Functional characterisation of neuropeptides that act as ligands for both calcitonin-type and pigment-dispersing factor-type receptors in a deuterostome

Xiao Cong [1, 2], Huachen Liu [1], Lihua Liu [1], Maurice R. Elphick [3]* and Muyan Chen [1]*

1 The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, China

2 Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, China

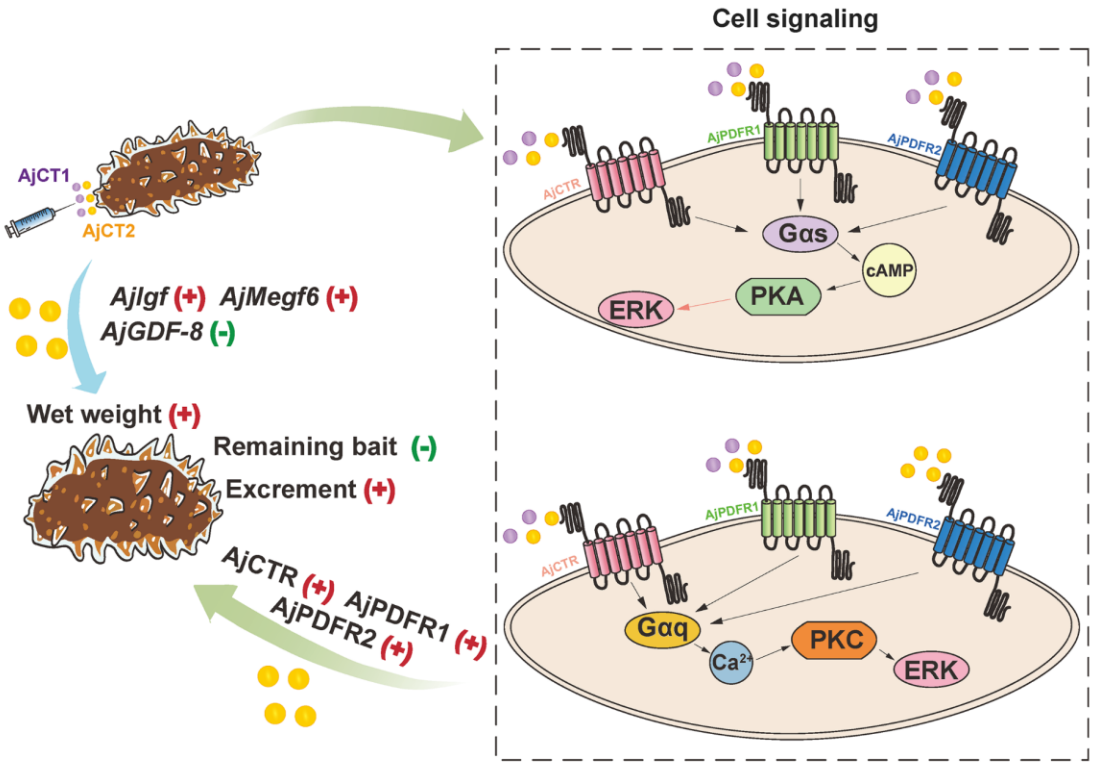
3 School of Biological & Behavioural Sciences, Queen Mary University of London, London, United Kingdom

* Correspondence: m.r.elphick@qmul.ac.uk; chenmuyan@ouc.edu.cn;

Abstract

The calcitonin (CT) family of related peptides exert their diverse physiological effects in mammals via two G-protein coupled receptors, CTR and the CTR-like receptor CLR. Analysis of the phylogenetic distribution of CT-type signaling has revealed the occurrence of CT-type peptides and CTR/CLR-type proteins in deuterostome and protostome invertebrates. Furthermore, experimental studies have revealed that in the protostome *Drosophila melanogaster* the CT-like peptide DH_{31} can act as a ligand for a CTR/CLR-type receptor and a pigment-dispersing factor (PDF) receptor. Here we investigated the signaling mechanisms and functions of CT-type neuropeptides in a deuterostome invertebrate, the sea cucumber *Apostichopus japonicus* (phylum Echinodermata). In *A. japonicus,* a single gene encodes two CT-type peptides (AjCT1 and AjCT2), both of which act as ligands for a CTR/CLR-type receptor (AjCTR) and two PDF-type receptors (AjPDFR1, AjPDFR2), but with differential activation of downstream cAMP/PKA, Gαq/Ca^{2+}/PKC and ERK1/2 signaling pathways. Analysis of the expression of the gene encoding AjCT1 and AjCT2 revealed transcripts in a variety of organ systems, but with highest expression in the circumoral nervous system. *In vitro* pharmacological experiments revealed that AjCT1 and/or AjCT2 cause dose-dependent relaxation of longitudinal body wall muscle and intestine preparations. Furthermore, *in vivo* pharmacological experiments and loss-of-function tests revealed a potential physiological role for AjCT2 signaling in promoting feeding and growth in *A. japonicus*. This is the first study to obtain evidence that CT-type peptides can act as ligands for both CTR/CLR-type and PDF-type receptors in a deuterostome. Furthermore, because of the economic importance of *A. japonicus* as a foodstuff, discovery of the potential role for CT-type peptides as regulators of feeding and growth in this species may provide a basis for practical applications in aquaculture.

39 **Graphical abstract**



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41 A schematic showing proposed molecular mechanisms by which CT-type neuropeptides regulate
42 feeding and growth in *A. japonicus*. AjCT1 and AjCT2 are represented by purple and yellow circles,
43 respectively. For cell signaling, black arrows indicate that this process is activated, whilst the pink
44 arrow indicates that the ERK1/2 is activated in the AjCT1/AjPDFR1, AjCT2/AjPDFR1 and
45 AjCT2/AjPDFR2 signaling pathways.

46

47 **Keywords:** calcitonin; neuropeptide; echinoderm; sea cucumber; calcitonin receptor;
48 pigment-dispersing factor receptor; feeding; growth

1. Introduction

The peptide calcitonin (CT) was first identified as a hormone that regulates blood calcium levels and subsequently a family of CT-related neuropeptides was discovered in mammals, including calcitonin gene related peptides (CGRPs), amylin (AMY), adrenomedullin (AM), and CT receptor-stimulating peptide (CRSP) (Copp et al., 1962; Sekiguchi et al., 2009; Sekiguchi et al., 2016). Members of the CT-type family of peptides typically have a disulfide bond in their N-terminal region (Cai et al., 2018) and exert their physiological effects in mammals by binding to two related G protein-coupled receptors: CTR (CT receptor) and CLR (CTR-like receptor) (Martins et al., 2014). However, receptor activity-modifying proteins (RAMPs) acting as accessory proteins are key determinants of ligand selectivity for CTR and CLR (Mclatchie et al., 1998). Thus, formation of a complex with a RAMP is essential for CLR function, whereas RAMPs are not required for CTR ligand recognition (Kuwasako et al., 2011; Bomberger et al., 2012). Furthermore, binding of CT to CTR in the absence of RAMPs (Katafuchi et al., 2009) can activate signaling via several downstream pathways, including cAMP accumulation, Ca^{2+} mobilization, and ERK activation (Chen et al., 1998; Pondel, 2000; Walker et al., 2010; Andreassen et al., 2014).

Transcriptome/genome sequencing has enabled analysis of the phylogenetic distribution of CT-type signaling systems, indicating that the evolutionary origin of CT-type peptides and CTR/CLR-related receptors can be traced back to the urbilaterian common ancestor of deuterostomes and protostomes (Mirabeau and Joly, 2013; Jekely, 2013). Furthermore, in protostomes two different types of CT-related peptides have been identified. Firstly, peptides that are structurally similar to vertebrate CT-type peptides in having an N-terminal disulphide bridge and secondly, diuretic hormone 31 (DH_{31})-type peptides that lack an N-terminal disulphide bridge. Furthermore, phylogenetic analysis indicates that gene duplication in a common ancestor of the protostomes gave rise to genes encoding CT-like peptides with a disulphide bridge and DH_{31}-type peptides, which were then differentially retained in protostome taxa (Cai et al., 2018). Receptors for CT-like peptides with a disulphide bridge have been characterised in a molluscan species, the oyster *Crassostrea gigas*, which has seven

79 CTR/CLR-type receptors. Two of these receptors, named Cg-CT-R and Cragi-CTR2,
80 were shown to be specifically activated by the CT-type neuropeptides Cragi-CT1b and
81 Cragi-CT2, respectively, thereby mediating intracellular Ca^{2+} mobilization and cAMP
82 accumulation (Schwartz *et al.*, 2019). CT-like peptides with a disulphide bridge are not
83 present in the insect *Drosophila melanogaster*. However, receptors that are activated by
84 DH_{31} have been identified in this species and, interestingly, DH_{31} can act as a ligand for
85 two types of receptors. Thus, DH_{31} acts as a ligand for a CTR/CLR-related protein
86 (CG17415), but it also acts as a ligand for a protein (CG13758) that is the receptor for
87 pigment-dispersing factor (PDF) (Mertens et al., 2005; Shafer et al., 2008). However,
88 the evolutionary and functional significance of the occurrence of dual receptor
89 pathways for DH_{31} in *Drosophila* are not known.

90 Currently, relatively little is known about CT-type signaling systems in
91 deuterostome invertebrates. Analysis of transcriptome/genome sequence data has
92 enabled identification of genes/transcripts encoding CT-type peptides and candidate
93 CTR/CLR-type receptors for these peptides in several deuterostome invertebrate taxa,
94 including urochordates (e.g. *Ciona intestinalis*), cephalochordates (e.g. *Branchiostoma*
95 *floridae*), hemichordates (e.g. *Saccoglossus kowalevskii*) and echinoderms (e.g.
96 *Strongylocentrotus purpuratus*) (Burke et al., 2006; Sekiguchi et al., 2009; Sekiguchi
97 et al., 2016; Rowe and Elphick, 2012; Martins et al., 2014). Furthermore, analysis of
98 the expression and action of CT-type peptides identified in the starfish *Asterias rubens*
99 and *Patiria pectinifera* has revealed that they act as muscle relaxants (Cai et al., 2018;
100 Semmens et al., 2016). However, experimental confirmation of ligand-receptor partners
101 has thus far only been reported for the cephalochordate *B. floridae*, where three CT-
102 type peptides were shown to act as ligands for a CTR/CLR-type receptor when it is co-
103 expressed with RAMP-type proteins (Sekiguchi et al., 2016). To gain further insights
104 into the mechanisms of CT-type signaling in invertebrate deuterostomes, the aim of this
105 study was to functionally characterise CT-type peptides and their receptors in an
106 echinoderm – the sea cucumber *Apostichopus japonicus*.

107 *A. japonicus* is an economically important species in Asian aquaculture
108 (Pangestuti and Arifin, 2018; Guo et al., 2020) and a species representative of the

109 phylum Echinodermata. As a phylum of deuterostome invertebrates, echinoderms form
110 a sister clade to the chordates together with hemichordates and xenoturbellids
111 (Bromham and Degnan, 1999; Bourlat et al., 2006; Dunn et al., 2008) and as such they
112 occupy a key "intermediate" phylogenetic position relative to the protostome
113 invertebrates and vertebrates (Zandawala et al., 2017), which make them attractive as
114 experimental systems to address evolutionary questions. In our previous studies, we
115 have established this species as a model system to obtain insights into the evolutionary
116 history and comparative physiology of neuropeptide signaling systems in bilaterians
117 (Chen et al., 2019; Li et al., 2022; Zheng et al., 2022; Cong et al., 2023; Zheng et al.,
118 2024). A gene encoding two CT-type peptides (AjCT1 and AjCT2) has been identified
119 in *A. japonicus* (Chen et al., 2019) and a candidate receptor (AjCTR) for these peptides
120 has also been predicted (Huang et al., 2021). Furthermore, relevant to the discovery that
121 the CT-like peptide DH_{31} can act as a ligand for both a CTR/CLR-type receptor and a
122 PDF-type receptor in *Drosophila*, the sequences of two PDF-type receptors have been
123 identified in *A. japonicus* (AjPDFR1 and AjPDFR2) (Huang et al., 2021).
124 The first aim of this study was to investigate if AjCT1 and/or AjCT2 act as ligands
125 for AjCTR, AjPDFR1 and AjPDFR2 and to characterise the downstream signaling
126 pathways that are activated when AjCT1 and/or AjCT2 bind to their receptor(s). The
127 second aim was to investigate the physiological roles of AjCT1 and AjCT2 in *A.*
128 *japonicus* by analysing the expression of the gene encoding these peptides, examining
129 the *in vitro* and *in vivo* pharmacological effects of these peptides and performing loss-
130 of-function tests. The functional characterisation of CT-type signaling in *A. japonicus*
131 reported in this study provides new insights into the evolution and comparative
132 physiology of neuropeptides and a basis for application of neuropeptide-based
133 strategies to improve aquaculture methods for this economically important species.

2. Results

2.1 Characterization and phylogenetic analysis of AjCT1/2

The complete cDNA sequences of the CT-type neuropeptide precursors *AjCTP1* and *AjCTP2* were confirmed. The coding sequence of *AjCTP2* is shorter than *AjCTP1* (Figure 1A) but both transcripts encode precursor proteins that comprise the same predicted 23-residue N-terminal signal peptide (Figure 1-figure supplement 1A). *AjCTP1* encodes both putative CT-type neuropeptides (AjCT1 and AjCT2), while *AjCTP2* only encodes AjCT2 (Figure 1A, Figure 1-figure supplement 1A and 1B). Exon and intron structure analysis showed that *AjCTP1* comprises four exons, with AjCT1 and AjCT2 encoded in the third and fourth exons, respectively. *AjCTP2* comprises three exons, excluding the third exon that encodes AjCT1 (Figure 1A). Phylogenetic analysis revealed that AjCTP1 and AjCTP2 are closely related to CT-type precursors from other sea cucumbers (*Holothuria glaberrima* and *Holothuria scabra*), and are positioned within a clade that also contains CT-type precursors from other echinoderms (sea urchin *S. purpuratus*, starfish *A. rubens*). Furthermore, with vasopressin/oxytocin-type neuropeptide precursors included as an outgroup, the echinoderm CT-type precursors are positioned within a clade that also comprises CT-type precursors from chordates and DH_{31}/CT-type precursors from protostomes (Figure 1B). The predicted tertiary structures of the AjCTP1 and AjCTP2 proteins are shown in Figure 1-figure supplement 1C. Sequence alignment of CT-related neuropeptides revealed that, with the exception of DH_{31}-type peptides, the N-terminal region of CT-related peptides has a pair of conserved cysteine residues that are known or predicted to form a disulfide bond. Another conserved feature is a C-terminal proline residue, with the exception of CGRPs (Figure 1-figure supplement 2). Accordingly, both AjCT1 and AjCT2 have these two conserved characteristics described above.

2.2 Characterization and phylogenetic analysis of three candidate receptors for AjCT1/2

The sequences of three candidate receptors for AjCT1 and AjCT2 were obtained by RACE and phosphorylation sites were predicted, as shown in Figure 1-figure

supplement 3A, 3B, and 3C. Phylogenetic analysis revealed that CTR/CLR-type receptors divided into two clades and AjCTR was positioned in a clade with other deuterostome CTR/CLR-type receptors, whilst the other clade comprised CTR/CLR-type receptors from protostomes. Similarly, AjPDFR1 and AjPDFR2 were positioned in a clade comprising PDF-type receptors from other deuterostomes, whilst another clade comprised PDF-type receptors from protostomes (Figure 1C). Domain prediction of the candidate receptors showed that AjCTR had HRM (Hormone receptor) and 7tm_GPCRs (seven-transmembrane G protein-coupled receptor) superfamily domains (Figure 1-figure supplement 4A), whereas AjPDFR1 and AjPDFR2 only presented the 7tm_GPCRs superfamily domain (Figure 1-figure supplement 4B). To further investigate an orthologous relationship of the candidate receptors with homologous receptors in other echinoderms, we conducted a gene synteny analysis in *A. japonicus*, *A. rubens* and *L. variegatus*. The neighboring genes of *AjCTR* included *GOLGA4*, *NeuroD*, *CRF2*, *ppGalNAc-T*, *URB3*, *SLC40A1*, *CMAH* and *MAGP*. The relative positions of these genes were different from those in *A. rubens* and *L. variegatus* (Figure 1-figure supplement 4C). The same situation was also observed for the synteny analysis of PDFRs.

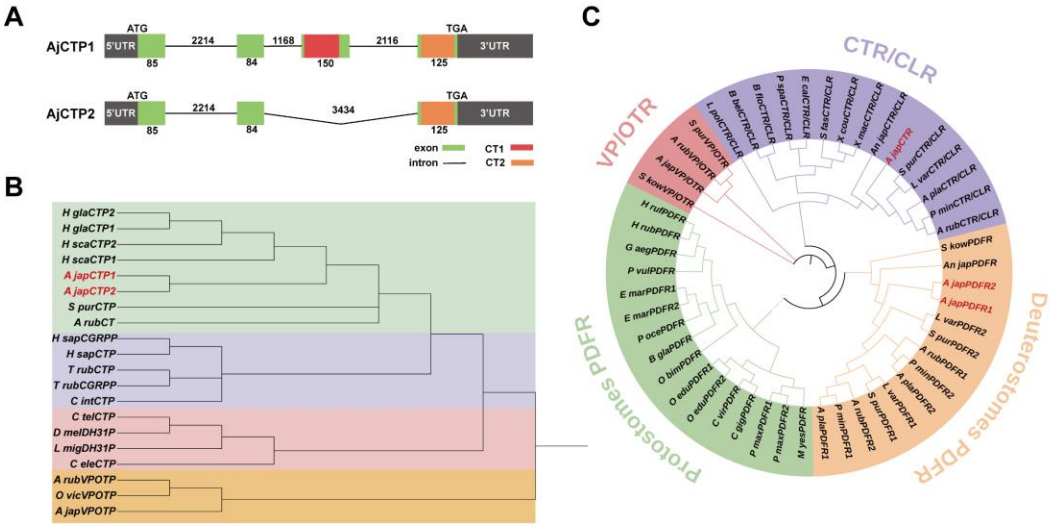

Figure 1. CT-type neuropeptide precursors and the candidate receptors in *A. japonicus.* (A) Alternative splicing schematic of *AjCTP1* and *AjCTP2*. Exons are represented by green rectangles, introns are represented by lines, AjCT1 and AjCT2 are represented by red and orange rectangles respectively, upstream and downstream non-coding regions are represented by gray rectangles. The numbers represent the length of exons or introns. (B) Phylogenetic analysis of *AjCTP1* and *AjCTP2*

and CT-related neuropeptide precursors from other bilaterians. CT-type precursors from deuterostomes are shown in green (echinoderms) and purple (chordates), CT-type precursors from protostomes are shown in pink and VP/OT-type neuropeptide precursors (outgroup) are shown in orange. (C) Phylogenetic analysis of relationships of *AjCTR*, *AjPDFR1* and *AjPDFR2* (red letters) with CT-type and PDF-type receptors in other taxa (black letters), using VP/OT-type receptors as an outgroup. CTR/CLR-type receptors are shown in purple, deuterostome PDFRs are shown in yellow, protostome PDFRs are shown in green and VP/OTRs are shown in pink. Full species names and accession numbers are listed in Supplementary Table 3.

2.3 AjCT1 and AjCT2 act as ligands for AjCTR, AjPDFR1 and AjPDFR2

To investigate if AjCT1 and/or AjCT2 act as ligands for AjCTR, AjPDFR1 and AjPDFR2, here, two receptor assay methods were employed. As shown in Figure 2A, both AjCT1 and AjCT2 (10^{-6} M) caused a significant increase in CRE luciferase activity in HEK293T cells transfected with AjCTR, AjPDFR1 or AjPDFR2 (Table 1). Furthermore, this effect was inhibited by pre-incubation of cells with the PKA inhibitor H89. The intracellular accumulation of cAMP was also measured, and this showed that AjCT1 and AjCT2 (10^{-9} – 10^{-5} M) caused a dose-dependent increase in cAMP in HEK293T cells transfected with AjCTR, AjPDFR1 or AjPDFR2 (Figure 2B). However, when exposed to AjCT1 or AjCT2, the maximum accumulation of intracellular cAMP was higher in cells transfected with *AjCTR* than in cells transfected with *AjPDFR1* and *AjPDFR2* (Figure 2B). Measurement of SRE luciferase activity revealed that AjCT1 and AjCT2 (10^{-6} M) administration evoked an increase in activity in HEK293T cells transfected with *AjCTR* and *AjPDFR1* (Figure 3A). However, in HEK293T cells transfected with *AjPDFR2*, an increase in SRE luciferase activity was only observed with AjCT2, not with AjCT1 (Table 1). Furthermore, as shown in Figure 3A, AjCT1- and AjCT2-induced increases in SRE luciferase activity were not observed in cells that had been pre-incubated with FR900359 (Gαq protein inhibitor) and Gö 6983 (PKC inhibitor). As shown in Figure 3-figure supplement 1, receptors localized in the cell membrane, but after exposure to AjCT1 and AjCT2 (10^{-6} M) for 15 min, internalization of pEGFP-N1/*AjCTR*, pEGFP-N1/*AjPDFR1* and pEGFP-N1/*AjPDFR2* was observed in transfected HEK293T cells (Figure 3B). Taken together, all these findings indicate that AjCT1 and AjCT2 can act as ligands for the three candidate receptors and then

activate cAMP/PKA signaling and Gαq/Ca^{2+}/PKC signaling in all combinations, with the exception of AjCT1/*AjPDFR2* which only activates the cAMP/PKA cascade.

To investigate whether the activated signaling pathways may exert downstream effects via the ERK–MAPK pathway, the phosphorylation levels of ERK1/2 were analysed in transfected HEK293T cells. pERK1/2 levels in cells transfected with *AjCTR* or *AjPDFR1*, but not *AjPDFR2,* were significantly increased after a 5 min incubation with AjCT1 (Figure 4A). Furthermore, this effect was blocked by the PKC inhibitor in AjCTR-expressing cells, and this effect was blocked by both PKA and PKC inhibitors in AjPDFR1-expressing cells (Figure 4-figure supplement 1A). pERK1/2 levels in cells transfected with *AjCTR* or *AjPDFR1* or *AjPDFR2,* were significantly increased after a 5 min incubation with AjCT2 (Figure 4B). Moreover, this effect persisted until 15 min after exposure to AjCT in both *AjCTR*-transfected cells and *AjPDFR1*-transfected cells but not in *AjPDFR2*-transfected cells (Figure 4B, Table 1). Furthermore, these effects were blocked by PKA and PKC inhibitors in AjPDFR1 and AjPDFR2 expressing cells, whereas in AjCTR expressing cells, peptide-induced pERK1/2 activity was only blocked by a PKC inhibitor (Figure 4-figure supplement 1B). These results indicate that, with the exception of AjCT1/*AjPDFR2,* both AjCT1 and AjCT2 can activate the ERK1/2 cascade by binding to the candidate receptors.

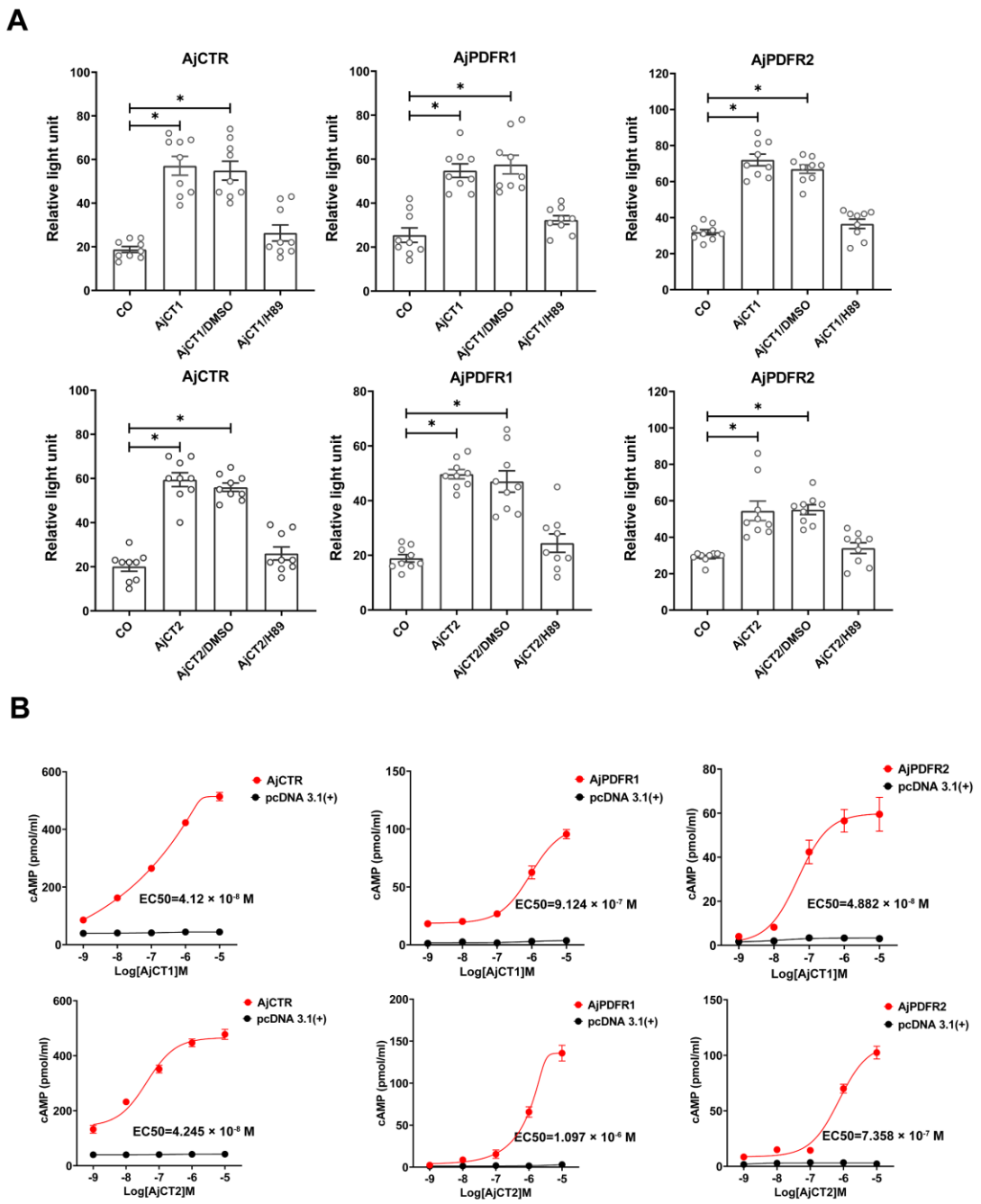

Figure 2. Pharmacological characterisation of AjCT1 and AjCT2 as ligands for the *A. japonicus* receptors AjCTR, AjPDFR1 and AjPDFR2. (A) CRE-driven luciferase activity measured in HEK293T cells transfected with one of the three candidate receptors after exposure to AjCT1or AjCT2 or neuropeptide + DMSO or neuropeptide + H89 (10 μM). The neuropeptide concentration used here was 10^{-6} M. (B) Measurement of cAMP accumulation after exposure to AjCT1 or AjCT2 (10^{-9} – 10^{-5} M). No cAMP elevation is observed in control experiments where HEK293T cells were transfected with empty pcDNA 3.1(+) (black circle). The EC_{50} values are shown in the figure. Error bars represent SEM for three independent experiments. * indicates a statistically significant difference with $p < 0.05$.

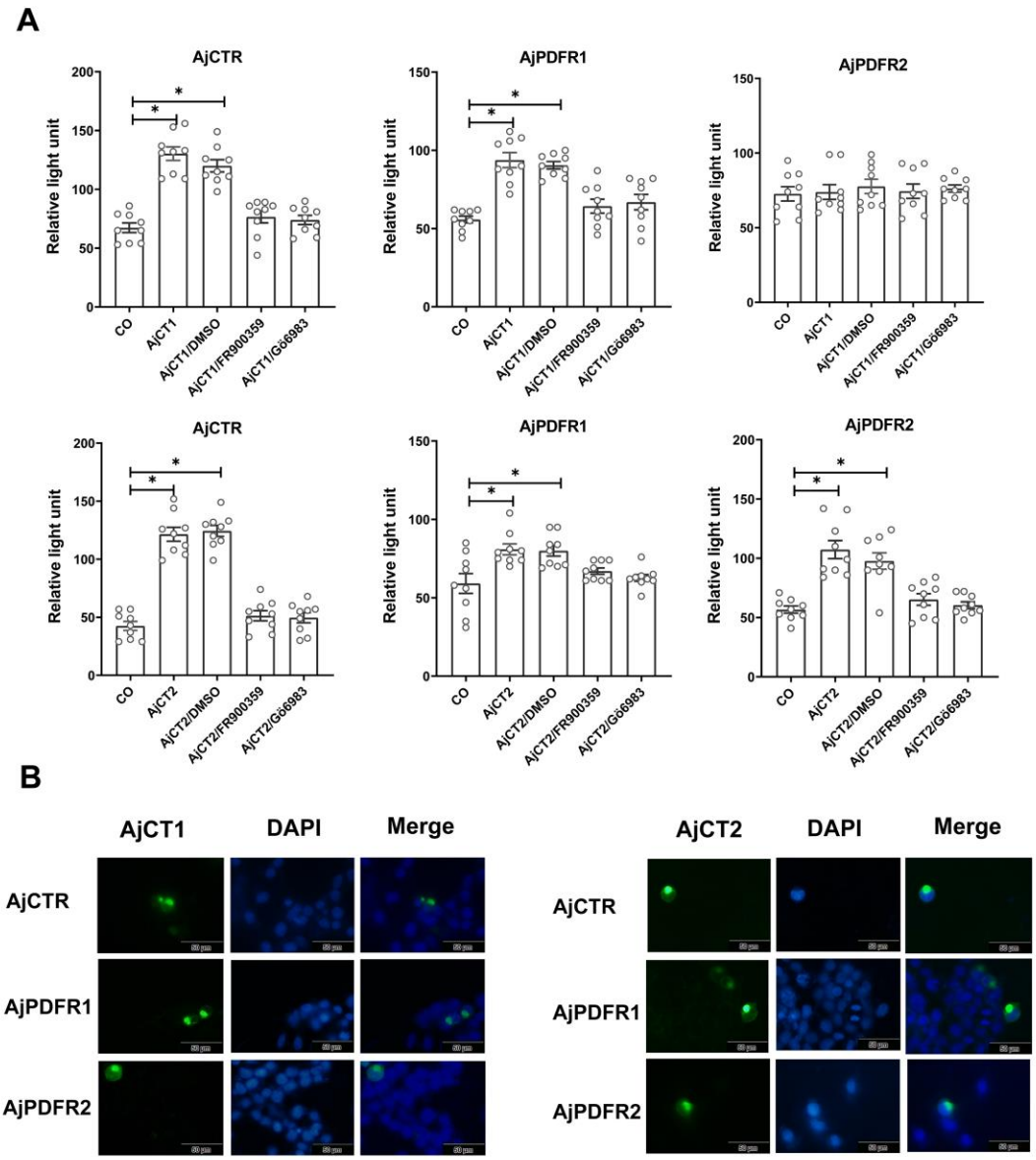

Figure 3. SRE-driven luciferase activity and receptor internalization in cells transfected with AjCTR, AjPDFR1 or AjPDFR2 and exposed to AjCT1 or AjCT2. (A) SRE-driven luciferase activity measured after incubation with AjCT1, AjCT2, neuropeptide (10^{-6} M)/DMSO, neuropeptide (10^{-6} M)/FR900359 (1uM) and neuropeptide (10^{-6} M)/Gö 6983 (1 μM). (B) Localization and internalization of AjCTR, AjPDFR1 and AjPDFR2 after 15 min treatment with 10^{-6} M AjCT1 or AjCT2. Green fluorescence represents the localization of pEGFP-N1/receptors in cells. The cell nucleus probe (DAPI) was used for cell nucleus staining. Scale bar: 50 μm. Error bars represent SEM for three independent experiments. * indicates a statistically significant difference with $p < 0.05$. ns, indicates no significant difference. All pictures are representative of at least three independent experiments.

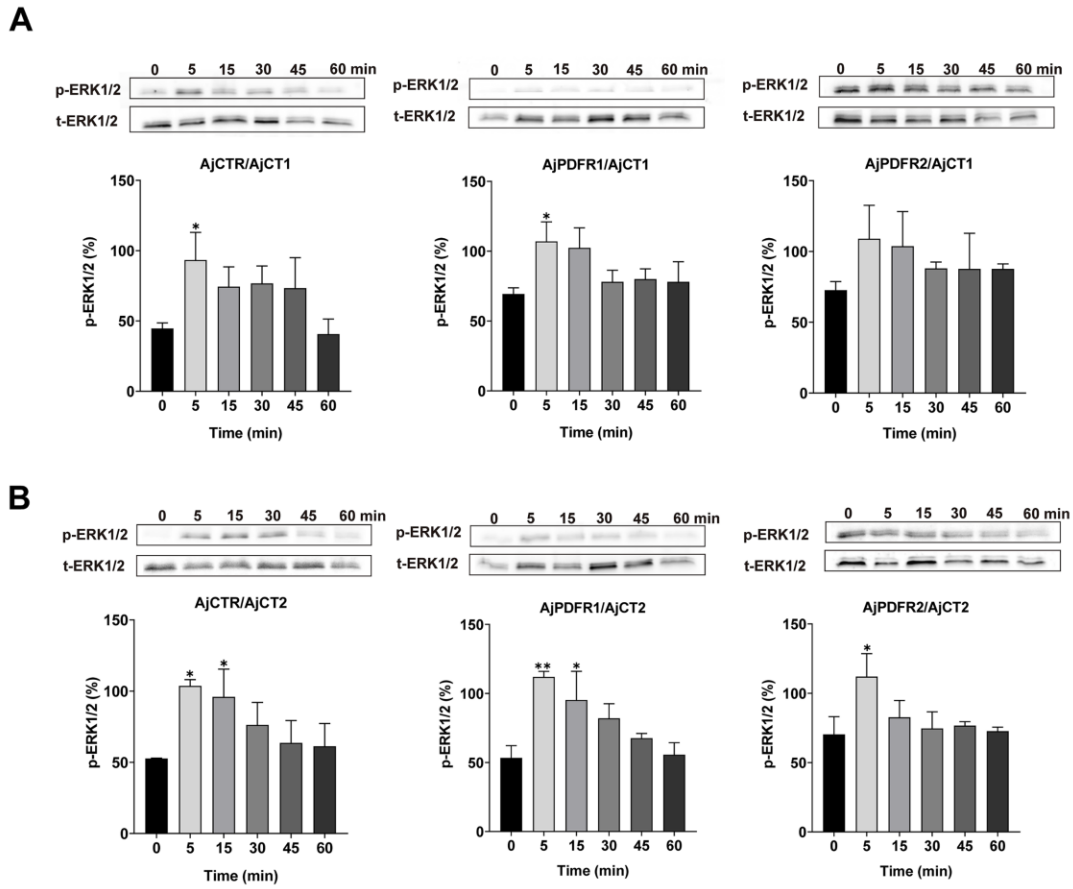

Figure 4. ERK1/2 activity in cells transfected with AjCTR, AjPDFR1 or AjPDFR2 and exposed to AjCT1 or AjCT2. (A) and (B) show the immunoblot intensity of representative phosphorylation bands. The transfected HEK293T cells were incubated with AjCT1 (10^{-6} M) or AjCT2 (10^{-6} M) for 0, 5, 15, 30, 45 and 60 min. The p-ERK1/2 was normalized based on t-ERK1/2. Error bars represent SEM for three independent experiments. Statistically significant differences are indicated as follows: *: $p < 0.05$, **: $p < 0.01$.

Table 1. Activation of signaling pathways

Assays / Receptors	cAMP/PKA		ERK1/2		Gαq/Ca^{2+}/PKC		ERK1/2	
	AjCT1	AjCT2	AjCT1	AjCT2	AjCT1	AjCT2	AjCT1	AjCT2
AjCTR	√	√	×	×	√	√	√	√
AjPDFR1	√	√	√	√	√	√	√	√
AjPDFR2	√	√	×	√	×	√	×	√

A tick indicates activation and the cross indicates no activation.

2.4 Localization of AjCTP1/2 expression in A. japonicus

qRT-PCR analysis revealed that *AjCTP1/2* had the highest expression level in the circumoral nervous system (CNS) compared with other tissues ($p < 0.05$) (Figure 5A).

Informed by these findings, mRNA *in situ* hybridization was used to visualize transcripts in the CNS and the *AjCTP1/2* positive signals were observed (Figure 5B). No positive staining was detected in the CNS incubated with the sense probe (Figure 5-figure supplement 1).

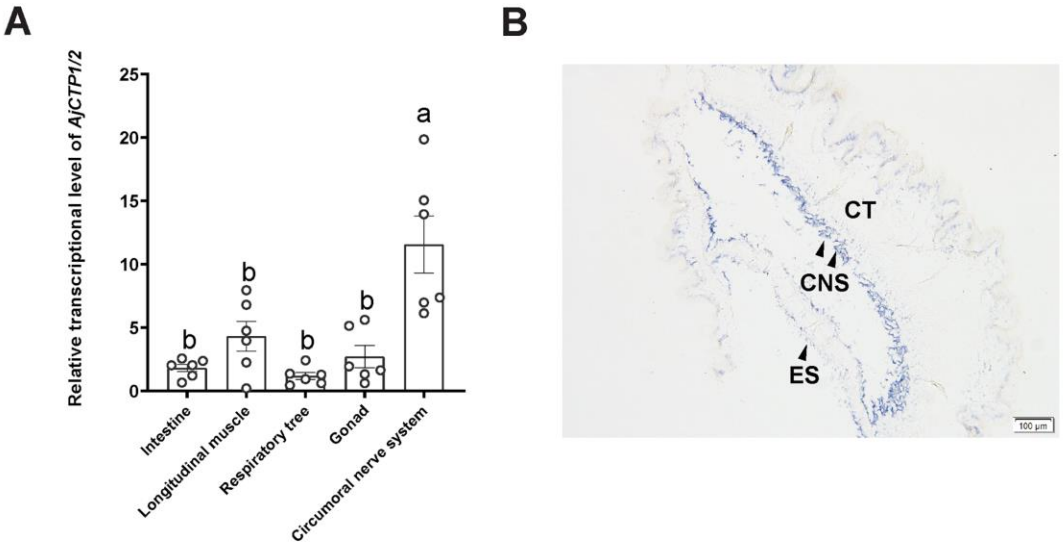

Figure 5. The expression profile of *AjCTP1/2* in *A. japonicus*. (A) Analysis of the transcript expression level of *AjCTP1/2* in different tissues. Lowercase letters a and b above columns indicate the statistical difference at $p < 0.05$; the expression level of *AjCTP1/2* in CNS was significantly higher than that in other tissues. Mean values with standard deviations (n = 5) are shown. (B) Localization of *AjCTP1/2* in CNS using mRNA *in situ* hybridization. CNS, circumoral nervous system; CT, connective tissue; Es, epidermis. Scale bar: 100 μm.

2.5 AjCT1 and AjCT2 causes dose-dependent relaxation of longitudinal muscle and intestine preparations from A. japonicus

Both AjCT1 and AjCT2 caused dose-dependent relaxation of longitudinal muscles from *A. japonicus* at 10^{-10}–10^{-6} M. At the highest concentration tested (10^{-6} M), AjCT1 and AjCT2 caused 30.07 ± 7.49% and 32.50 ± 14.49% reversal of ACh-induced contraction, respectively (Figure 6A, 6B). AjCT1 had no effect on the contractile state of intestine preparations, even at concentrations as high as 10^{-5} M (Figure 6C). Unlike AjCT1, AjCT2 caused dose-dependent relaxation of intestine preparations, with 26.33 ± 8.75% reversal of ACh-induced contraction observed at 10^{-5} M (Figure 6D).

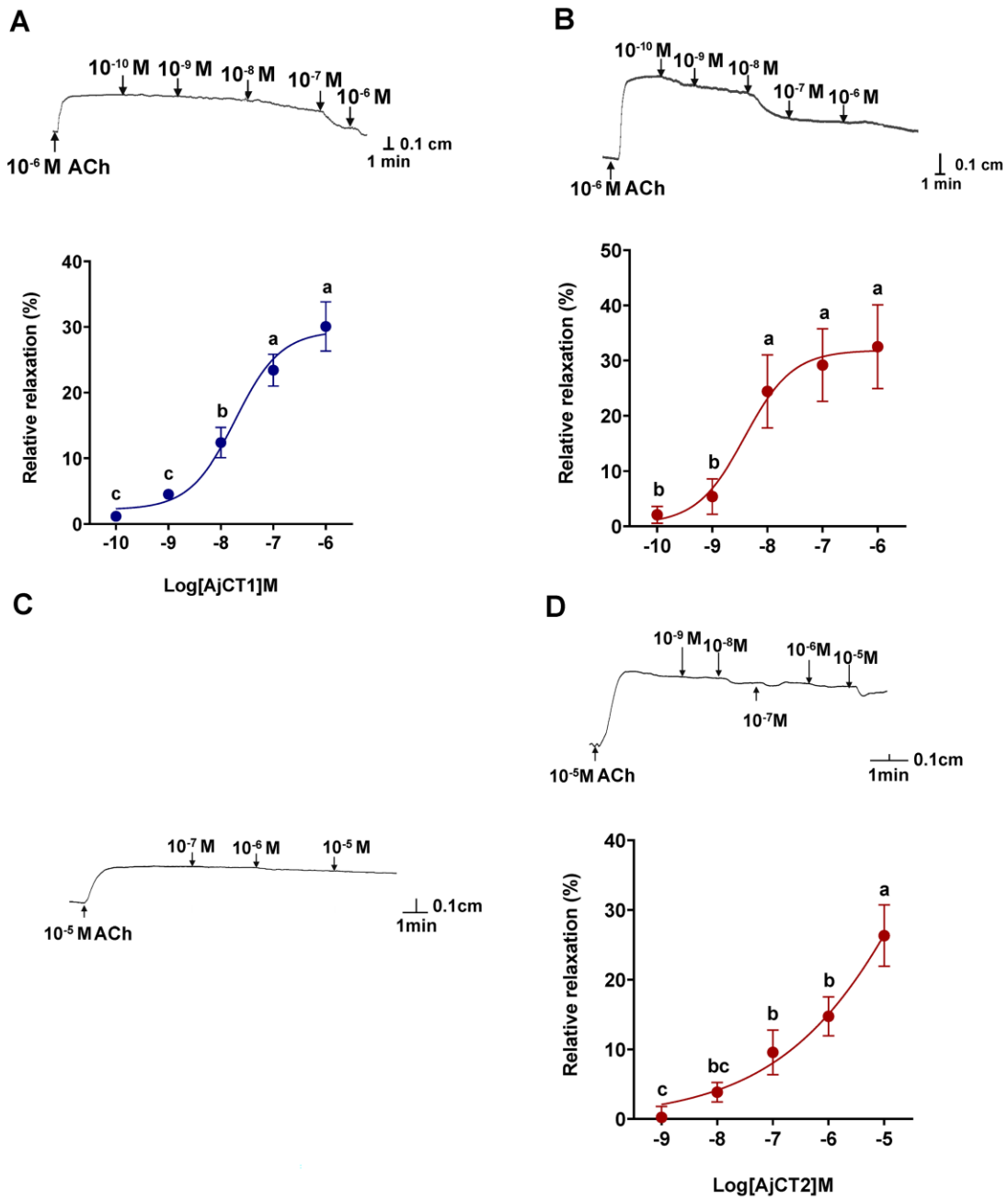

Figure 6. Pharmacological effects of AjCT1 and AjCT2 on longitudinal muscle and intestine preparations from *A. japonicus*. (A) and (B) show representative recordings of the relaxing effects of AjCT1 and AjCT2 on longitudinal muscle preparations. The graphs show the dose-dependent relaxing effects of AjCT1 and AjCT2 (10^{-10} – 10^{-6} M), calculated as the percentage reversal of the contracting effect of 10^{-6} M ACh. (C) and (D) show representative recordings of experiments in which AjCT1 (10^{-7} – 10^{-5} M) and AjCT2 (10^{-9} – 10^{-5} M) were tested on intestine preparations. AjCT1 had no effect, but AjCT2 caused relaxation and the graph shows the dose-dependent relaxing effect of AjCT2 (10^{-9} – 10^{-5} M), calculated as the percentage reversal of the contracting effect of 10^{-5} M ACh. Mean values ± SEM were determined from three preparations. Lowercase letters a, b and c above columns indicate the statistical difference at $p < 0.05$, whilst there is no significant difference between bc and c.

304

305 ***2.6 AjCT2 affects feeding and growth in A. japonicus***

306 To investigate the effects of AjCT1 and AjCT2 *in vivo*, a long-term injection
307 experiment was carried out. As shown in Figure 7A, there were no significant changes
308 in wet mass in the CT1L, CT1H and CT2L groups ($p \geqslant 0.05$), but in the CT2H group
309 there was a significant increase in wet weight after 24 days of injection in comparison
310 with the control group (CO) ($p < 0.05$). Correspondingly, the mass of faeces increased
311 and the residual bait mass decreased (Figure 7A-figure supplement 1). Furthermore, the
312 relative intestinal expression levels of several growth factors were investigated and the
313 results showed that *AjGDF-8*, a growth inhibitory factor, was significantly down-
314 regulated in all the experimental groups. In contrast, the relative expression levels of
315 *AjIgf* and *AjMegf6,* growth promoting factors, were significantly up-regulated in
316 intestines (Figure 7B).

317 Subsequently, the relative intestinal expression levels of AjCT1 and AjCT2
318 receptors were further tested for the CT2H group. The results showed that the relative
319 expression levels of all three receptors were significantly up-regulated, with the ranked
320 fold change being *AjCTR* > *AjPDFR1* > *AjPDFR2* (Figure 7C). Conversely, the relative
321 expression levels of the three receptor genes were significantly down-regulated in
322 si*AjCTP1*-1 treated animals (Figure 8A, 8B and 8C). The interference rate for
323 si*AjCTP1/2*-1 was 66 % (Figure 8-figure supplement 1).

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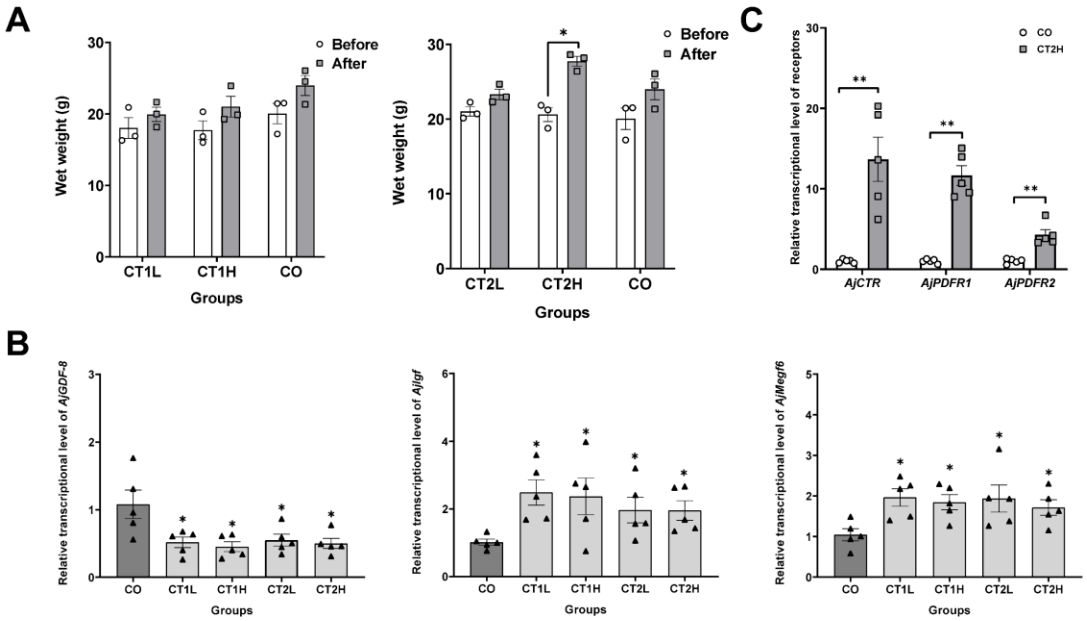

Figure 7. *In vivo* pharmacological tests revealed that AjCT1 and AjCT2 affect feeding and growth-related gene expression in *A. japonicus*. (A) Body mass in different groups before and after injection of AjCT1, AjCT2 or sterilized seawater. (B) The relative intestinal transcript expression levels of *AjGDF-8*, *AjIgf* and *AjMegf6* in different groups after injection of AjCT1 or AjCT2 or sterilized seawater (CO) *in A. japonicus*. (C) The relative intestinal expression levels of AjCT1/2 receptors (*AjCTR*, *AjPDFR1* and *AjPDFR2*) in CT2H and CO groups after 24 days injection experiment. Values were mean ± SEM (n = 5). * indicates statistically significant differences with $p < 0.05$, ** indicates statistically significant differences with $p < 0.01$. CO: control group (sterilized seawater); CT1L/CT2L: AjCT1/AjCT2 low concentration group (5×10^{-3} mg/mL); CT1H/CT2H: AjCT1/AjCT2 high concentration group (5×10^{-1} mg/mL).

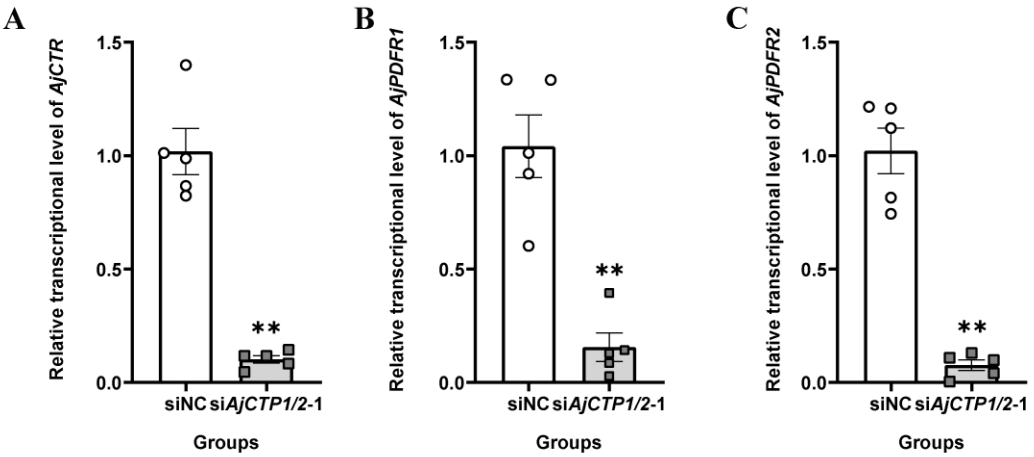

Figure 8. The relative intestinal expression levels of AjCT1/2 receptors after *AjCTP1/2*-1 knockdown. (A), (B) and (C) show the relative expression levels of *AjCTR*, *AjPDFR1* and *AjPDFR2* respectively. Values are means ± SEM (n = 5). ** indicates statistically significant differences with $p < 0.01$.

342 **3. Discussion**

343 Previous studies have revealed that the CT-type neuropeptide DH_{31} can act as a
344 ligand for a CTR/CLR-type receptor and a pigment-dispersing factor (PDF) receptor in
345 a protostome – the insect *D. melanogaster* (Mertens et al., 2005; Shafer et al., 2008;
346 Schwartz et al., 2019). Here we show that two CT-type peptides (AjCT1 and AjCT2)
347 act as ligands for a CTR/CLR-type receptor and two pigment-dispersing factor (PDF)
348 type receptors in a deuterostome – the sea cucumber *A. japonicus* (phylum
349 Echinodermata). This is an important finding because it indicates that the ability of CT-
350 type neuropeptides to act as ligands for both CTR/CLR-type receptors and PDF-type
351 receptors is an evolutionarily ancient property that originated in a common ancestor of
352 the Bilateria. However, further studies on a wider variety of both protostome (e.g.
353 molluscs, annelids) and deuterostome taxa (e.g. other echinoderms, hemichordates) will
354 be needed to establish if this is an evolutionarily conserved property of CT-type
355 neuropeptides. Here, we also investigated the signal transduction pathways that are
356 activated by AjCT1 and AjCT2 when these peptides bind to their receptors and then
357 proceeded to investigate the expression and actions of these peptides to gain insights
358 into their physiological/behavioral roles in *A. japonicus*, as discussed below.

359

360 ***3.1 Alternative RNA splicing gives rise to transcripts encoding one (AjCT2) or two***
361 ***(AjCT1, AjCT2) CT-type neuropeptides in A. japonicus***

362 In vertebrates, alternative RNA splicing gives rise to transcripts encoding CT or
363 CGRP (Amara et al., 1982; Morris et al., 1984). Analysis of transcriptome/genome
364 sequence data from *A. japonicus* has revealed that in this species alternative RNA
365 splicing gives rise to transcripts encoding one (AjCT2) or two (AjCT1, AjCT2) CT-type
366 neuropeptides (Rowe et al., 2014; Zandawala et al., 2017; Chen et al., 2019; this study).
367 A similar pattern of alternative RNA splicing has been reported for CT-type precursors
368 in other sea cucumbers (*H. scabra* and *H. glaberrima*; class Holothuroidea) and in
369 brittle stars (*Amphiura filiformis* and *Ophiopsila Aranea*; class Ophiuroidea)
370 (Zandawala et al., 2017; Suwansa-Ard et al., 2018). In contrast, CT-type precursors
371 identified in other echinoderm classes (Asteroidea, Echinoidea and Crinoidea)

comprise a single CT-type neuropeptide (Rowe and Elphick, 2012; Mirabeau and Joly, 2013; Rowe et al., 2014; Semmens et al., 2016; Cai et al., 2018; Aleotti et al., 2022). However, it remains to be determined if this feature of CT-type precursors in sea cucumbers and brittle stars evolved independently or originated in a common ancestor of eleutherozoan echinoderms but with subsequent loss in Asteroidea and Echinoidea (Figure 9).

Analysis of the sequences of AjCT1 and AjCT2 revealed the presence of a two cysteine residues in their N-terminal regions, which is an evolutionarily conserved feature of CT-type peptides. Furthermore, structural analysis of CT-type peptides in vertebrates and in starfish has revealed that these cysteines form a disulphide bridge in the mature peptides (Cai et al., 2018) and therefore it is inferred that this post-translational modification also occurs in AjCT1 and AjCT2. Furthermore, the C-terminal residue of the AjCT1 and ACT2 is predicted to be an amidated proline, which is a conserved post-translational modification of CT/DH31-type peptides in many taxa, but not vertebrate CGRPs (Cai et al., 2018).

3.2 Identification and characterisation of receptors for CT-type neuropeptides in A. japonicus

Informed by previous genome-wide analysis of GPCRs in *A. japonicus* (Huang et al., 2021), we identified three candidate receptors for AjCT1 and/or AjCT2. Firstly, AjCTR, which is a homolog of CT/DH_{31}-type receptors that have been identified in other taxa, including vertebrates and insects (Garelja et al., 2022; Johnson et al., 2005; Toribio et al., 2003). Secondly, AjPDFR1 and AjPDF2, which are homologs of the PDF-type receptor that has been shown to be activated by DH_{31} in *D. melanogaster* (Mertens et al., 2005; Zandawala, 2012).

In mammals, CTR and CLR mediate effects of all CT-related neuropeptides, but their ligand specificity relies on the presence and types of receptor activity-modifying proteins (RAMPs) (McLatchie et al., 1998; Purdue et al., 2002; Conner et al., 2004; Hay et al., 2018). Thus, CTR acts as a functional receptor for CT-type peptides without an associated RAMP (Katafuchi et al., 2009), whereas activation of downstream

signaling via CLR requires an associated RAMP (Hay et al., 2018). Here, we investigated the occurrence of RAMPs in *A. japonicus* using the tblastn method employed by Sekiguchi *et al.*, (2016), which enabled identification of RAMPs in the cephalochordate *B. floridae*. However, no RAMPs were identified in *A. japonicus*, suggesting that AjCTR may be functionally more similar to CTR than CLR. Accordingly, tblastn analysis of other available echinoderm genomes (*A. rubens*, *S. purpuratus* and *A. japonica*), as well as transcriptome databases for brittle stars (*O. victoriae*, *O. aranea* and *A. filiformis*; no published genomes), also failed to identify RAMPs. Likewise, RAMPs have also not been found in protostomes (Schwartz et al., 2019). Therefore, RAMPs may be unique to chordates, although interestingly RAMPs have also not been identified in the urochordate *C. intestinalis* (Sekiguchi et al., 2009).

As highlighted above, here we show that the CT-type peptides AjCT1 and AjCT2 both act as ligands for a CTR/CLR-type receptor (AjCTR) and two PDF-type receptors (AjPDFR1, AjPDFR2) in *A. japonicus*. Furthermore, characterization of downstream signaling mechanisms revealed that both AjCT1 and AjCT2 trigger AjCTR-mediated cAMP accumulation, Ca^{2+} mobilization, and ERK signaling. These findings are consistent with previous studies on CT-type receptors in vertebrates and in the mollusc *Crassostrea gigas* (Pondel, 2000; Walker et al., 2010; Andreassen et al., 2014; Schwartz et al., 2019). Both AjCT1 and AjCT2 trigger cAMP accumulation, Ca^{2+} mobilization, and ERK signaling mediated by AjPDFR1. Both AjCT1 and AjCT2 triggered cAMP accumulation mediated by AjPDF2, but only AjCT2 (not AjCT1) triggered Ca^{2+} mobilization and ERK signaling mediated by AjPDFR2. These differences in the signal transduction mechanisms activated by AjCT1 and AjCT2 may account for differences in the pharmacological activities and physiological roles of these peptides, as discussed below.

3.3 AjCT1 and AjCT2 act as muscle relaxants in A. japonicus

Analysis of the expression of the gene encoding the precursors comprising AjCT2 or AjCT1 and AjCT2 revealed the occurrence of transcripts in the circumoral nervous system as well as in peripheral tissues/organs, including the longitudinal body wall

muscle, digestive system and gonads. Accordingly, analysis of CT-type neuropeptide expression in other echinoderms has revealed a widespread pattern of expression in larval and adult starfish (*A. rubens*) and in juvenile and adult featherstars (*Antedon mediterranea*) (Mayorova et al., 2016; Cai et al., 2018; Aleotti et al., 2022). Several CT-related peptides act as muscle relaxants in vertebrates (Steiner et al., 1991; Pinto et al., 1996; Yoshimoto et al., 1998; Edvinsson et al., 2001; Golpon et al., 2001; Kandilci et al., 2008) and, accordingly, previous studies have revealed that CT-type neuropeptides cause relaxation of muscle preparations in starfish, including locomotory organs (tube feet) and the apical muscle, which is functionally similar to the longitudinal body wall muscle in sea cucumbers (Cai et al., 2018). Therefore, it was of interest to investigate if AjCT1 and/or AjCT2 act as muscle relaxants in *A. japonicus*. Both AjCT1 and AjCT2 caused dose-dependent relaxation of longitudinal muscle preparations, consistent with the relaxing effect of CT-type peptides on starfish apical muscle preparations. Furthermore, AjCT2, but not AjCT1, caused dose-dependent relaxation of intestine preparations from *A. japonicus*. Interestingly, the CT-type peptide ArCT does not to cause relaxation of cardiac stomach preparations from the starfish *A. rubens* (Cai et al., 2018). Therefore, from a functional perspective ArCT is more like AjCT1 than AjCT2 and we speculate that this may reflect loss of an exon encoding a AjCT2-like peptide in the Asteroidea lineage. Further insights into this issue and the evolution of CT-type neuropeptide function echinoderms could be obtained by investigation of the actions of CT-type peptides in brittle stars and sea urchins. Differences in the effects of AjCT1 and ArCT2 on the intestine may be explained by differences in their signal transduction mechanisms. Thus, only AjCT2, not AjCT1, triggered AjPDFR2-mediated acivation of Ca^{2+} mobilization and ERK signaling and therefore we speculate that the relaxing effect of AjCT2 on the intestine may be mediated by activation of AjPDFR2.

3.4 AjCT2 stimulates feeding and growth in A. japonicus

In vivo pharmacological experiments revealed that treatment with AjCT2 at a high concentration (5×10^{-1} mg/ml) caused a significant increase in body mass, and this was

462 accompanied by a decrease in residual bait and an increase in feacal mass. However,
463 these effects were not observed with AjCT2 at a low concentration (5×10^{-3} mg/ml) or
464 with AjCT1 at low or high concentrations. It is noteworthy that AjCT2, but not AjCT1,
465 has a relaxing effect on the intestine in *A. japonicus* and therefore the effects of AjCT2
466 on feeding, defecation and growth may, at least in part, be mediated via binding of this
467 peptide to receptors in the intestine. Accordingly, upregulation of the expression of the
468 AjCT1/AjCT2 receptors AjCTR, AjPDFR1 and AjPDFR2 was observed in the intestine
469 of animals injected with AjCT2 at a high concentration. Furthermore, changes in
470 intestinal expression of genes encoding growth-related factors were also observed in
471 animals injected with AjCT2 at a high concentration, with up-regulation of *AjIgf* and
472 *AjMegf6* expression and down-regulation of *AjGDF-8* (Appendix 1—figure 1).
473 Consistent with these changes in gene expression and the growth-promoting effect of
474 AjCT2, it has been reported that *GDF-8* inhibits growth in *A. japonicus* (Li et al., 2016),
475 whilst *Megf6* and *Igf* promote growth in vertebrates (Párrizas and LeRoith, 1997;
476 Deribe et al., 2009).

477 Collectively, these findings indicate that AjCT2 has a physiological role in
478 stimulating feeding and growth in *A. japonicus.* To the best of our knowledge, this is
479 the first study to report orexigenic and growth-promoting effects of a CT-type
480 neuropeptide in bilaterian animals. Interestingly, previous studies have reported the
481 expression of CT-type neuropeptides in the digestive system of invertebrate chordates
482 (Sekiguchi et al., 2009; Sekiguchi et al., 2016). However, in contrast to the stimulatory
483 effect of AjCT2 on feeding/growth in *A. japonicus*, CT-type neuropeptides have been
484 found to inhibit feeding and growth in vertebrates (Del et al., 2002; Hay et al., 2018;
485 Nakamura et al., 2018). Thus, it appears that opposing roles of CT-type neuropeptide
486 signaling systems as regulators for feeding/growth have evolved in deuterostomes, with
487 a stimulatory role reported here in an echinoderm and an inhibitory role reported
488 previously in vertebrates. Further research is now needed to investigate if the
489 stimulatory effect of AjCT2 on feeding/growth observed in here in *A. japonicus* also
490 applies to other sea cucumber species. Moreover, our discovery of this effect of AjCT2
491 on *A. japonicus* may provide a basis for development of novel approaches to improve

aquaculture of this economically important species.

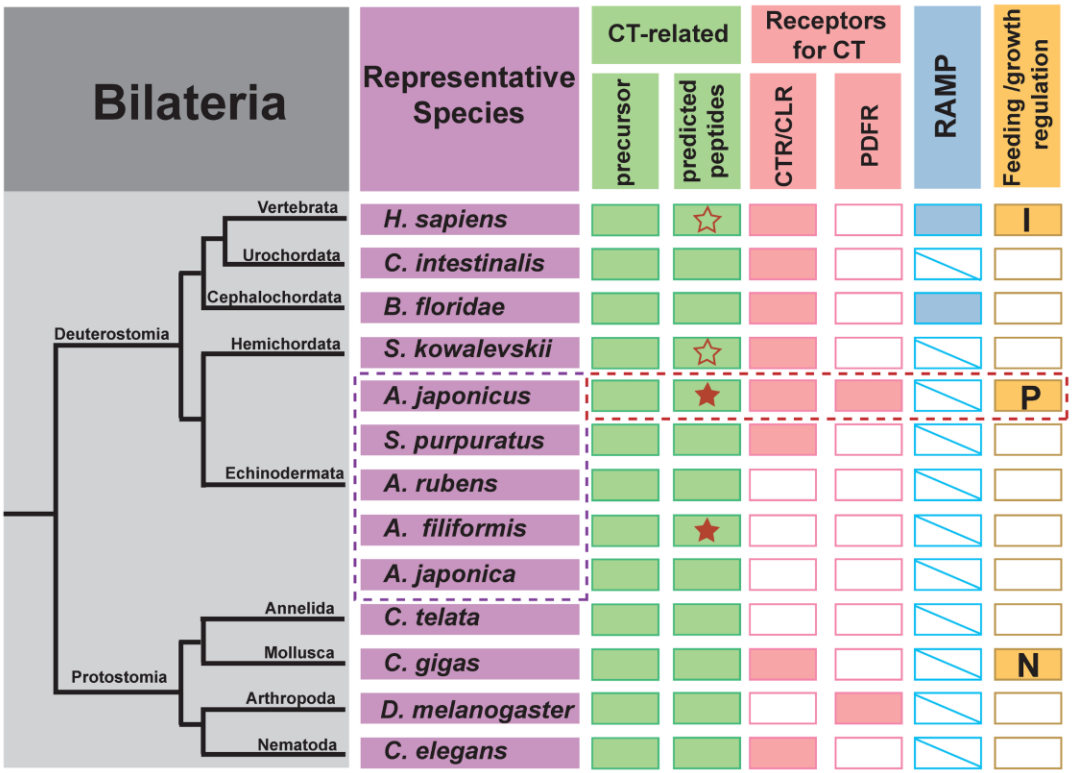

Figure 9. Summary of CT-related neuropeptides and their corresponding precursors, receptors, RAMPs and feeding/growth regulation in representative species. Echinoderms are boxed in purple dotted lines. The summary of *A. japonicus* is framed in red dotted lines. A green rectangle indicates the presence of neuropeptides or precursors. The red pentagram indicates that neuropeptides are generated by alternative splicing of precursor transcripts, unfilled pentagram represents a single gene encoding one neuropeptide, the filled pentagram represents a gene encoding two neuropeptides in tandem. For the receptors for CT, pink filled rectangles show that the presence of the corresponding receptors has been reported and unfilled rectangles indicate that their receptors have not been reported. Blue filled rectangles indicate that RAMPs have been identified in this species, blue rectangles with a line show that RAMPs have not been identified in this species. Yellow filled rectangles indicate that feeding/growth regulation by CT-type neuropeptides has been investigated and unfilled rectangles showed that there was no relevant study in this species. I: Inhibition; P: Promotion; N: Ineffective.

508 **4. Materials and methods**

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (Homo sapiens)	HEK293 cell line	Procell	CL-0005	
Antibody	Phospho-p44/42 MAPK ERK1/2(Thr202/ Tyr204) (goat)	Cell Signaling Technology	CAT#4930	IF (1:2000)
Antibody	p44/42 MAPK ERK1/2 (137F5) (goat)	Cell Signaling Technology	CAT#4695T	IF (1:1000)
Antibody	HRP-conjugated goat anti-rabbit IgG (goat)	Absin	Abs20040ss	IF (1:2000)
sequence-based reagent	Primers for pcDNA3.1(+) plasmid construction--AjCTR	This paper	PCR PRIMER	FORWARD CGCGGATCCAAAATGCAGGAGA ACGATTC BamH I
sequence-based reagent	Primers for pcDNA3.1(+) plasmid construction--AjCTR	This paper	PCR PRIMER	REVERSE CCGGAATTCTTAAACTACCGTT GTTTTAT EcoR I
sequence-based reagent	Primers for pcDNA3.1(+) plasmid construction—AjPDFR1	This paper	PCR PRIMER	FORWARD CGCGGATCCACAATGAGTGTCG GGAACGT BamH I
sequence-based reagent	Primers for pcDNA3.1(+)	This paper	PCR PRIMER	REVERSE CCGGAATTCTCATTTCCCACCCC

	plasmid construction—AjPDFR1			ACTCAC EcoR I
sequence-based reagent	Primers for pcDNA3.1(+) plasmid construction—AjPDFR2	This paper	PCR PRIMER	FORWARD CGCGGATCCAAGATGGCGAACAATGAAAC BamH I
sequence-based reagent	Primers for pcDNA3.1(+) plasmid construction—AjPDFR2	This paper	PCR PRIMER	REVERSE CCGGAATTCTCATTCATTAAATTTGCCGT EcoR I
sequence-based reagent	Plasmid construction AjCTR–EGFP	This paper	PCR PRIMER	FORWARD CCGGAATTCTGAAAATGCAGGAGAACGATTC EcoR I
sequence-based reagent	Plasmid construction AjCTR–EGFP	This paper	PCR PRIMER	REVERSE CGCGGATCCCGAACTACCGTTGTTTTATCTG BamH I
sequence-based reagent	Plasmid construction AjPDFR1–EGFP			CCGGAATTCTGACAATGAGTGTCGGGAACGT EcoR I
sequence-based reagent	Plasmid construction AjPDFR1–EGFP			CGCGGATCCCGTTTCCCACCCCACTCACTGT BamH I
sequence-based reagent	Plasmid construction AjPDFR2–EGFP			CCGGAATTCTGAAGATGGCGAACAATGAAAC EcoR I
sequence-based reagent	Plasmid construction AjPDFR2–EGFP			CGCGGATCCCGTTCATTAAATTTGCCGTTCA BamH I
sequence-based reagent	Primers for qPCR assays β -Actin	This paper	PCR PRIMER	FORWARD AAGGTTATGCTCTTCCTCACGCT
sequence-based reagent	Primers for qPCR assays	This paper	PCR PRIMER	REVERSE GATGTCACGGACGATTTCACG

	β -Actin			
sequence-based reagent	Primers for qPCR assays β -Tubulin	This paper	PCR PRIMER	FORWARD GAAAGCCTTACGACGGAACA
sequence-based reagent	Primers for qPCR assays β -Tubulin	This paper	PCR PRIMER	REVERSE CACCACGTGGACTCAAAATG
sequence-based reagent	Primers for qPCR assays *AjCTP1/2*	This paper	PCR PRIMER	FORWARD GTTTGGCGGTACCCATTAC
sequence-based reagent	Primers for qPCR assays *AjCTP1/2*	This paper	PCR PRIMER	REVERSE GGCGTTCGGTCTGTTAATAG
sequence-based reagent	Primers for qPCR assays *AjMegf6*	This paper	PCR PRIMER	FORWARD CGGTCCTTACTGTGCGGAGA
sequence-based reagent	Primers for qPCR assays *AjMegf6*	This paper	PCR PRIMER	REVERSE CGTCCTCCCAGCTGGACATC
sequence-based reagent	Primers for qPCR assays *AjIgf*	This paper	PCR PRIMER	FORWARD TGGAAGGCAGTAAGGCTA
sequence-based reagent	Primers for qPCR assays *AjGDF-8*	This paper	PCR PRIMER	REVERSE TAGGCTGATGTGGTTGGA
sequence-based reagent	Primers for qPCR assays *AjGDF-8*	This paper	PCR PRIMER	FORWARD ATACGCCCATACTCACATCC
sequence-based reagent	Primers for qPCR assays *AjIgf*	This paper	PCR PRIMER	REVERSE TGGCAGGAGTCAACCTTCAT
sequence-based reagent	Primers for qPCR assays *AjCTR*	This paper	PCR PRIMER	FORWARD GACGAATGGAGGAGAAGG
sequence-based reagent	Primers for qPCR assays	This paper	PCR PRIMER	REVERSE CGCCGCAATTAGAGTAGT

	AjCTR			
sequence-based reagent	Primers for qPCR assays *AjPDFR1*	This paper	PCR PRIMER	FORWARD TTATCAACCGCCGTCTTC
sequence-based reagent	Primers for qPCR assays *AjPDFR1*	This paper	PCR PRIMER	REVERSE CAGGAGGAGGAACACATTC
sequence-based reagent	Primers for qPCR assays *AjPDFR2*	This paper	PCR PRIMER	FORWARD TGTTCTCCTGCCTCTTCT
sequence-based reagent	Primers for qPCR assays *AjPDFR2*	This paper	PCR PRIMER	REVERSE GTCCTTCTCATCGGTGTG
sequence-based reagent	Primers for 3'RACE *AjCTP1* *AjCTP2*	This paper	PCR PRIMER	GATTACGCCAAGCTTCGAGCTG TGTCGATAACATTACGCATGG
sequence-based reagent	Primers for 3'RACE AjPDFR1	This paper	PCR PRIMER	GATTACGCCAAGCTTGAGGGAA AGCCAAACCAGCGAGACG
sequence-based reagent	Primers for 3'RACE AjPDFR2	This paper	PCR PRIMER	GATTACGCCAAGCTTGTGCAAC CGGACGAAGATCCACGTG
sequence-based reagent	Primers for 5'RACE AjPDFR1	This paper	PCR PRIMER	GATTACGCCAAGCTTCGTCTCG CTGGTTTGGCTTTCCCTC
sequence-based reagent	Primers for 5'RACE AjPDFR2	This paper	PCR PRIMER	GATTACGCCAAGCTTGCCTTCC ACAAACATCCATGCGAACGC
sequence-based reagent	Primers for 5'RACE AjCTR	This paper	PCR PRIMER	GATTACGCCAAGCTTACGCCGG GCTCTCAAGAAGTCATTC
sequence-based reagent	Primers for in situ hybridization *AjCTP1/2*	This paper	PCR PRIMER	FORWARD CGAGCTGTGTCGATAACATTAC GCATGG

sequence-based reagent	Primers for in situ hybridization AjHOP	This paper	PCR PRIMER	REVERSE AATGGGTACCGCCAAACTGGCCTTC
sequence	RNAi	This paper	si*AjCTP1*-1	GGAAUUAUCAAGACAGUAU(dT)(dT) AUACUGUCUUGAUAAUUCC(dT)(dT)
sequence	RNAi	This paper	si*AjCTP1*-2	GAAAGUCGCUAAUGCAGUA(dT)(dT) UACUGCAUUAGCGACUUUC(dT)(dT)
sequence	RNAi	This paper	siNC	UUCUCCGAACGUGUCACGU(dT)(dT) ACGUGACACGUUCGGAGAA(dT)(dT)
commercial assay or kit	SMARTer RACE 5'/3' Kit	Takara	Cat No. 634858	
commercial assay or kit	FastPure Gel DNA Extraction Mini Kit	Vazyme	DC301-01	
commercial assay or kit	Hifair® III 1st Strand cDNA Synthesis SuperMix	YEASEN	Cat No. 11141ES60	
commercial assay or kit	Hieff UNICON Universal Blue qPCR SYBR Green Master Mix	YEASEN	Cat No. 11184ES08	
commercial assay or kit	DIG RNA Labeling Kit (SP6/T7)	Roche	Cat No.11175025910	
commercial assay or kit	DIG Nucleic Acid Detection Kit	Roche	Cat No.11175041910	
commercial assay or kit	Firefly luciferase reporter gene assay kit	MK	MF4001	

commercial assay or kit	cAMP Assay kit	R&D systems	Cat No. KGE002B	

509

510 ***4.1 Animals***

511 Adult sea cucumbers were collected from an aquaculture breeding farm in Weihai,
512 Shandong, China. All the animals were acclimated in breeding aquaria (18 °C, 32 ppt)
513 for at least seven days and fed daily with a commercially formulated diet. Tissues used
514 for cDNA cloning and qRT-PCR were obtained from five adult *A. japonicus* (~120 g)
515 and kept at -80 °C until use. Circumoral nervous tissues from three adult sea cucumbers
516 (~120 g) were fixed with 4% paraformaldehyde (PFA, G-CLONE, Beijing, China) and
517 used for *in situ* hybridization analysis. *In vitro* functional analysis and *in vivo*
518 experiments were carried out using twelve (~120 g body weight) and 168 (~20 g) sea
519 cucumbers, respectively.

520

521 ***Ethical approval***

522 All methods were carried out in accordance with the ARRIVE guidelines
523 (https://arriveguidelines.org) for animal experiments. All animal care and use
524 procedures were approved by the Institutional Animal Care and Use Committee of
525 Ocean University of China (Permit Number: 20141201). They were performed
526 according to the Chinese Guidelines for the Care and Use of Laboratory Animals (GB/T
527 35892-2018).

528

529 ***4.2 Molecular structure and phylogenetic analysis of CT-type neuropeptides and***
530 ***precursors***

531 *A. japonicus* CT-type neuropeptide precursor sequences (*AjCTP1*, GenBank
532 accession number: MF401985; *AjCTP2*, GenBank accession number: MF401986) were
533 identified in our previous study (Chen et al., 2019). Based on the published sequences,
534 transcript-specific primers for *AjCTP1* and *AjCTP2* (Supplementary Table 1) were
535 designed using Primer3Plus (https://www.primer3plus.com/) and sequence accuracy

536 was confirmed by SMARTer RACE 5'/3' Kit (TaKaRa, Kusatsu, Japan) according to
537 the manufacturer's instruction. A sequence translation tool (http://www.bio-
538 soft.net/sms/index.html) was used to obtain the corresponding amino acid sequences
539 encoded by the cloned cDNAs. To identify the conserved features of CT-type
540 neuropeptides in Bilateria, sequences were aligned using Clustal-X with default settings
541 and then predicted using the online tool Multiple Sequence Comparison Display Tool
542 (http://www.bio-soft.net/sms/index.html). Tertiary structures were investigated using
543 SWISS-MODEL (https://swissmodel.expasy.org/interactive). A neighbor joining tree
544 was constructed to investigate relationships of *AjCTP1*, *AjCTP2* and CT-type
545 precursors from other Bilateria. Amino acid sequences were aligned using MEGA7
546 (v.7170509) with default settings and then modified by iTOL
547 (https://itol.embl.de/login.cgi). The accession numbers or citations for all sequences
548 used for analysis are listed in Supplementary Table 2.
549

550 ***4.3 Cloning, sequence analysis and phylogenetic tree construction for candidate***
551 ***AjCT1/2 receptors in A. japonicus***

552 Five potential receptors for AjCT1 and AjCT2 in *A. japonicus* (GenBank accession
553 number: PIK49998.1, PIK49999.1, PIK49604.1, PIK34007.1 and PIK62458.1) were
554 identified based on genome-wide analysis (Huang et al., 2021). Sequence comparison
555 revealed that both PIK49998.1 and PIK62458.1 were partial sequences of PIK34007.1.
556 Thus, three candidate receptors named as *AjCTR* (Calcitonin receptor, GenBank
557 accession number: PIK49604.1), *AjPDFR1* (Pigment-dispersing factor receptor 1,
558 GenBank accession number: PIK34007.1), and *AjPDFR2* (Pigment-dispersing factor
559 receptor 2, GenBank accession number: PIK49999.1) were predicted and then analyzed.
560 To confirm the accuracy of these sequences, cDNAs were cloned using the SMARTer
561 RACE 5'/3' Kit (Takara, Cat No. 634858) and then sequenced (Beijing Genomics
562 Institution, China). Receptor sequence analysis and phylogenetic analysis were
563 performed as described in 4.2. In addition, the online tool NetPhos-3.1
564 (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1) was used to predict
565 potential phosphorylation sites. The conserved domains of protein sequences were

566 predicted by the Batch SMART plugin of TBtools (Toolbox for biologists, v.1.098652)
567 software. For analysis of gene synteny, neighboring genes of *AjCTR*, *AjPDFR1* and
568 *AjPDFR2* were found based on an *A. japonicus* chromosome-level genome (Wang et
569 al., 2022) and the corresponding genes in the starfish *Asterias rubens* and the sea urchin
570 *Lytechinus variegatus* were also identified by performing BLASTp in the NCBI
571 database. Specific primers for cloning of cDNAs encoding the candidate receptors are
572 shown in Supplementary Table 1 and the details of the sequences used for constructing
573 the phylogenetic tree are listed in Supplementary Table 3.
574

575 ***4.4 Testing AjCT1 and AjCT2 as ligands for candidate receptors and exploration of***
576 ***downstream signaling pathways***

577 AjCT1 and AjCT2 were custom synthesized by GL Biochem Ltd. (Shanghai,
578 China). Subsequently, CRE (cAMP response element) -Luc (Luciferase) detection,
579 cAMP accumulation, SRE (serum response element) -Luc detection, receptor
580 translocation assays, and Western blotting were carried out.

581 *AjCTR*, *AjPDFR1* and *AjPDFR2* incorporating enhanced green fluorescent protein
582 (EGFP) were constructed respectively according to the method described by Li *et al.*
583 (2022). Briefly, the complete ORFs of candidate receptors were cloned and sequenced.
584 Then, the PCR products were digested with EcoRI and BamHI (TaKaRa, Kusatsu,
585 Japan) and then incorporated into the pEGFP-N1 vector (YouBio, Changsha, China)
586 that was subject to the same double-digestion. Finally, the constructed plasmid with
587 correct sequences was transfected into HEK293T cells seeded on glass coverslips in
588 12-well plates. After incubation with AjCT1 (10^{-6} M), AjCT2 (10^{-6} M) or serum-free
589 DMEM (Biosharp, Hefei, China) for 15 min, cells were washed with PBS (Biosharp,
590 Hefei, China) and fixed with 4% PFA for 10 min. Finally, cell nuclei were stained with
591 DAPI (G-CLONE, Beijing, China) for 5 min and an Olympus bright field light
592 microscope was used for image capture. The pcDNA 3.1(+) vectors (YouBio, Changsha,
593 China) comprising the complete ORF of the three candidate receptors were also
594 constructed as described by Li *et al.* (2022). Then, they were co-transfected with the
595 cAMP response element, pCRE-Luc (YouBio, Changsha, China) or the pSRE-Luc

596 plasmid including PKC reporter gene (YouBio, Changsha, China) into HEK293T cells.
597 Incubation was carried out with AjCT1 (10^{-6} M), AjCT2 (10^{-6} M) or serum-free DMEM
598 (negative control) for 4 h at 37 °C. Finally, luciferase activity was tested using the firefly
599 luciferase assay kit (MK, Shanghai, China). When required, transfected cells described
600 above were co-incubated with H89 (PKA inhibitor) (Absin, Shanghai, China),
601 FR900359 (Gαq protein inhibitor) (Cayman Chemical, Ann Arbor, Michigan) or Gö
602 6983 (PKC inhibitor) (Cayman Chemical, Ann Arbor, Michigan) before the experiment
603 to explore the potential signaling pathways. To confirm the concentration of
604 intracellular cAMP after stimulation, HEK293T cells expressing pcDNA 3.1(+)/*AjCTR*,
605 pcDNA 3.1(+)/*AjPDFR2* or pcDNA 3.1(+)/*AjPDFR1* were treated with AjCT1 or
606 AjCT2 (10^{-9} – 10^{-5} M) respectively for 15 min after 2 h starvation in serum-free medium.
607 Subsequently, cAMP was measured using the ELISA-based cAMP assay kit (R&D
608 systems, USA&Canada) following the manufacturer's instruction. HEK293T cells
609 transfected with empty pcDNA3.1(+) were used as a negative control. The specific
610 primers used for constructing plasmids are listed in Supplemental Table 1.
611 Western blotting was performed as described previously (Li et al., 2022). Briefly,
612 HEK293T cells transfected with the constructed pcDNA 3.1(+) plasmid were starved
613 in serum-free medium for 2 h, and then incubated with AjCT1 or AjCT2 (10^{-6} M). PVDF
614 membranes containing proteins were blocked with 5% BSA (G-CLONE, Beijing, China)
615 and incubated overnight with phosphorylated ERK1/2 kinases (CST, Boston, China).
616 After that, goat anti-rabbit horseradish peroxidase-conjugated secondary antibody
617 (Absin, Shanghai, China) was applied and incubated, followed by the addition of Omni-
618 ECL™ Femto Light Chemiluminescence reagents (EpiZyme, Shanghai, China) .
619 Finally, RVL-100-G (ECHO, America) was applied for visualization of the PVDF
620 membrane. When required, transfected cells were co-incubated with PKA or PKC
621 inhibitor before the experiment to identify signaling pathways that activate downstream
622 ERK1/2 cascade.
623
624 ***4.5 Total RNA extraction and Quantitative Real-Time PCR (qRT-PCR) detection***
625 qRT-PCR was employed to detect *AjCTP1* and *AjCTP2* transcript expression in

626 five tissues from *A. japonicus*, including the intestine, longitudinal muscle, respiratory
627 tree, gonad and circumoral nervous system. Because it was difficult to design specific
628 primers for *AjCTP1* and *AjCTP2* (Zheng et al., 2022), total transcript expression levels
629 for *AjCTP1/2* (*AjCTP1* + *AjCTP2*) were investigated. Total RNA was extracted from
630 the collected tissues using Trizol RNA isolation reagent (Vazyme, Nanjing, China),
631 cDNA was synthesized using Hifair® III 1st Strand cDNA Synthesis SuperMix
632 (YEASEN, Shanghai, China) according to manufacturer's instructions. For qRT-PCR,
633 amplification was performed in 20 μL volume reactions based on Hieff UNICON
634 Universal Blue qPCR SYBR Green Master Mix (YEASEN, Shanghai, China) and
635 expression levels were assessed by a Corbett Rotoe-Gnen Q (Qiagen, Germany). β-
636 actin (GenBank accession number: PIK61412.1) and β-tubulin (GenBank accession
637 number: PIK51093) were used as house-keeping genes for normalization (Zhao et al.,
638 2014). The $2^{-\Delta\Delta CT}$ method was applied to analyze the relative expression levels and
639 comparison between tissues was carried out using one-way analysis of variance
640 (ANOVA) followed by a Tukey post hoc test (IBM SPSS 25.0 software, Chicago, IL,
641 USA). A p value < 0.05 was considered as a statistically significant difference. Specific
642 sequences of primers used for qRT-PCR are listed in Supplemental Table 1.
643
644 ***4.6 Localization of AjCTP1/2 expression in A. japonicus using in situ hybridization***
645 Digoxigenin-labeled RNA antisense probes and corresponding sense probes were
646 synthesized using DIG RNA Labelling Kit (Roche, Basel, Switzerland) and the specific
647 primers are listed in Supplementary Table 1. Then, localization of the total *AjCTP1/2*
648 transcripts was detected in the circumoral nervous system. The detailed method used
649 was described by Li *et al.* (2022). Briefly, the tissue was prehybridized after rehydration
650 and proteinase K treatment, following the addition of antisense RNA probes that bind
651 specifically to mRNA (sense RNA probes were used for negative control). Blocking
652 buffer was used for blocking non-specific binding sites and then alkaline phosphatase-
653 conjugated anti-digoxigenin antibodies were added according to the manufacturer's
654 instruction, which could specifically bind to the hybridized nucleic acid probe. Finally,
655 the chromogenic substrate NBT/BCIP was added for visualization of *AjCTP1/2*

656 expression. Images were taken using an Olympus bright field light microscope
657 (Olympus, Tokyo, Japan).
658
659 ***4.7 Analysis of the in vitro activity of AjCT1 and AjCT2 on preparations from A.***
660 ***japonicus***
661 Longitudinal muscles and intestines (20 mm in length) from *A. japonicus* were
662 dissected and then suspended in a 50 mL glass organ bath containing 20 mL sterilized
663 seawater. Both ends of the preprations were tied with cotton ligatures and then one end
664 was tied to a metal hook and the other end was connected with a High Grade Isotonic
665 Transducer (ADinstruments MLT0015, Oxford, UK). Before testing, the preparation
666 was stabilized for about 20 min, and the resting tension was adjusted to 1.0 g. Informed
667 by previous findings from Cai et al. (2018), we investigated if AjCT1 and AjCT2 caused
668 relaxation of preparations. First, acetylcholine (ACh) was applied to induce the
669 contraction of preparations and then when a stable state of contraction was achieved,
670 20 uL AjCT1 or AjCT2 at a range of concentrations was added and relaxation responses
671 were recorded. Output signal (cm) from PowerLab data acquisition hardware
672 (ADinstruments PowerLab 4/26, Oxford, UK) was calculated using LabChart (v8.0.7)
673 software.
674
675 ***4.8 Investigation of the in vivo pharmacological effects of AjCT1 and AjCT2 in A.***
676 ***japonicus***
677 A total of 150 adult sea cucumbers were randomly assigned to five groups (30
678 individuals per group), including two high concentration groups (CT1H and CT2H, 5
679 $\times 10^{-1}$ mg/ml), two low concentration groups (CT1L and CT2L, 5×10^{-3} mg/ml) and
680 the control group (CO, sterilized seawater), with each group consisting of three parallel
681 experiments comprising ten animals. Before the experiments, sea cucumbers were
682 starved for a day to normalize their physiological status and their wet weights were
683 measured. AjCT1 and AjCT2 were dissolved in sterilized seawater and then injected
684 through the peristome at noon every other day (1 μl/g wet weight) and the CO group
685 were injected in the same way but with sterilized seawater (1 μl/g wet weight). Faeces

and residual bait were collected and weighed after drying every day. After 24 days, wet weights of sea cucumbers were measured again. Subsequently, animals were dissected and intestines were collected for detecting the expression levels of the three receptor genes (*AjCTR, AjPDFR1, AjPDFR2*) and several growth factors, including multiple epidermal growth factor 6 (*AjMegf6*, GenBank accession number: MG018199.1), insulin-like growth factor (*AjIgf*, GenBank accession number: PIK50518.1) and growth and differentiation factor-8 (*AjGDF-8*, GenBank accession number: KP100064.1) (Li et al., 2016; Wang et al., 2018). IBM SPSS Statistics 25.0 was applied for statistical analysis and a p-value < 0.05 was considered as significant difference. The specific primers for qRT-PCR analysis are listed in Supplementary Table 1.

4.9 Loss-of-function experiments by RNA interference (RNAi)

To investigate how receptor expression is affected by knock-down of ArCTP expression, an RNAi experiment was carried out. It was difficult to design *AjCTP1*- and *AjCTP2*- specific interference oligonucleotides and therefore, we designed and customized generic interference oligonucleotides targeting both *AjCTP1* and *AjCTP2* (si*AjCTP1/2*-1 and si*AjCTP1/2*-2, containing the extra 2′ ome modification) (Beijing Tsingke Biotech Co., Ltd. China). Meanwhile, a negative control siRNA (siNC) was also customized for the experiment (Supplementary Table 1). The si*AjCTP1/2*-1, si*AjCTP1/2*-2 or siNC dry powder was diluted to 20 μM with DEPC water following the manufacturer's instruction, then 60 μl sterilized seawater was mixed with 20 μl si*AjCTP1/2*-1, si*AjCTP1/2*-2 or siNC, and 20 μl Lipo6000 transfection reagent (Beyotime, Shanghai, China) as the transfection solution. Eighteen sea cucumbers were randomly divided into three groups and injected with three different transfection solutions respectively. The mixed solutions were injected through the peristome once a day (1 μl/g wet weight) and intestines were dissected and frozen in liquid nitrogen 3 days after injection. The expression level of *AjCTP1/2* was investigated by qRT-PCR and the interference rate was calculated to determine the inhibition efficiency of siRNAs. Finally, the relative expression levels of *AjCTR*, *AjPDFR1* and *AjPDFR2* were investigated using the selected *A. japonicus* intestines.

Competing Interests

The authors declare that no competing interests exist.

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Author Contributions

Xiao Cong, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing—original draft preparation; Huachen Liu, Methodology, Validation; Lihua Liu, Methodology, Validation; Maurice R. Elphick, Formal analysis, Validation, Writing—review & editing; Muyan Chen, Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—review & editing

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Supplementary figures

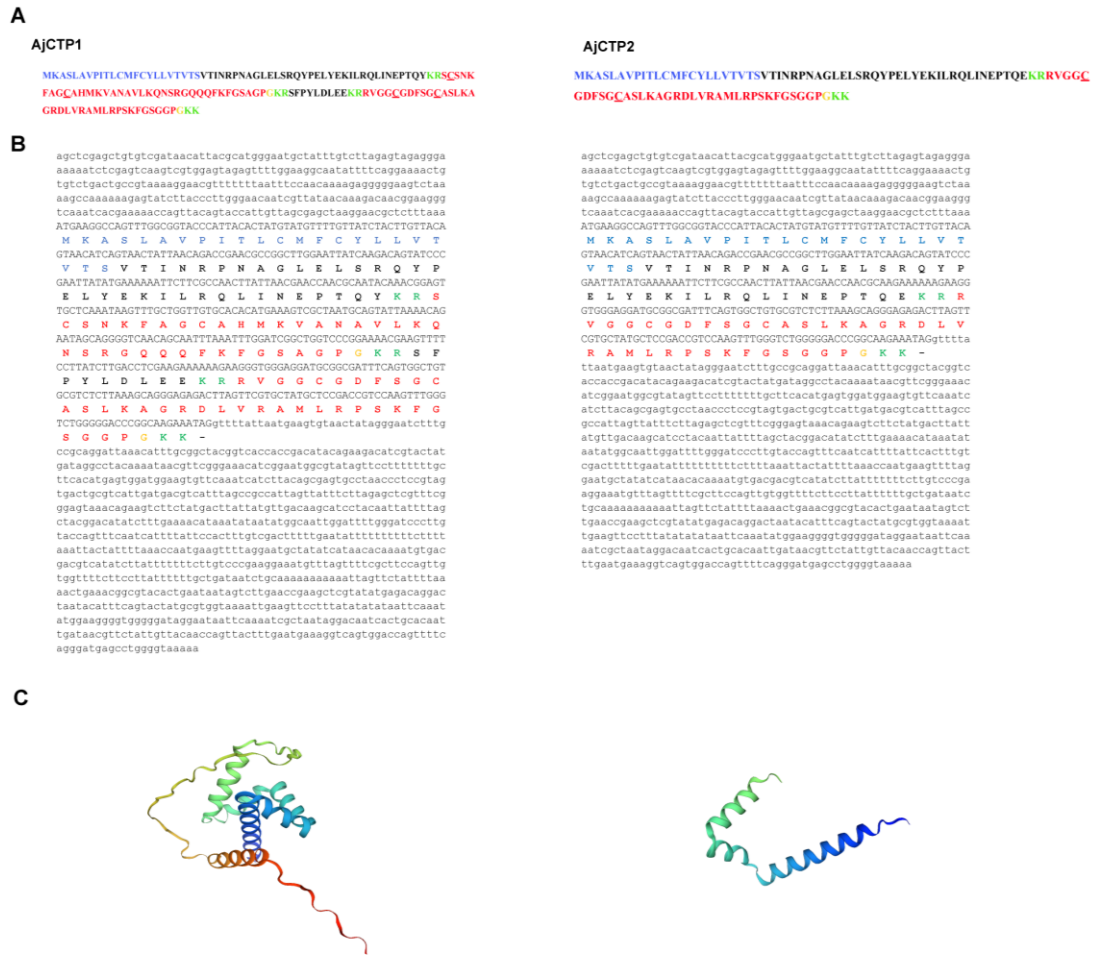

Figure 1-figure supplement 1. Calcitonin-type (CT-type) neuropeptide precursors in *A. japonicus*. (A) *AjCTP1* comprises two putative CT-type neuropeptides, AjCT1 and AjCT2, whereas *AjCTP1* generated by alternative splicing comprises only AjCT2. The predicted signal peptide is shown in blue, the predicted cleavage sites (KR or KK) are shown in green, CT-type neuropeptides are shown in red and C-terminal glycines that are potential substrates for amidation are shown in orange. Predicted disulfide bridges in AjCT1 and AjCT2 are formed between the two underlined cysteines. (B) Nucleotide sequences encoding CT-type neuropeptide precursors and the deduced amino acid sequences in *A. japonicus*. 3'UTR and 5'UTR were shown in lowercase and ORF was shown in uppercase. Blue uppercase labeled the predicted signal peptide, the green uppercase showed the predicted cleavage site, AjCT1 and AjCT2 were shown in red and the putative substrate for amidation was shown in orange. (C) The tertiary structures of AjCTP1 and AjCTP2 as predicted by SWISS-MODEL

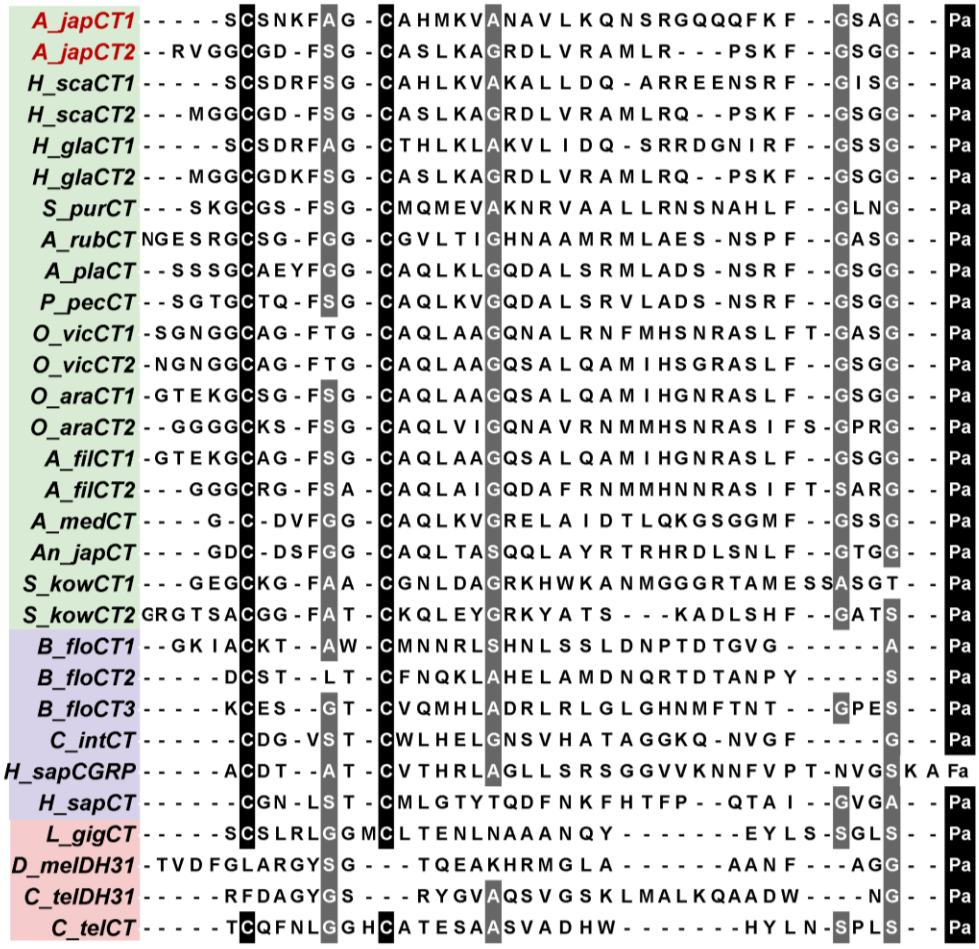

Figure 1-figure supplement 2. Comparison of CT-type neuropeptides from Bilateria: deuterostomes-ambulacraria (green), chordata (purple) and protostomes (pink). The conserved residues are shown with white lettering highlighted in gray and black. The accession numbers or citations are shown in the Supplementary Table 2.

A

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   1 cagaatcctttcaccggacctctgcagctgaaatcaaagtcgaattcaggagtggtggat
   1                                           M  Q  E  N  D  S  Y  Y
  61 gtcaaaacggacactggcagtacgaaaggatatcgaaaATGCAGGAGAACGATTCATATT

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 269  R  C  W  H  N  A  N  E  I  E  W  V  V  G  G  H  I  I  A  V
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938

B

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1981 cgtcgaattgatctgtttgttgttaacaaagtaatcatttgctattacgaattggtaaat
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C

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```

Figure 1-figure supplement 3. Amino acid sequences and phosphorylation sites of AjCTR, AjPDFR1 and AjPDFR2 in *A. japonicus*. (A), (B) and (C) show the cDNA sequences encoding the receptors and their deduced amino acid sequences. ORFs are shown in uppercase; 3'UTR and 5'UTR are shown in lowercase. The stop codon is shown by the asterisk (*). Predicted potential phosphorylation sites are shown with squares.

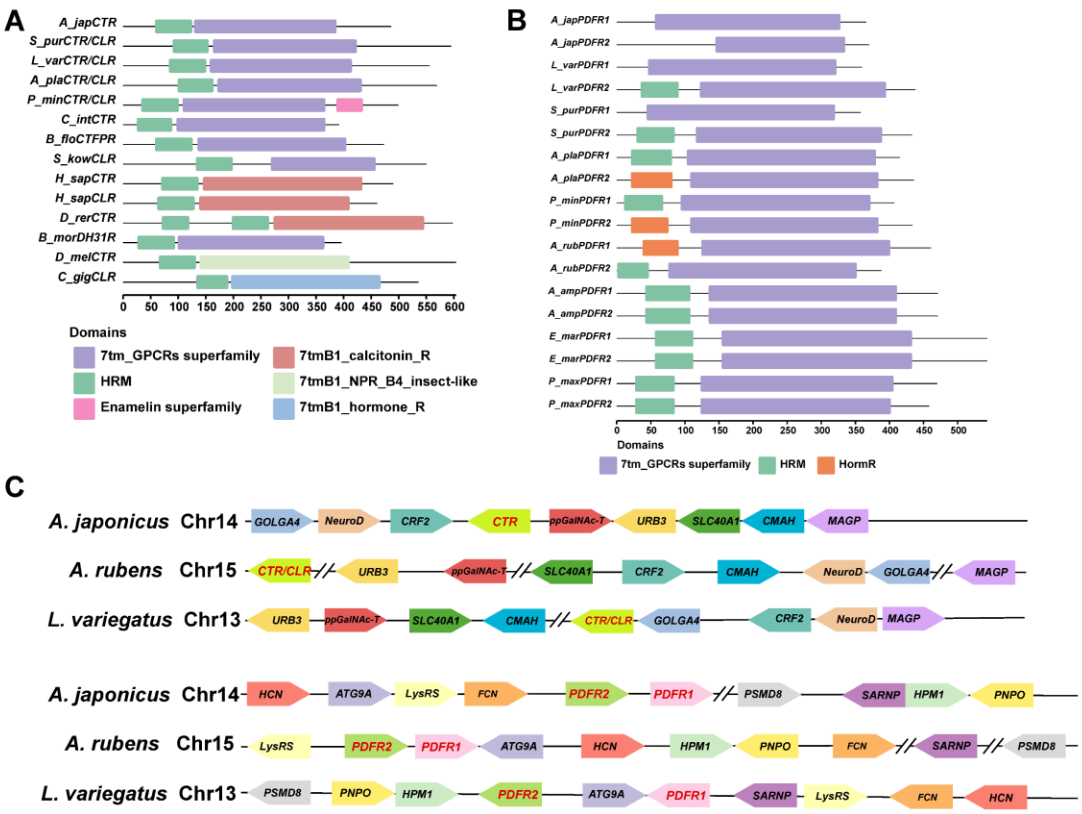
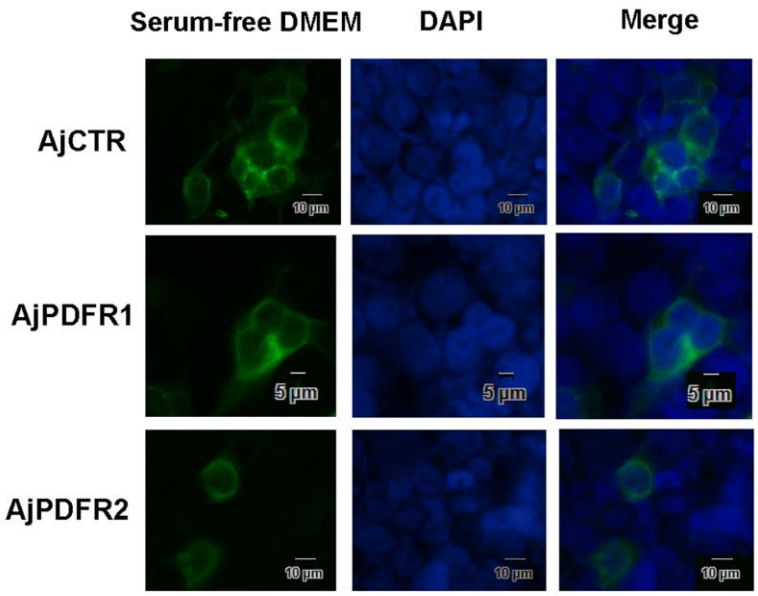


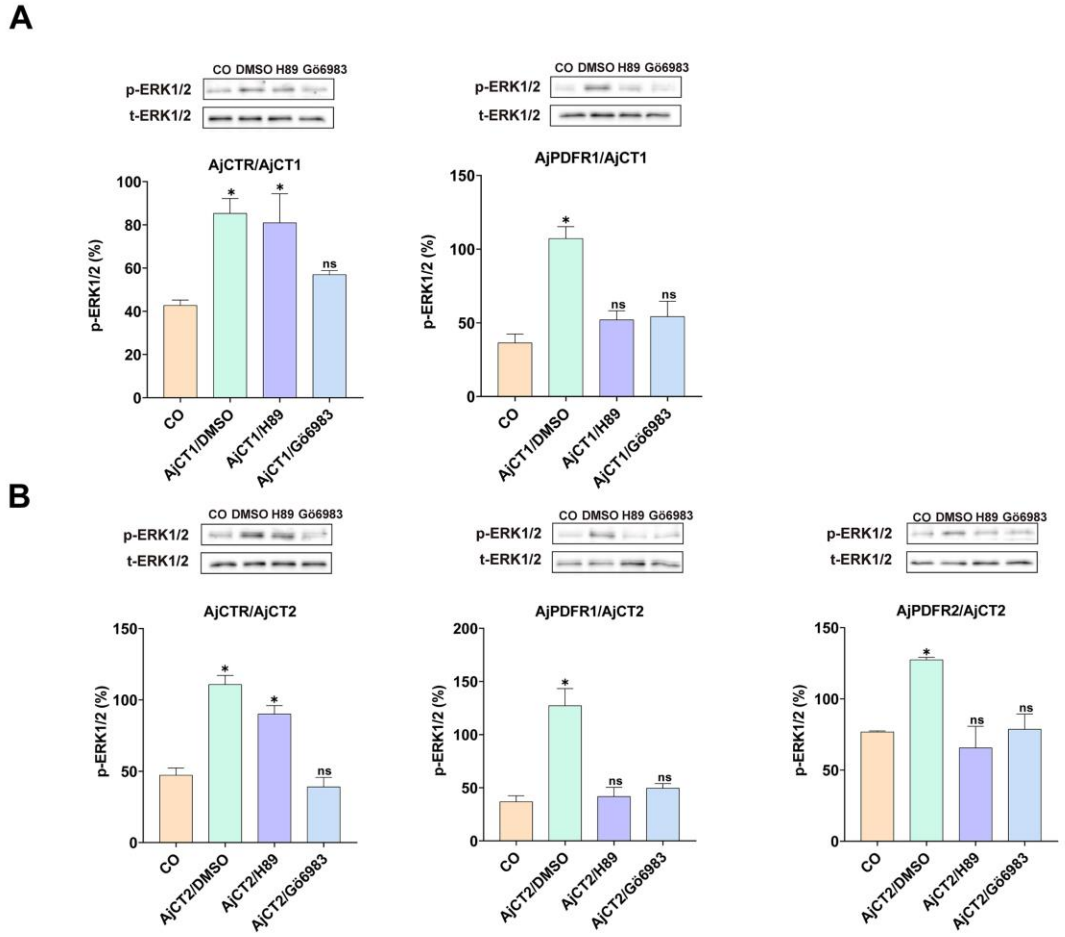
Figure 1-figure supplement 4. Characterization of AjCT1 and AjCT2 receptors (AjCTR, AjPDFR1 and AjPDFR2). (A) Conserved domains of CTR/CLR-type protein sequences. Black line represents the length of proteins and different domains are represented by rectangles. (B) Conserved domains of PDFRs in Bilateria. Black line represents the length of proteins and different domains are represented by rectangles. (C) Synteny analysis of AjCT1 and AjCT2 receptors (AjCTR, AjPDFR1 and AjPDFR2). The chromosomal locations of AjCT1 and AjCT2 receptor genes from *A. rubens*, *L. variegatus* and *A. japonicus* are shown, with different colors used to mark neighboring genes and with their transcriptional direction shown by arrows. *GOLGA4*, golgin subfamily A member 4; *NeuroD*, neurogenic differentiation factor; *CRF2*, corticotropin-releasing factor receptor 2; *CTR*, calcitonin type receptor; *CLR*, calcitonin gene-related peptide type 1 receptor; *ppGalNAc-T*, polypeptide N-acetylgalactosaminyltransferase; *URB3*, E3 ubiquitin-protein ligase ubr3; *SLC40A1*, solute carrier family 40 member 1; *CMAH*, cytidine monophosphate-N-acetylneuraminic acid hydroxylase; *MAGP*, microfibril-associated glycoprotein; *HCN*, hyperpolarization-gated and cyclic nucleotide regulated channel; *ATG9A*, autophagy-related protein 9A-like; *lysRS*, lysine--tRNA ligase; *FCN*, ficolin; *PDFR1*, pigment-dispersing factor receptor 1; *PDFR2*, pigment-dispersing factor receptor 2; *PSMD8*, 26S proteasome regulatory subunit 8; *SARNP*, SAP domain-containing ribonucleoprotein; *HPM1*, histidine protein methyltransferase 1; *PNPO*, pyridoxine-5'-phosphate oxidase.



964

965 Figure 3-figure supplement 1. Localization of AjCTR, AjPDFR1 and AjPDFR2 . Green
966 fluorescence represents the localization of pEGFP-N1/receptors in cell membrane. Cell nucleus
967 probe (DAPI) was applied for cell nucleus staining.

968



969

970 Figure 4-figure supplement 1. Effects of PKA or PKC inhibitor on AjCT1 or AjCT2 stimulated
971 ERK1/2 phosphorylation in AjCTR, AjPDFR1 or AjPDFR2 expressing HEK293T cells. (A)
972 ERK1/2 phosphorylation, activated by AjCT1, is blocked by PKC inhibitor Gö 6983 in AjCTR
973 expressing HEK293T cells and is blocked by PKA inhibitor H89 and PKC inhibitor in AjPDFR1
974 expressing HEK293T cells. (B) ERK1/2 phosphorylation, activated by AjCT2, is blocked by PKC
975 inhibitor in AjCTR expressing HEK293T cells and is blocked by PKA inhibitor and PKC inhibitor
976 in AjPDFR1 and AjPDFR2 expressing HEK293T cells. Serum-starved HEK293 cells were pre-
977 treated with DMSO, PKA inhibitor, or PKC inhibitor before AjCT1 or AjCT2 stimulation. All
978 pictures and data are representative for at least three independent experiments.

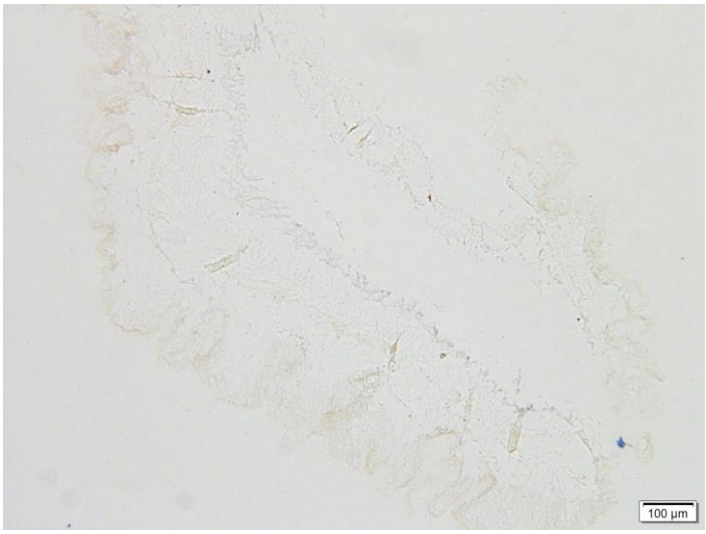


Figure 5-figure supplement 1. Negative control of circumoral nervous system *in situ* hybridization. The sense probe for AjCTP1/2 was incubated with sections of tissue from adult *A. japonicus* instead of an antisense probe. Scale bar: 100 μm.

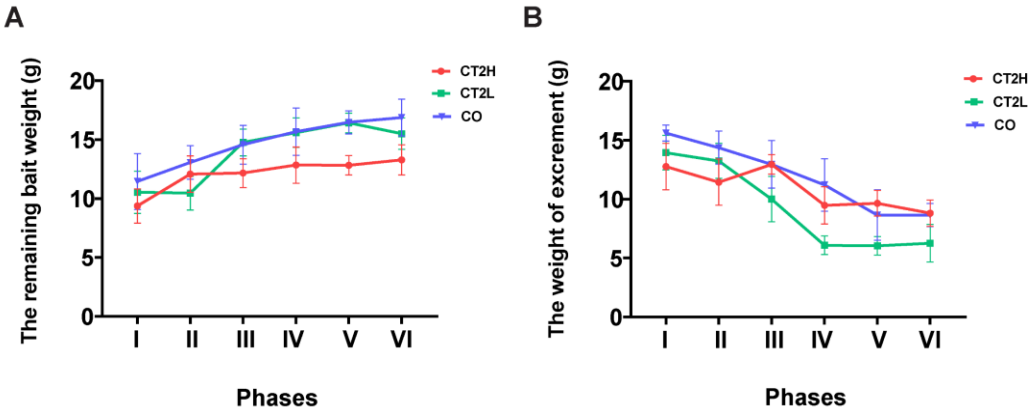


Figure 7A-figure supplement 1. The mass of remaining bait and the excrement. (A) Calculation of the remaining bait mass in different phases. (B) The mass of excrement in different phases. Every four days was divided into one phase (I-VI) for statistics. CO: control group (sterilized seawater); CT2L: AjCT2 low concentration group (5×10^{-3} mg/mL); CT2H: AjCT2 high concentration group (5×10^{-1} mg/mL).

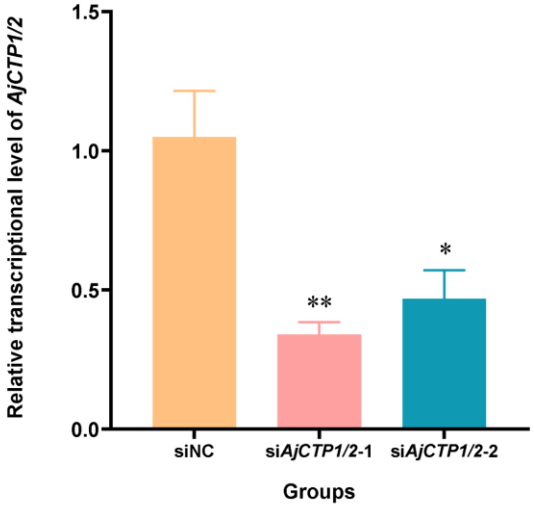


Figure 8-figure supplement 1. The relative expression level of *AjCTP1/2* in siNC, si*AjCTP1/2*-1 and si*AjCTP1/2*-2. Values are means ± SEM (n = 5). Asterisks represent the significant difference as follows: *: $p < 0.05$, **: $p < 0.01$. siNC: negative control group; si*AjCTP1/2*-1 and si*AjCTP1/2*-2: experimental groups targeting *AjCTP1/2*.