatatttgtgttatgctgtttataa
1751 1383 atataaacccatggttaagaacctgtattttagaacgc **aagcagttggtgacgcctt**
1752 1443 **ttggaagtctgcaactctgtaactgtatgtgggtaccattgaagctatatgccaatc**caa
1753 1503 **tcactgcttctctatcataaccttttggaaaaacacacattttataggcaaaatagtaa**
1754 1563 agttatgagttaaaccaaacggttgctgtgcaaatcggagcgtttcaatccgctcgatgtt
1755 1623 ttattggtttgaactgactgttgtttctcaataataacaaatctgtgcaactactgtgga
1756 1683 tttcgttgctcattctgaatattgtttcttttgtttaaactccaatgttgacaaactta
1757 1743 acgttaatccattgtaaatattttatgcataaccgtcttttgagtcacttcgtaagcc
1758 1803 cgatgttttattttgttaagtgtatataccttgaacatgaactagcagaaaagagttac
1759 1863 ttagcacgatttgcagatttgaatcatgttaggaagataaacacatttataatagttctcaa
1760 1923 caatgcttactaatctatctaaggaaagttgctgttctgaaaagaagcaggcttgagat
1761 1983 ggaggtatttctgttctactcacaacctctcttctgtgtgacagattaataataacat
1762 2043 agttgtcaacctgacattataaacctgctgttaatttgattggcgaacacgcgctcacg
1763 2103 tgtcatactttacagatattttgccaagtaaaactgctccaacactttatatttttagaa
1764 2163 gagttctataattattaaatattgtacgcttttgacgtttcgttaacttcgctttgagattt
1765 2223 acattgagaatcgtttgcattgattgctcagcagcaggttaggctatggacgccgaac
1766 2283 gtgctttaaatagcatacttgtttatacacagctttatcgatcatttcatacagtaatgt
1767 2343 cgaaataggtcatatagtggttcaaaacagtgattgaatttataaataatgtcaattgac
1768 2403 aaggatttatccatgggcaactcacatatatactgcattttattcataataaagcagatca
1769 2463 caatccctagttgaaatctgaaactgttttatttaacttgagctcgtgatttctaataatgac
1770 2523 actctttacgcatcacacaatattgtatcgtagccctgacgcaaatgaatacaagtggt
1771 2583 cttatgtattatgcaaaattatagttgagttgttatccaaagattagtttaacattatttg
1772 2643 tcctatatcattagcgcctaaaatgtaacatttactttgaagtgataaaaacgtgtctctat
1773 2703 tcctttctgactgagcacttgggtagatacagtgctgcttaaatttcaacgtgctgactat
1774 2763 gacattaaacaataacgacttgcttaaatgaattttcaggtgataaatgcatcaccaaa
1775 2823 tgttgatattacaaagtctattaaattccgaaaccctaaatgtcaatttttgtgaa
1776 2883 caaattagttccaaaagttgtaacagaccaggttgccattgtggcaacagttgtcaact
1777 2943 tttacggatacagtggtcattgtgttgtttactaaaatgtttgaagtctcgaagcaaaag
1778 3003 ctggaataaaaaagtttaaaaaacgattcataaaaaacatttatactgtatattttattca
1779 3063 ttttaactgttttgttgcgttgaagctaagtgtgttgcttatcaattgaaaacagtaaca
1780 3123 gtacaatgaaacatccagcaaaacaaaaatcgacagtaaaccaaaaaatcgaacaaacga

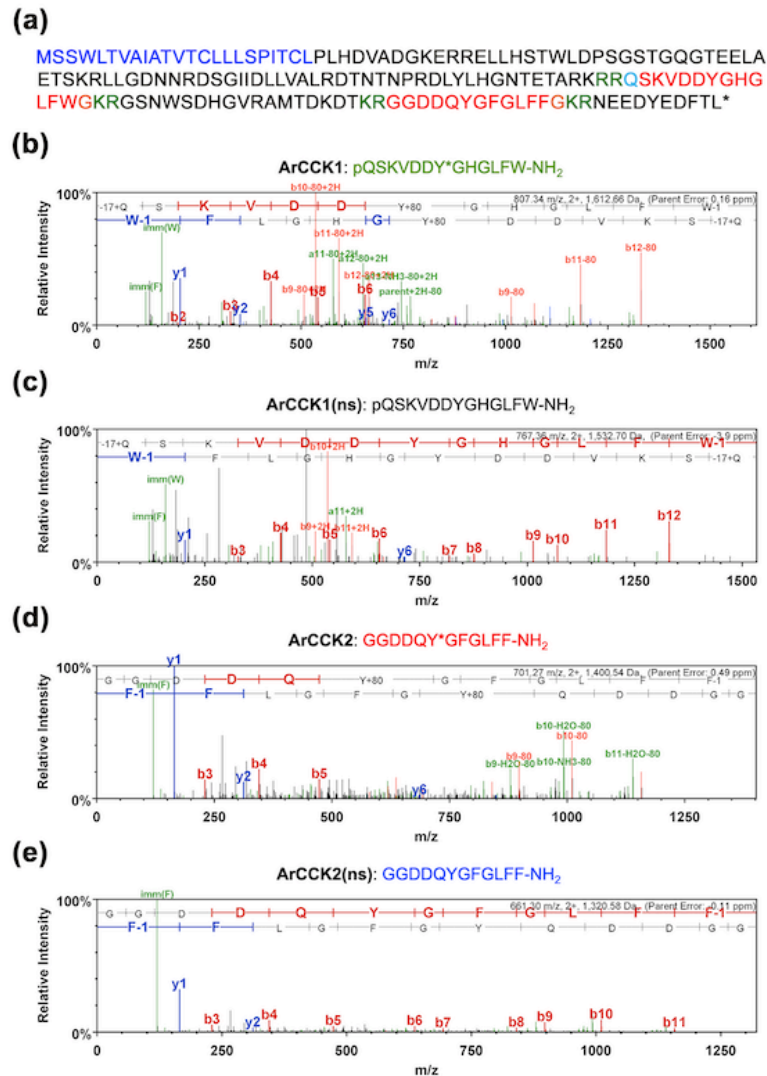
CCK-type signalling in an echinoderm

1781 3183 gcaaacaaacaaacaatgaaaaaaaaacaaaatcaagtctaaggcaaaagttttcaatcaaa
1782 3243 ttaacgaaggatgatggcctaaattaagtaagcgattttaacgaacattgaaaacgaac
1783 3303 ttactcttcctaacttcaaactcgaaactaaccaataagaacaataaattataacataat
1784 3363 atcccttaaatttactggacacacaccatttactagtaaagacactggacgcttttggtaa
1785 3423 ttgtcaaagacc

1786

1787 **Figure 1 – figure supplement 1. The *A. rubens* CCK-type precursor (ArCCKP).** The
1788 nucleotide sequence of contig 1124413 derived from radial nerve cord transcriptome data
1789 (Semmens et al., 2016; GenBank accession number KT601716) is shown in lowercase (3434
1790 bases) and the encoded the precursor protein sequence is shown in uppercase (163 residues).
1791 The predicted signal peptide is shown in dark-blue, two putative CCK-type peptides are shown
1792 in red but with an N-terminal glutamine residue in first peptide shown light blue to indicate
1793 that it is a potential substrate for pyroglutamination. Putative dibasic cleavage sites are shown
1794 in green. The asterisk shows the position of the stop codon. The sequences of primers used for
1795 PCR cloning of a cDNA encoding ArCCKP are highlighted in yellow. The sequence of the
1796 cloned cDNA was found to be identical to the corresponding sequence of contig 1124413.
1797

CCK-type signalling in an echinoderm



1798
 1799
 1800
 1801
 1802
 1803
 1804
 1805
 1806
 1807
 1808
 1809
 1810
 1811
 1812
 1813
 1814
 1815
 1816
 1817
 1818
 1819

Figure 1 – figure supplement 2. Determination of the structures of peptides derived from the ArCCKP by mass spectrometric (LC-MS-MS) analysis of *A. rubens* radial nerve cord extract. (a) Amino acid sequence of ArCCKP, with the predicted signal peptide shown in dark blue, predicted dibasic cleavage sites shown in green and predicted CCK-type neuropeptides shown in red. C-terminal glycine residues that are predicted substrates for amidation are shown in orange and an N-terminal glutamine residue that is a potential substrate for pyroglutamination (pQ) is shown in light blue. The nucleotide sequence of a cDNA encoding ArCCKP is shown in **Figure 1 – figure supplement 1. (b – d)** MS/MS data for four CCK-type peptides derived from ArCCKP detected in *A. rubens* radial nerve cord extracts: **(b)** pQSKVDDY(SO₃H)GHGLFW-NH₂ [ArCCK1, which has a sulphated tyrosine], **(c)** pQSKVDDYGHGLFW-NH₂ [ArCCK1(ns), which is not sulphated], **(d)** GGDDQY(SO₃H)GFGLFF-NH₂ [ArCCK2, which has a sulphated tyrosine] and **(e)** GGDDQYGFGLFF-NH₂ [ArCCK2(ns), which is not sulphated]. The b series of peptide fragment ions are shown in red, the y series are shown in blue and additional identified peptide fragment ions are shown in green. The amino acid sequence identified in each mass spectrum is shown above it, with -17+Q representing an N-terminal pyroglutamate residue (pQ), W-1 representing an amidated C-terminal tryptophan residue (W-NH₂), F-1 representing an amidated C-terminal phenylalanine residue (F-NH₂) and Y+80 representing a sulphated tyrosine residue (Y*). The observed m/z of the precursor ion for each peptide is with a charge state of +2 and with errors between the experimentally determined and predicted values ranging from -3.9 ppm to +0.49 ppm.

CCK-type signalling in an echinoderm

1820 **Figure 2 – source data 1. Accession numbers for the receptor sequences used for the**
 1821 **phylogenetic tree in Figure 2.**

1822

Receptor	Species name	Accession number	Phylum	References (DOI or journal link) for papers reporting experimental identification of neuropeptide ligands for receptors
Acal CCKR2	<i>Aplysia californica</i>	XM_013090996.1	Lophotrochozoa	
Ajap CCKR	<i>Apostichopus japonicus</i>	GHCH01030881.1	Ambulacraria	
Arub CCKR	<i>Asterias rubens</i>	MW261740	Ambulacraria	This study
Cbri CKR1	<i>Caenorhabditis briggsae</i>	XP_002640196.1	Nematoda	
Cbri CCKR2	<i>Caenorhabditis briggsae</i>	XP_002642853.1	Nematoda	
Cele CKR1	<i>Caenorhabditis elegans</i>	NP_491918.3	Nematoda	
Cele CCKR2	<i>Caenorhabditis elegans</i>	ACA81683.1	Nematoda	10.1210/en.2007-1772
Ctel CCKR	<i>Capitella teleta</i>	ELT89517.1	Lophotrochozoa	
Cint CioR1	<i>Ciona intestinalis</i>	Q70SX9	Urochordata	10.1530/JOE-11-0410
Cint CioR2	<i>Ciona intestinalis</i>	H7CE69	Urochordata	10.1530/JOE-11-0410
Cgig CCKR1	<i>Crassostrea gigas</i>	MF787221	Lophotrochozoa	10.1038/s41598-018-34700-4
Cgig CCKR2	<i>Crassostrea gigas</i>	MF787222	Lophotrochozoa	10.1038/s41598-018-34700-4
Drer CCKR1	<i>Danio rerio</i>	XP_697493.2	Vertebrata	
Drer CCKR2	<i>Danio rerio</i>	XP_017213239.1	Vertebrata	
Dpul SKR	<i>Daphnia pulex</i>	EFX77608.1	Arthropoda	
Dmel SKR1	<i>Drosophila melanogaster</i>	NP_001097023.1	Arthropoda	10.1006/bbrc.2002.6459
Dmel SKR2	<i>Drosophila melanogaster</i>	NP_001097021.1	Arthropoda	10.4161/fly.21534

CCK-type signalling in an echinoderm

Ggal CCKR1	<i>Gallus gallus</i>	BAJ46148.1	Vertebrata	
Ggal CCKR2	<i>Gallus gallus</i>	NP_001001742.1	Vertebrata	10.1016/S0167-0115(03)00068-5
Gpau CCKR	<i>Glossoscolex paulistus</i>	GBIL01035016.1	Lophotrochozoa	
Hsap CCKR1	<i>Homo sapiens</i>	NP_000721.1	Vertebrata	10.1006/bbrc.1993.1610
Hsap CCKR2	<i>Homo sapiens</i>	NP_795344.1	Vertebrata	https://www.jbc.org/content/268/11/8164.long
Lgig CCKR1	<i>Lottia gigantea</i>	XP_009047144.1	Lophotrochozoa	
Lgig CCKR2	<i>Lottia gigantea</i>	XP_009047126.1	Lophotrochozoa	
Mmus CCKR1	<i>Mus musculus</i>	NP_033957.1	Vertebrata	https://jpet.aspetjournals.org/content/282/3/1206
Mmus CCKR2	<i>Mus musculus</i>	NP_031653.1	Vertebrata	10.1038/sj.bjp.0702448
Ovic CCKR	<i>Ophionotus victoriae</i>	MW261741	Ambulacraria	
Pcau CCKR	<i>Priapululus caudatus</i>	XM_014813624.1	Priapulida	
Spur CCKR	<i>Strongylocentrotus purpuratus</i>	XP_782630.3	Ambulacraria	
Skow CCKR1	<i>Saccoglossus kowalevskii</i>	XP_006814715.1	Ambulacraria	
Skow CCKR2	<i>Saccoglossus kowalevskii</i>	XP_006814705.1	Ambulacraria	
Tcas SKR1	<i>Tribolium castaneum</i>	XP_015835017.1	Arthropoda	
Tcas SKR2	<i>Tribolium castaneum</i>	XP_972750.1	Arthropoda	
Pame SKR1	<i>Periplaneta americana</i>	AAX56942.1	Arthropoda	
Hsap OrexinR 1	<i>Homo sapiens</i>	NP_001516.2	Vertebrata	
Mmus OrexinR 1	<i>Mus musculus</i>	NP_945197.2	Vertebrata	

1823

1824

CCK-type signalling in an echinoderm

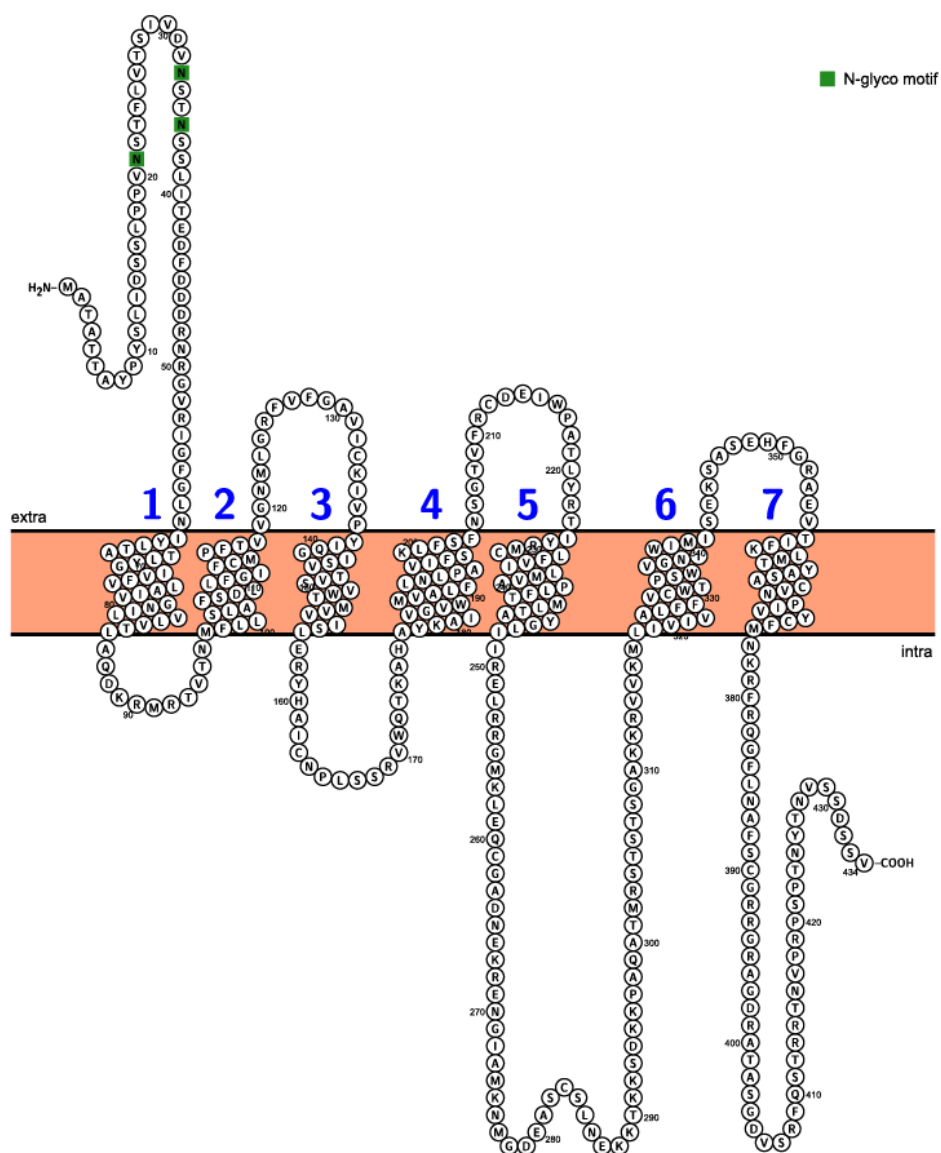
```
1825
1826     1  aaaaaacaacctcgcacagtgaagtggcgatttgattaacttggatataatttgacagtggt
1827     61  gtaaagtcacaactacatttcgtcttgtgaagaaggatacttttcaacaagaactctcgt
1828    121  ctaagcatcatggcgactgcgaccaccgcctacccgtactcgttatagatagcagcctt
1829           M A T A T T A Y P Y S L I D S S L   17
1830    181  ccaccgggtcaattctacttttcttagtgaccagtattgtggatgtaaactcgacgaactct
1831           P P V N S T F L V T S I V D V N S T N S   37
1832    241  tcgttgatcacggaggattttgacgatgaccgtaacagggcgctccggatcgggttcggg
1833           S L I T E D F D D D R N R G V R I G F G   57
1834    301  ttgaatatctacctgaccgctacgctgtacggatcgtcttcgtgctggccatcgtgggc
1835           L N I Y L T A T L Y G I V F V L A I V G   77
1836    361  aacatcttggttctcgtcacgctggcccaggataagaggatgcgtacggtgaccaacatg
1837           N I L V L V T L A Q D K R M R T V T N M   97
1838    421  ttctgctgagctcggcctttagcgatctcctcttttggtatattctgcatgccgtttacg
1839           F L L S L A F S D L L F G I F C M P F T   117
1840    481  gtggttgggaacatgcttggacgattcgtcttcggagcgtgtatttgcaaaatcgtaccg
1841           V V G N M L G R F V F G A V I C K I V P   137
1842    541  tacattcaaggatataatcagtcacagtggtccgtatggaccatggtcgtcatatcactggag
1843           Y I Q G I S V T V S V W T M V V I S L E   157
1844    601  aggtatcatgctatctgcaaccctctgtcgtcacggtctggcagacaaaagcgcgatgcg
1845           R Y H A I C N P L S S R V W Q T K A H A   177
1846    661  tacaaggccatagtcggggtgtggatggtggctttgtttctcaatctaccagcggtaatc
1847           Y K A I V G V W M V A L F L N L P A V I   197
1848    721  ttcagcaagttattctcgttcaacagcggcaccgtattcagatgcatgagatttgacct
1849           F S K L F S F N S G T V F R C D E I W P   217
1850    781  gctacactctatcgaacaatttataggatgtgtttgtttgtgattctaattggtggctcca
1851           A T L Y R T I Y R M C L F V I L M V A P   237
1852    841  ctcttcacgatgctcactgcttatggccttatcatccgagagctacgtagaggcatgaag
1853           L F T M L T A Y G L I I R E L R R G M K   257
1854    901  cttgaacaatgtggagctgataatgagaaaaagggagaacggaatagcaatgaagaacatg
1855           L E Q C G A D N E K R E N G I A M K N M   277
1856    961  ggagcgaagcctcctgtagcctcaatgagaaaaaaactaagaaatccgacaaaaagccg
1857           G D E A S C S L N E K K T K K S D K K P   297
1858   1021  gcacaagctacgatgcggagcacctcaaccagcggggccaagaaacgcgtcgtcaagatg
1859           A Q A T M R S T S T S G A K K R V V K M   317
1860   1081  ctcatcgtcatcgtggcgctgttctttgtctgctggacaccatcttgggtcggcaacatc
1861           L I V I V A L F F V C W T P S W V G N I   337
1862   1141  tggatcatgatctctgagaagagcgcagcagcacttcggccggggccgaggtgaccatc
1863           W I M I S E K S A S E H F G R A E V T I   357
1864   1201  ttcaagctgatgacgtacgctcggcatgtgtcaaccccatcgtctactgcttcatgaat
1865           F K L M T Y A S A C V N P I V Y C F M N   377
1866   1261  aagcgtttccgacagggcctcctcaacgcgttctcatgcggccggagaggacgcgcgggg
1867           K R F R Q G F L N A F S C G R R G R A G   397
1868   1321  gaccgagccacggcgagcgggtgacgtcagccgatttcagtcgacacggcgcacaaatgtg
1869           D R A T A S G D V S R F Q S T R R T N V   417
1870   1381  ccgcgacctagcccaacgaattacactaacgtctcgtcggactcttcgggtgtagcttggc
1871           P R P S P T N Y T N V S S D S S V *   434
1872   1441  gtcgggagaggctaactagcagtccttggaaacttcatcttttgaccttgttcaaaaggcat
1873   1501  cgccagtttcatTTTTGCAAAGGGCATTTC
```

1874
1875

1876 **Figure 2 - figure supplement 1. The *A. rubens* CCK receptor (ArCCKR).** The nucleotide
1877 sequence of contig 1110296 derived from radial nerve cord transcriptome data (GenBank
1878 accession number MW261740) is shown in lowercase (1530 bases; numbering on the left) and
1879 the encoded ArCCKR protein sequence is shown in uppercase (434 residues). The start and
1880 stop codons are highlighted in yellow. The coding sequence was synthesized (GenScript) to
1881 enable testing of the ArCCKP-derived CCK-type peptides as ligands for ArCCKR (see **Figure**
1882 **3**).

CCK-type signalling in an echinoderm

1883



1884

1885

1886

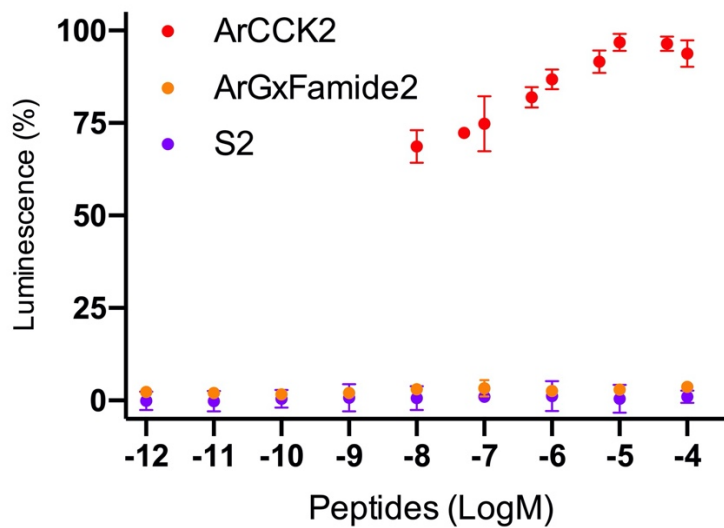
1887

1888

Figure 2 – figure supplement 2. Topology of ArCCKR. Predicted topology of ArCCKR inferred by the Protter tool (Omasits et al. 2014) with seven transmembrane domains numbered in blue and predicted N-glycosylation sites shown in green.

CCK-type signalling in an echinoderm

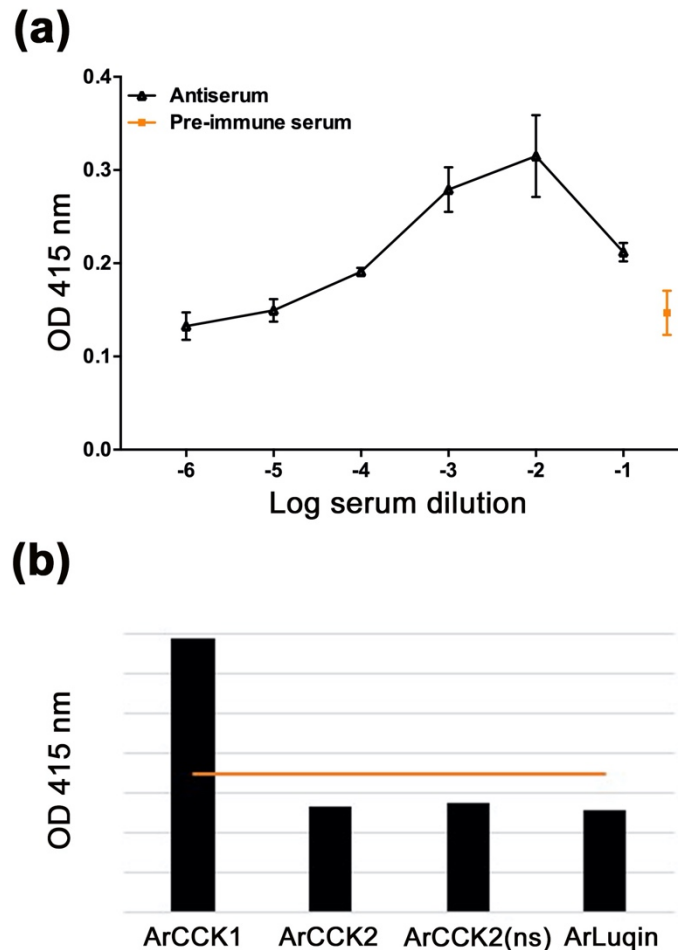
1889 **Figure 3 – source data 1.** Data for the graphs shown in **Figure 3** and **Figure 3 – figure**
1890 **supplement 1.** (not included here – available as separate file)
1891



1892

1893 **Figure 3 – figure supplement 1.** Graph showing the selectivity of ArCCKR as a receptor for
1894 CCK-type peptides. The *A. rubens* CCK-type peptide ArCCK2 (red) triggers luminescence in
1895 CHO-K1 cells expressing the receptor ArCCKR, the promiscuous G-protein G α 16 and the
1896 calcium-sensitive luminescent GFP-apoaequorin fusion protein G5A. In these experiments
1897 ArCCK2 was tested at concentrations between 10⁻⁸ and 10⁻⁴ M; see **Figure 3b** for experiments
1898 showing complete concentration-response curves. The receptor is not activated by the
1899 SALMFamide-type neuropeptide S2 (SGPYSFNLSGLTF-NH₂; purple) or by the *A. rubens*
1900 tachykinin-like peptide ArGxFamide2 (GGGVPHVVFQSGGIF-NH₂; orange). Each point
1901 represents mean values (\pm s.e.m) from at least three independent experiments done in triplicate.

CCK-type signalling in an echinoderm



1902

1903

1904 **Figure 5 – figure supplement 1. Characterisation of a rabbit antibodies to ArCCK1 using**

1905 **an enzyme-linked immunosorbent assay (ELISA) (a).** By comparison with pre-immune

1906 (orange), 0.1 nmol of the ArCCK1 antigen peptide is detected by the ArCCK1 antiserum (black

1907 line) at dilutions between 1:10 and 1:10000. All data points are mean values from two

1908 replicates. **(b)** Graph showing the results of ELISA tests using the TEA fraction of affinity-

1909 purified antibodies to ArCCK1 (dilution 1:10). The red line indicates the mean optical density

1910 (OD) value of negative control experiments without peptides. The four peptides tested were

1911 applied at a concentration of 0.1 nmol but only the mean OD value for ArCCK1 is above the

1912 OD value for the negative control. All data points are mean values from six replicates. These

1913 experiments demonstrate that specific antibodies to ArCCK1 were successfully generated.

1914

CCK-type signalling in an echinoderm

1915 **Figure 6 – source data 1.** Data for graphs shown in **Figure 6b, d and f.** (not included here –
1916 available as separate file)

1917

1918 **Figure 7 – source data 1.** Data for graphs shown in **Figure 7a, b and c.** (not included here –
1919 available as separate file)

1920

1921 **Figure 7 - video 1. ArCCK1 (10 μ l 1 mM) induced retraction of the cardiac stomach in**
1922 **the starfish *A. rubens*.** (not included here – available as separate file)

1923

1924 **Figure 7 - video 2. ArCCK2 (10 μ l 1 mM) induced retraction of the cardiac stomach in**
1925 **the starfish *A. rubens*.** (not included here – available as separate file)

1926

1927 **Figure 8 – source data 1.** Data for graphs shown in **Figure 8.** (not included here – available
1928 as separate file)

1929

CCK-type signalling in an echinoderm

1930
1931
1932

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Oligonucleotide primers for cloning of ArCCKP cDNA (<i>Asterias rubens</i>)	5'-TCGCTACTGTTTCTCTCGCA-3' 5'-AAAGGCGTCAACAACACTGCTT-3'			
Recombinant DNA reagent	Zero Blunt® Topo	ThermoFisher	Cat. no. 450159	
Recombinant DNA reagent	pBluescript SKII (+)	Agilent Technologies	Cat. no. 212205	
Recombinant DNA reagent	pcDNA 3.1+ vector with neomycin selectable marker (mammalian expression vector)	Invitrogen	Cat. no. V790-20	
Transfected construct (<i>Asterias rubens</i>)	<i>Asterias rubens</i> ArCCK receptor cDNA cloned in expression vector pcDNA 3.1+	This paper	GenBank: MW261740	
Cell line (<i>Cricetus griseus</i>)	Chinese hamster ovary cells (CHO-K1)	Sigma-Aldrich	RRID: CVCL_0214	Cat. no. 85051005
Antibody (<i>Asterias rubens</i>)	Antibody to ArCCK1	This paper	RRID: AB_2877176	
Antibody (sheep)	Alkaline phosphatase (AP)-conjugated anti-DIG antibody	Roche	Cat. no. 11093274910	
Antibody (goat)	Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase conjugated	Jackson ImmunoResearch	Cat. no. 111-035-003	
Commercial assay, kit	AminoLink Plus Immobilization Kit	ThermoFisher Sci.	Cat. no. 44894	
Software, algorithm	MSConvert	ProteoWizard Toolkit	Version 3.0.5759	doi: 10.1093/bioinformatics/btn323
Software, algorithm	Scaffold	Proteome Software Inc	Version 4.6.1	
Software, algorithm	MAFFT	MAFFT	Version 7	http://mafft.cbrc.jp/alignment/server/
Software, algorithm	IQ-tree web server			http://iqtree.cibiv.univie.ac.at
Software, algorithm	MUSCLE	EMBL-EBI, Hinxton		https://www.ebi.ac.uk/Tools/msa/muscle/

CCK-type signalling in an echinoderm

Software, algorithm	Volocity®	PerkinElmer	Version 6.3.1	
Software, algorithm	Adobe Photoshop CC	Adobe	Version 19.1.4, x64	
Software, algorithm	LabChart	ADInstruments	Version 8.0.7	
Software, algorithm	ImageJ		Version 1.0	http://rsb.info.nih.gov/ij
Software, algorithm	Prism	GraphPad	Version 6.0	

1933

1934