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RESEARCH ARTICLE

Characterization of an evolutionarily conserved calcitonin signalling system in a lophotrochozoan, the Pacific oyster (*Crassostrea gigas*)

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ABSTRACT

In Protostoma, the diuretic hormone 31 (DH31) signalling system was long considered as the orthologue of the chordate calcitonin (CT) signalling system. Using the Pacific oyster (*Crassostrea gigas*) transcriptomic database GigaTON, we characterized seven G-protein-coupled receptors (GPCRs) named Cragi-CTR1–7 and phylogenetically related to chordate CT receptors (CTRs) and to protostome DH31 receptors. Two CT precursors (Cragi-CTP1 and Cragi-CTP2) containing two CT-type peptides and encoded by two distinct genes with a similar organization were also characterized. These oyster neuropeptides (Cragi-CT1/2) exhibit the two N-terminal paired cysteine residues and, except CTP2-derived peptide (Cragi-CTP2dp), show the C-terminal proline-amide motif typical of deuterostome CT-type peptides. All mature Cragi-CTs except Cragi-CTP2dp were detected in visceral ganglion extracts using mass spectrometry. Cell-based assays revealed that the previously characterized oyster receptors Cg-CT-R and Cragi-CTR2 were specifically activated by Cragi-CT1b and Cragi-CT2, respectively. This activation does not require the co-expression of receptor activity-modifying proteins (RAMPs). Thus, oyster CT signalling appears functionally more closely related to vertebrate CT/CTR signalling than to calcitonin gene-related peptide/calcitonin receptor-like receptor (CGRP/CLR) signalling. Gene expression profiles in different adult tissues and in oysters acclimated to brackish water suggest the potential implication of both Cg-CT-R/Cragi-CT1b and Cragi-CTR2/Cragi-CT2 in water and ionic regulations, although with apparently opposite effects. The present study represents the first comprehensive characterization of a functional CT-type signalling system in a protostome and provides evidence for its evolutionarily ancient origin and its early role in osmotic homeostasis.

KEY WORDS: Neuropeptide, Osmotic homeostasis, Diuretic hormone 31, GPCR, Lophotrochozoa

INTRODUCTION

Neuropeptides play roles as neurotransmitters, neuromodulators or neurohormones, and thereby participate in the regulation of many physiological functions in metazoans via the activation of different types of receptors, mostly G-protein-coupled receptors (GPCRs). Understanding the evolution of neuropeptide signalling systems is a significant challenge and basically involves as a first step the study of neuropeptide–receptor pairs. Global analyses have indeed shown that neuropeptides and their cognate receptors co-evolved over large evolutionary distances (Jekely, 2013; Mirabeau and Joly, 2013). Because of the lack of available genomic and transcriptomic data in several taxa, neuropeptide signalling systems have mainly been studied in vertebrates and Ecdysozoa model species. However, recent studies have identified and characterized the neuropeptide repertoire of several species of the protostome ecdysozoan sister clade, i.e. Lophotrochozoa, including annelids (Bauknecht and Jékely, 2015) and molluscs (Veenstra, 2010; Adamson et al., 2015; Zatylny-Gaudin et al., 2016; Ahn et al., 2017; Zhang et al., 2018) such as the oyster *Crassostrea gigas* (Stewart et al., 2014). In this species, large-scale genomic and transcriptomic resources (Zhang et al., 2012; Riviere et al., 2015) are now available, and facilitate the study of signalling pathways (Bigot et al., 2014; Dubos et al., 2018; Li et al., 2016; Schwartz et al., 2018). The present study investigated the evolution of the calcitonin (CT)/diuretic hormone 31 (DH31) signalling system in the bivalve mollusc *C. gigas*.

In chordates, CT belongs to the large CT/CGRP family composed of several neuropeptides including CT, the calcitonin gene-related peptide (CGRP), the calcitonin receptor-stimulating peptide (CRSP), amylin, adrenomedullin 1 (AM1), and intermedin/adrenomedullin 2 (IMD/AM2). Although all these peptides have a disulfide bridge between two cysteine residues in the N-terminal region that forms a cyclic structure, they share only low sequence identity. These peptides transduce signals through only two related receptor types: the CT receptor (CTR) and the CGRP receptor known as the calcitonin-like receptor (CLR). The complexity of the signalling pathway of the CT/CGRP peptide family relies on the involvement of accessory proteins named RAMP (receptor activity-modifying protein) and RCP (receptor component protein), which form heterotrimeric complexes with the receptors (Weston et al., 2016). CTR and CLR can bind different RAMPs (RAMP1, RAMP2 and RAMP3) and thus change their ligand specificity. CLR becomes a CGRP receptor in association with RAMP1 but an AM1 receptor in association with RAMP2 and RAMP3 (Mclatchie et al., 1998). CTR alone transduces the CT signal and possibly the CRSP signal (Katafuchi et al., 2009), but its interaction with RAMPs generates an AM receptor that also interacts with CGRP (Dickerson, 2013; Prado et al., 2001). The RCP accessory protein appears to be critical only for the efficient coupling of CLR/RAMP complexes to Gαs proteins (Dickerson, 2013). In mammals, the variety of the CT/CGRP family of peptides is associated with a great diversity of sometimes overlapping functions (Muff et al., 1995)

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such as calcaemia and phosphoraemia regulation for CT (Copp, 1963), vasodilation for CGRP (Brain et al., 1985) or AM (Kitamura et al., 1993), pain mediation for CGRP (Russell et al., 2014), inhibition of insulin-induced glucose uptake for AM, or suppression of food intake for CRSP (Katafuchi et al., 2003, 2009; Sawada et al., 2006). This complex family of peptides, CRSP excluded (Katafuchi et al., 2009), is well conserved among vertebrates (Chang et al., 2004). More recently, CT-type peptides have also been characterized in more basal deuterostome species such as cephalochordates (Sekiguchi et al., 2016) and urochordates (Fritsch et al., 1979, 1980; Sekiguchi et al., 2009), but also echinoderms where they play the role of a muscle relaxant (Cai et al., 2018). In Protostoma, peptides immunologically related to vertebrate CT were initially identified in insects (Langvad Hansen et al., 1982) and crustaceans (Arlot-Bonnemains et al., 1986). Later, a diuretic peptide composed of 31 amino acid residues and named dippu-DH31 (DH for diuretic hormone) was characterized in the cockroach *Diploptera punctata* (Furuya et al., 2000). Dippu-DH31 shows a small amount of sequence similarity with the CT family of peptides, but lacks the typical N-terminal pair of cysteine residues. As insect DH31 homologs specifically activate CTR/CLR-related receptors (Iga and Kataoka, 2015; Johnson, 2004; Zandawala et al., 2013), they were initially considered as the insect evolutionary counterparts of the deuterostome CT/CGRP family of peptides. However, recent mining of the termite and locust genomes revealed the existence of a gene encoding typical CT-related peptides with an N-terminal disulfide bridge in addition to the DH31-encoding genes present in all insect genomes (Veenstra, 2014). Up to now, only a CT-type peptide has been identified in nematodes (Mirabeau and Joly, 2013). Interestingly, in Lophotrochozoa and more precisely in the annelid *Platynereis dumerlii*, the two CT-like and DH31-like types of neuropeptides coexist (Conzelmann et al., 2013). Moreover, annelid DH31 activates two DH31-related receptors (Bauknecht and Jékely, 2015). This infers the occurrence of a duplication event of a CT/DH31 ancestral gene in the common ancestor of protostomes (Conzelmann et al., 2013). However, in molluscs, only typical CT-like peptides have been identified among the predicted neuropeptides of *Patinopecten yessoensis* (Zhang et al., 2018). Interestingly a CTR/CLR homolog named Cg-CT-R and potentially involved in ionic regulation in the pacific oyster *C. gigas* was previously characterized but remained orphan (Dubos et al., 2003). Thanks to the recent emergence of genomic (Zhang et al., 2012) and transcriptomic (Riviere et al., 2015) data in *C. gigas*, we have identified several other potential CT/DH31 receptors as well as putative CT peptide precursors. Therefore, this study demonstrates for the first time the existence of a functional CT-type signalling system distinct from a DH31 signalling system in a protostome.

MATERIALS AND METHODS

Animals and tissue sampling

Two-year old adult oysters *Crassostrea gigas* (Thunberg 1793), purchased from a local farm (in Normandy, France), were used for peptide characterization and transcription analyses. Adult tissues [mantle, mantle edges, gills, labial palps, digestive gland, gonad (mix of all stages), heart, adductor muscle] were sampled, and the visceral ganglia were carefully dissected out, thus limiting any contamination from the adjacent adductor muscles. To study the influence of osmotic conditions, oysters were transferred from seawater (33‰) to brackish water (8‰) at 17°C. Two conditioning experiments were carried out. An acute osmotic conditioning (AC) experiment corresponding to the addition of distilled water once in the seawater tank or a mild osmotic conditioning (MC) experiment consisting of diluting the seawater with a continuous flow ($0.5\ l\ h^{-1}$) of distilled water over a period of 3 days. Tissues (visceral ganglia, mantle and gills) were sampled after 12 h of incubation of the oysters in brackish water at the final salinity.

Peptide synthesis

Materials

All Fmoc (fluorenylmethyloxycarbonyl) amino acid residues and *O*-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Christof Senn Laboratories (Dielsdorf, Switzerland) or Novabiochem (Darmstadt, Germany). PEG-PS resins were from Applied Biosystems (Applera, France) and α-cyano-4-hydroxycinnamic acid from LaserBio Labs (Sophia-Antipolis, France). Piperidine, triisopropylsilane (TIS), *tert*-butylmethylether (TBME), thallium(III) trifluoroacetate and anisole were supplied from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Trifluoroacetic acid (TFA), *N*-methylpyrrolidone (NMP) and dimethylformamide (DMF) were from Biosolve (Dieuze, France). Dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIEA) and acetonitrile were from Fisher Scientific (Illkirch, France).

Peptide synthesis

Peptides (Table 1) were synthesized on a 433A Applied Biosystems automated peptide synthesizer following the standard manufacturer's procedures (0.1 mmol scale) on low loading Fmoc-PAL-PEG-PS resin (Cragi-CT1a, -CT1b, -CT2) or Fmoc-Val preloaded PEG-PS resin (Cragi-CTP2dp) as previously described (Chatenet et al., 2006). All Fmoc amino acids (1 mmol, 10 equiv) were coupled by *in situ* activation with HBTU (1.25 mmol, 12.5 equiv) and DIEA (2.5 mmol, 25 equiv) in NMP. Reactive side chains were protected as follow: Asn, His, Gln, trityl (Trt) amine; Asp, Glu, *tert*-butyl (OtBu) ester; Ser, Thr, Tyr, *tert*-butyl (*t*Bu) ether; Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide; Lys, *tert*-butyloxycarbonyl (Boc) carbamate; Cys, acetamidomethyl (Acm) thioether. To minimize the aggregation phenomenon, pseudoproline residues were used for synthesis of Cragi-CT1a [Fmoc-Leu-Ser(Ψ(Me,Me)Pro)-OH] and Cragi-CT1b {Fmoc-Asn-Ser[Ψ(Me,Me)Pro]-OH, Fmoc-Leu-Thr[Ψ(Me,Me)Pro]-OH and Fmoc-Gln-Thr[Ψ(Me,Me)Pro]-OH}. The formation of the disulfide bond was carried out on resin by selective deprotection of the cysteines and *in situ* cyclization with thallium(III) trifluoroacetate (1.2 equiv) in 15 ml of a DMF/anisole (9.5:0.5 v/v) solution at 0°C for 4 h. After filtration and washes with 3×5 ml of DMF/anisole solution, 3×5 ml of DMF and 3×5 ml of DCM, the cyclic peptides were cleaved from the resin and deprotected by adding 10 ml of an ice-cold mixture of TFA/TIS/H_2O (9.5:0.25:0.25 v/v/v) and agitating for 3 h at room temperature. Peptides were then obtained by precipitation in TBME. Crude peptides were purified by semipreparative reversed-phase HPLC (RP-HPLC) on a Kinetex XB-C_{18} column (250×21.2 mm; Phenomenex, Le Pecq, France) using a linear gradient (20–50% or 20–40% over 50 min) of acetonitrile (0.1% TFA) at a flow rate of $10\ ml\ min^{-1}$ as previously described (Leprince et al., 2001).

Table 1. Amino acid sequences of Cragi-CT peptides

Peptide name	Peptide sequence
Cragi-CT1a	TFTQREACALSLNVDICTEKYIEETADHQSKLQNLIEGNP-amide
Cragi-CT1b	SCTLNLAYHCQTSEYAGLTDLYNYLNSNASP-amide
Cragi-CTP2dp	ETQERQIKLCRGMGPNNHPCGLTSFDVRNV
Cragi-CT2	TCAVELGGACRTEWASAIADQYYYLMGPHGP-amide

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Absorbance was monitored at 215 and 280 nm using a UV detector (Gilson, Villiers Le Bel, France). Analytical RP-HPLC analysis, performed on a Kinetex XB-C_{18} column (250×4.6 mm; Phenomenex) revealed that the purity of all peptides was higher than 97%. The authenticity of each peptide was verified by MALDI-TOF-MS on an UltrafleXtreme (Bruker Daltonik, Bremen, Germany) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix.

Mass spectrometry characterization of endogenous oyster calcitonin neuropeptides

Peptide extraction

Fifteen visceral ganglia, frozen and crushed in liquid nitrogen, were extracted in acetonitrile/water/TFA/0.1% (90/10 v/v) 30 min at 4°C. After centrifugation for 20 min at 15,000 ***g*** at 4°C, supernatant was concentrated on C18 Sep-Pak cartridges. Half of the recovered peptide sample was reduced with 100 mmol l^{-1} DTT at 55°C for 60 min and alkylated with 50 mmol l^{-1} iodoacetamide at 55°C for 45 min. Both mature and reduced/alkylated peptides were analysed by mass spectrometry.

Mass spectrometry

Sample and standard peptides were resuspended in 3% (v/v) acetonitrile and 0.1% (v/v) formic acid (FA) buffer before mass spectrometry analyses. Mature peptides were analysed with a nano-LC1200 system coupled to a Q-TOF 6545XT AdvanceBio mass spectrometer equipped with a nanospray source and an HPLC-chip cube interface (Agilent Technologies, Les Ulis, France). Briefly, peptides were enriched and desalted on a 40 nl Zorbax RP-C18 trap column (300 Å pore size, 5 µm particle size stationary phase) and then a 35 min linear gradient (3–75% acetonitrile in 0.1% FA), at a flow rate of 300 nl min^{-1}, was used to separate peptides on a Zorbax 300SB-Chip C18 column (150 mm long×75 µm i.d.). Full autoMS1 scans from 290 to 1700 *m/z* and autoMS2 from 59 to 1700 *m/z* were recorded. In each cycle, a maximum of 5 precursors sorted by charge state (2+ preferred and single-charged ions excluded) were isolated and fragmented in the collision cell that was automatically adjusted depending on the *m/z*. Active exclusion of these precursors (sorted by abundance only) was enabled after four spectra within 1.5 min, static exclusion from 100 to 300 *m/z* was applied and the absolute threshold for precursor selection was set to 1000 counts (relative threshold 0.1%). Scan speed varied based on precursor abundance (22,000 counts per spectrum) and with 100% purity stringency and 30% purity cut-off. Masshunter Analysis Navigator version B.08.00 service pack 1 was used for data processing. An additional analysis was performed using a NanoElute nLC-TIMS-TOF pro mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Reduced and alkylated peptides were fractionated with a 100 min gradient using a reversed-phase Reprosil column (25 cm long×75 µm i.d.) and analysed in PASEF mode. The fragmentation pattern was used to determine the sequence of the peptide using Peaks X software and the GigaTON database (http://ngspipelines-sigenae.toulouse.inra.fr/).

Molecular identification of oyster calcitonin signalling components

Oyster CT putative precursors were obtained by performing a BLAST search using the GigaTON database resource (http://ngspipelines-sigenae.toulouse.inra.fr/) and the *Mytilus californianus* EST (ES402507) as the query sequence (Conzelmann et al., 2013). Oyster RCP transcript was obtained using *Drosophila* RCP sequence (NP_573175.2) as query. Candidate oyster CT/CL/DH31-related receptors were retrieved from an in-house *C. gigas* GPCR database generated by selecting, from the GigaTON database, the sequences indexed with the IPR000832 (secretin-like GPCRs) and IPR017452 (rhodopsin-like receptors) InterPro family labels. Then, GPCRs annotated as calcitonin receptors (CALCRs and CALRLs) were selected. Gene structure was deduced by aligning the cDNA sequences with the genome sequence (Zhang et al., 2012). A multiple sequence alignment was performed with CTR/CLR/DH31Rs from various species using Clustal Omega (Sievers et al., 2011). Seaview (Gouy et al., 2010) was used for selecting the conserved protein regions and for manual correction of the alignment. PhyML was used for generating the trees. The reliability of the inferred trees was estimated by applying the bootstrap procedure with 100 replications. FigTree (http://tree.bio.ed.ac.uk/software/figtree/) was used to draw the tree.

Pharmacological characterization of oyster calcitonin receptors

Molecular cloning of the *Cragi-CTR*s and *Cragi-RCP* and transfection of mammalian cells

Full-length sequences of the incomplete transcripts found by *in silico* analysis were acquired by a (5′ or 3′) PCR primer walking strategy. PCR was performed using 50 ng of a *C. gigas* 'all development stages and adult central nervous system' directional and normalized cDNA library inserted into the Pal 17.3 vector (Evrogen) (Fleury et al., 2009) in a 50 µl reaction volume containing 1.5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} dNTPs, 1 µmol l^{-1} of each sequence-specific primer and Pal 17-specific primers (Table S1), 1.25 units of Go-Taq polymerase and the appropriate buffer (Promega, Madison, WI, USA) in nuclease-free water. Samples were subjected to the following cycling parameters (95°C, 2 min; 30 cycles of: 95°C for 45 s, annealing temperature for 30 s, 72°C for 1 min, followed by 5 min at 72°C). Each PCR reaction was diluted 10 times and 1 µl was used as a template for nested PCR run under the same conditions using a nested sequence-specific primer and Pal 17 nested specific primers (Table S1). PCR amplified products were cloned into the pGEM®-T Easy Vector (Promega) and sequenced. The CDS of the candidate Cragi-CTRs and the Cragi-RCP transcripts were then amplified by PCR (Pfu DNA polymerase, Promega) using gene-specific sense primers harbouring a Kozak consensus sequence and antisense primers (Table S1) and 10 ng of cDNA library inserted into the Pal 17.3 vector (Evrogen) as the template. The resulting PCR products were directionally cloned into the pTARGET expression vector (Promega). The correct insertion of the PCR products was confirmed by sequencing. Human embryonic kidney (HEK293T, Sigma-Aldrich) cells were transiently transfected with the *Cragi-CTR*/pTARGET or *Cragi-CTR*/*Cragi-RCP*/pTARGET constructs using Fugene HD (Promega) according to the manufacturer's instructions. As a first step, co-transfection was done with a pTARGET expression construct for the human $G\alpha_{16}$ subunit, a promiscuous G-protein that can direct intracellular signalling of GPCRs to the release of calcium via the phospholipase C_β pathway, regardless of the endogenous G-protein coupling of the receptor (Offermanns and Simon, 1995). To assess receptor activity independent of $G\alpha_{16}$, calcium responses were measured in cells expressing only the Cragi-CTRs. Cells for negative control experiments were transfected with empty pTARGET and $G\alpha_{16}$/pTARGET constructs.

Calcium fluorescence assay

Activation of the Cragi-CTRs by candidate peptide ligands was monitored using a fluorescence-based calcium mobilization assay. Briefly, transfected HEK293T cells were loaded with Fluo-4 Direct (Invitrogen, Carlsbad, CA, USA) plus probenecid (q.s.

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2.5 mmol l^{-1} final in the cell; Molecular Probes) for 1 h (45 min at 37°C and 15 min at room temperature). Excitation of the fluorophore was done at 488 nm. The calcium response was measured for 2 min at 525 nm using a FLEXstation 3 (Molecular Devices) at 37°C. Data were analysed using SoftMax Pro (Molecular Devices). Candidate ligands were first tested at a final concentration of 10^{-5} mol l^{-1}. Concentration–response measurements of activating ligands were conducted in triplicate and for at least three independent experiments. Half-maximal effective concentrations (EC_{50} values) were calculated from concentration–response curves that were constructed using non-linear regression analysis with a sigmoidal dose–response equation using Prism 5.0 (GraphPad Software, USA).

cAMP luminescence assay

Cragi-CTR- and *Cragi-CTR*/*Cragi-RCP*-transfected HEK293T cells were incubated with Glosensor cAMP reagent (q.s. 4% final in the medium; Promega) for 2 h at room temperature prior to injection of the candidate ligands. cAMP luminescence response was measured for 30 min after injection using a FLEX station 3 (Molecular Devices) at room temperature. Data were analysed using SoftMax Pro (Molecular Devices). Candidate

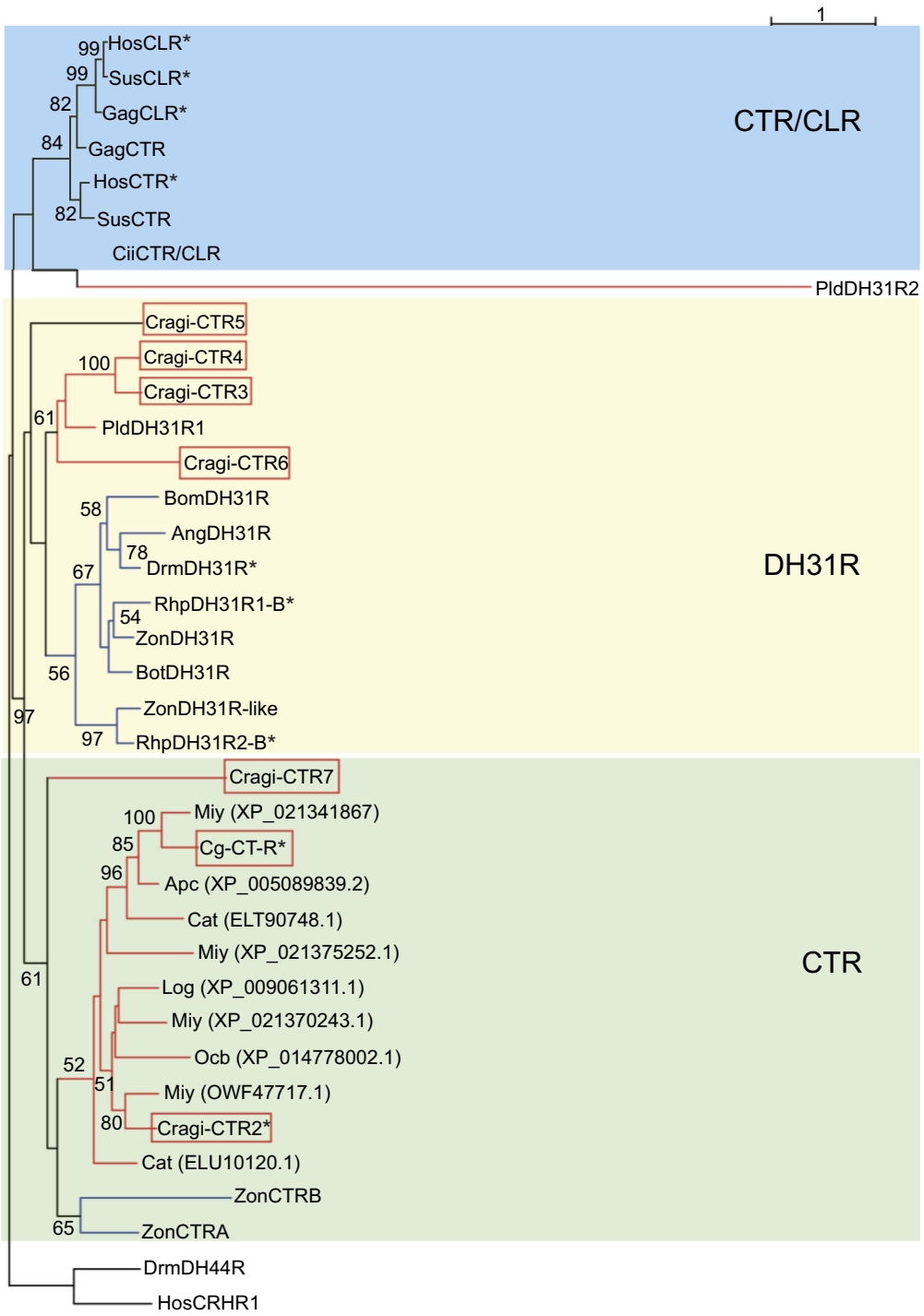

Fig. 1. Phylogenetic representation of the relationship between Cragi-CTRs, Cg-CT-R and other CTR/DH31R family members. Phylogenetic and molecular evolutionary analyses were conducted using Seaview based on the maximum-likelihood method. The accession numbers and the sequences used to construct the tree are listed in Tables S2 and S3. Asterisks indicate receptors for which peptides that act as ligands have been identified and demonstrated experimentally. Blue, yellow and green rectangles respectively include deuterostome CTR (calcitonin receptor)/CLR (calcitonin-like receptor), protostome DH31R (diuretic hormone 31 receptor) and protostome CTR. Black, red and blue branches respectively represent chordate, lophotrochozoan and ecdysozoan species. *Crassostrea gigas* receptors are boxed in red. Hos, *Homo sapiens*; Sus, *Sus scrofa*; Gag, *Gallus gallus*; Cii, *Ciona intestinalis*; Pld, *Platynereis dumerilii*; Cragi, *Crassostrea gigas*; Bom, *Bombyx mori*; Ang, *Anopheles gambiae*; Drm, *Drosophila melanogaster*; Rhp, *Rhodnius prolixus*; Zon, *Zootermopsis nevadensis*; Bot, *Bombus terrestris*; Miy, *Mizuhopecten yessoensis*; Apc, *Aplysia californica*; Cat, *Capitella teleta*; Log, *Lottia gigantean*; Ocb, *Octopus bimaculoides*.

peptide ligands were first tested at a final concentration of 10^{-5} mol l^{-1}.

Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR analysis was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Recovered RNA was then further purified on Nucleospin RNAII columns (Macherey-Nagel, Düren, Germany). After treatment for 20 min at 37°C with 1 U of DNase I (Sigma-Aldrich) to remove genomic DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotide primers (Promega), 0.5 mmol l^{-1} dNTPs and 200 U MMLV reverse transcriptase (Promega) at 37°C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70°C for 10 min. The GoTaq® qPCR Master Mix (Promega) was used for real-time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95°C/15 s, 60°C/15 s) with gene-specific primers. A parallel amplification of *C. gigas* elongation factor 1 α (*EF1 α*) transcript (BAD15289) was carried out to normalize the expression data of the studied transcripts. EF1 α was found to be a reliable normalization gene as no significant difference (P<0.05) of Ct values was observed between the different samples compared. The coefficient of variation of EF1 α was less than 5%. Thus, the relative expression level of each gene was calculated for one copy of the EF1 α reference gene by using the following formula: $N=2^{(\text{Ct EF1 }\alpha-\text{Ct }Cg\text{-cDNA})}$. The PCR amplification efficiency ($E=10^{(-1/\text{slope})}$) for each primer pair was determined by linear regression analysis of a dilution series to ensure that E ranged from 1.98 to 2.02. The specificity of the primer pairs was confirmed by melting curve analysis at the end of each qPCR run.

Statistical analysis

Expression levels between differentially conditioned animals were compared using an unpaired Student's t-test. Significance was set at P<0.05.

RESULTS

Molecular characterization of oyster CT/DH31-related receptors and RCP

In silico analysis of the GigaTON database (Riviere et al., 2015) resulted in the identification of six full-length cDNAs encoding Cragi-CTR3 (CHOYP_CALCR.1.6, GenBank MK290746), Cragi-CTR4 (CHOYP_CALCR.6.6), Cragi-CTR5 (CHOYP_CALCR.2.6), Cragi-CTR6 (CHOYP_CALRL.2.3), Cragi-CTR7 (CHOYP_CALCR.3.6) and CHOYP_CALRL.3.3 (GenBank AJ551182) corresponding to the formerly characterized

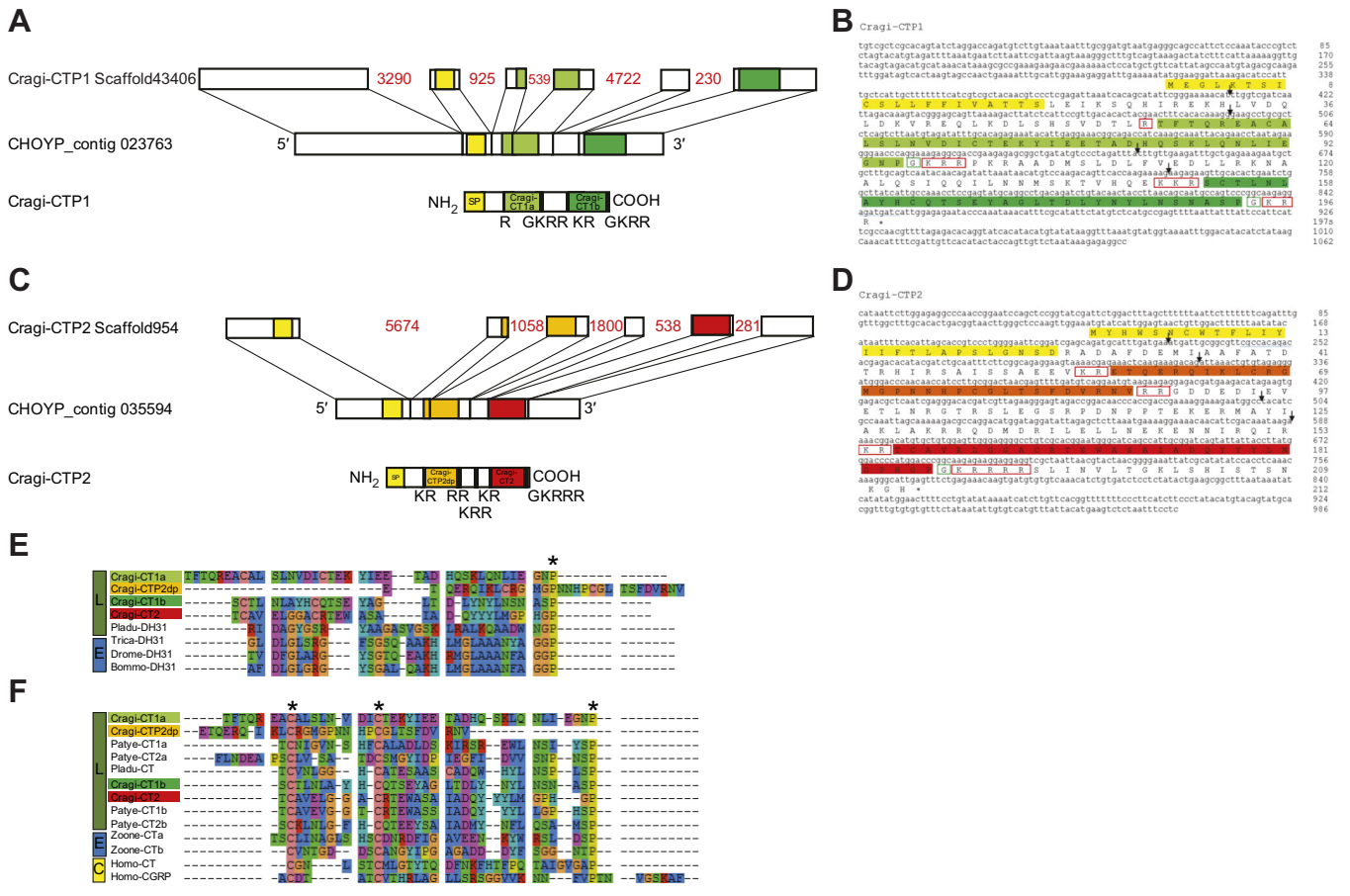

Fig. 2. Schematic representation of oyster CT genes and precursors. (A–D) Cragi-CTP1 (A,B) and Cragi-CTP2 (C,D) genes and sequence of the precursors. (E,F) Sequence alignment of Cragi-CT peptides with protostome and deuterostome DH31 (E) and CT (F). SP, signal peptide. Amino acids with similar physico-chemical properties are in the same colour. Pladu, *Platynereis dumerilii*; Trica, *Tribolium castaneum*; Drome, *Drosophila melanogaster*; Bommo, *Bombyx mori*; Patye, *Patinopecten yessoensis*; Zoone, *Zootermopsis nevadensis*; Homo, *Homo sapiens*; L, Lophotrochozoa; E, Ecdysozoa; C, Chordata. Arrows in B and D indicate intron positions. Asterisks in E and F indicate conserved amino acids. Numbers in red indicate the length in nucleotides of the introns. The accession numbers and the sequences used to construct the alignment are listed in Table S4.

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Cg-CT-R (Dubos et al., 2003). The full-length sequence of the partial cDNA encoding Cragi-CTR2 (CHOYP_CALCR.4.6, GenBank MK290745) was obtained by a RACE procedure. In the GigaTON database, all these receptors have a discrete tissue expression pattern. The phylogenetic analysis clearly shows that both vertebrate CTRs and CLRs cluster separately from protostome DH31R and CTR sequences (Fig. 1). The protostome sequences segregate into two main subgroups: the first one corresponds to DH31R sequences including Cragi-CTR5 and also Cragi-CTR3 and Cragi-CTR4, which seem closer to *Platynereis dumerilii* DH31R1; the second cluster corresponds to CT-type receptors and comprises lophotrochozoan receptors, especially Cragi-CTR2, Cg-CT-R and the more distant Cragi-CTR7. This cluster also contains the termite putative receptors CTRA and CTRB. Intriguingly, the functionally characterized *P. dumerilii* DH31 receptor 2 (PldDH31R2) showed an ambiguous placement as a result of the long branches.

Molecular characterization of Cragi-CTs

A BLAST search of the GigaTON database resulted in the identification of two transcripts coding for the precursors of Cragi-CT1 (Cragi-CTP1; CHOYP contig_023763) and Cragi-CT2 (Cragi-CTP2; CHOYP contig_035594). The two Cragi-CTPs share a similar organization, starting with a signal peptide and harbouring two potential CT/DH31-like peptides (a and b) cleaved N-terminally at monobasic/dibasic processing sites and C-terminally, except for Cragi-CT2-derived peptide (CTP2dp), at a tribasic/tetrabasic processing site typically found in CT/DH31 precursors. Because of the presence of a glycine residue preceding the C-terminal cleavage sites, mature peptides (except Cragi-CTP2dp) are probably amidated (Fig. 2 and Table 1). *Cragi-CTP1* and *Cragi-CTP2* genes, both composed of six exons, also display an analogous arrangement with introns at similar positions. Cragi-CT1a and Cragi-CTP2dp are encoded by the second/third and the third/fourth exon of the respective genes and Cragi-CT1b and Cragi-CT2 are encoded by the last exons (Fig. 2A–D). Alignment of

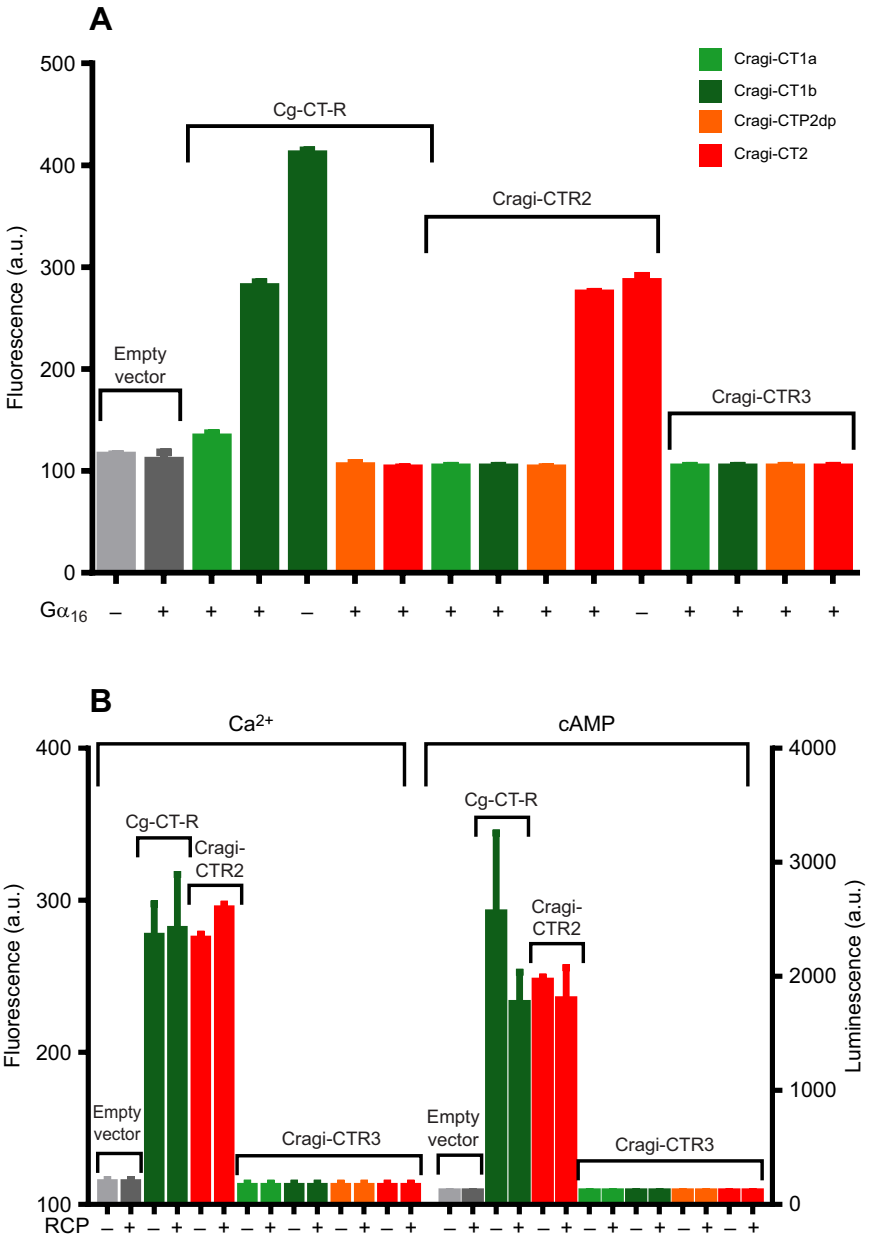

Fig. 3. Activation of Cg-CT-R, Cragi-CTR2 and Cragi-CTR3 by Cragi-CTs. (A) Fluorescence signal induced by Cg-CT-R, Cragi-CTR2 and Cragi-CTR3 expressed in HEK293T cells and challenged by Cragi-CT1s (Cragi-CT1a and Cragi-CT1b) and Cragi-CT2s (Cragi-CTP2dp and Cragi-CT2) at a concentration of 10^{-5} mol l^{-1} in the absence (–) or presence (+) of the promiscuous protein $G\alpha_{16}$. (B) Intensity signal (fluorescence: Ca^{2+}, luminescence: cAMP) induced by Cg-CT-R, Cragi-CTR2 and Cragi-CTR3 expressed in HEK293T cells and challenged by, respectively, Cragi-CT1b, Cragi-CT2 and all Cragi-CTs at a concentration of 10^{-5} mol l^{-1} in the absence (–) or presence (+) of *C. gigas* receptor component protein (Cragi-RCP). Cells transfected with an empty vector or with $G\alpha_{16}$/RCP expressed alone were used as negative controls. Vertical bars represent the s.e.m., *n* (number of replicates)=3.

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Cragi-CTs with CT and DH31 peptides from other species showed that oyster peptides only shared subtle sequence identity but, except for Cragi-CTP2dp, displayed the conserved C-terminal 'proline-amide' motif common to insect DH31 and vertebrate CTs (Fig. 2E, F). Moreover, all of these peptides are similar in length at around 30 amino acids. Cragi-CT1a, Cragi-CT1b, Cragi-CTP2dp and Cragi-CT2 also exhibited the typical two cysteine residues for disulfide bond formation common to all deuterostome and protostome CT-type peptides. Two distinct cysteine spacing patterns differentiate CTa-type peptides (C9/8XC motif) from CTb-type peptides (C7XC motif) (Fig. S2).

Analysis of extracts of *C. gigas* visceral ganglia using nano-LC coupled to mass spectrometry (LC-MS) uncovered the presence of peptides with retention times and molecular masses corresponding to those of synthetic Cragi-CT1a [904.65*m*/*z* (*z*=5+)], Cragi-CT1b [1141.8489*m*/*z* (*z*=3+)] and Cragi-CT2 [1109.84*m*/*z* (*z*=3+)] under native conditions. No signal corresponding to Cragi-CTP2dp molecular mass was detected from visceral ganglia extracts (Fig. S1). Because of low levels of Cragi-CTs, the analysis of reduced and alkylated peptides from visceral ganglia extracts by tandem mass spectrometry only generated an interpretable MS/MS spectrum for Cragi-CT2 (peak score of 37.09, false discovery rate <1%).

Distinct Cragi-CT forms specifically activate Cragi-CTR2 and Cg-CT-R

A calcium mobilization assay was used to identify the cognate ligands of Cragi-CTRs. Only Cg-CT-R, Cragi-CTR2 and Cragi-CTR3 were tested. The receptors were transiently transfected into HEK293T cells with the promiscuous $G\alpha_{16}$ protein, with or without Cragi-RCP (CHOYP-CRCP.1.1, GenBank MK290747), and challenged with high concentrations (10^{-5} mol l^{-1}) of synthetic Cragi-CT1s and Cragi-CT2s. No signal was obtained with cells transfected with an empty vector or with a $G\alpha_{16}$-expressing vector. Only Cg-CT-R and Cragi-CTR2 were specifically activated with a high dose (10^{-5} mol l^{-1}) of Cragi-CT1b and Cragi-CT2, respectively, in the presence or absence of the promiscuous $G\alpha_{16}$ protein. In addition, no activation of Cragi-CTR3 was observed with any of the four Cragi-CTs (Fig. 3A). Co-expression of Cragi-CTRs with Cragi-RCP had no effect on the activation of the receptors, as assessed in both calcium and cAMP assays (Fig. 3B). Thus, dose-dependent activation of Cg-CT-R and Cragi-CTR2 was recorded by omitting the $G\alpha_{16}$ and the RCP proteins (Fig. 4). In a calcium mobilization assay, Cragi-CT1b activated Cg-CT-R with a half-maximal effective concentration (EC_{50}) of 3.37×10^{-7} mol l^{-1} and Cragi-CT2 activated Cragi-CTR2 with an EC_{50} of 1.70×10^{-7} mol l^{-1} (Table 2). The transduction via Gαs was investigated using a cAMP luminescence assay. Cragi-CT1b activated Cg-CT-R with an EC_{50} of 1.75×10^{-7} mol l^{-1} and Cragi-CT2 activated Cragi-CTR2 with an EC_{50} of 0.18×10^{-7} mol l^{-1} (Table 2).

Gene expression of Cragi-CTR signalling components

The expression of genes encoding Cg-CT-R, Cragi-CTR2, Cragi-CTP1 and Cragi-CTP2 was investigated by RT-qPCR in several adult tissues (Fig. 5). As mentioned previously (Dubos et al., 2003), *Cg-CT-R* was mainly expressed in the mantle, the gills and the heart (Fig. 5C). *Cragi-CTR2* gene was ubiquitously expressed in adult tissues with a higher expression pattern in visceral ganglia (Fig. 5D). *Cragi-CTP1* and *Cragi-CTP2* genes were also majorly expressed in visceral ganglia but also in peripheral tissues like the mantle edges, the mantle and also the gills for *Cragi-CTP2* and the digestive gland, the gonads but not the gills for *Cragi-CTP1* (Fig. 5A,B). As

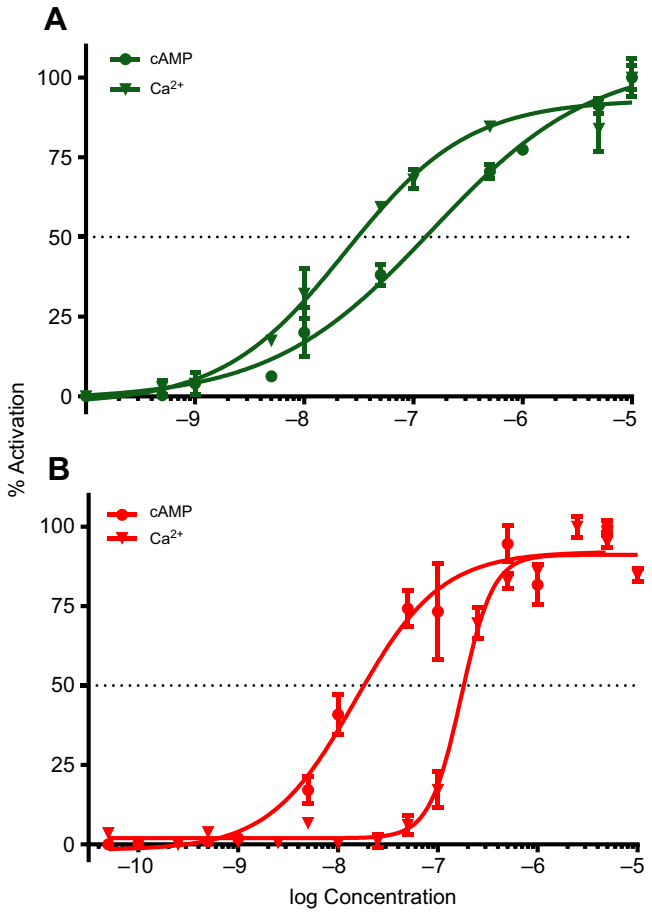

Fig. 4. Dose-dependent activation of Cg-CT-R and Cragi-CTR2 by Cragi-CTs. Activity of Cragi-CT1b on Cg-CT-R (A) and Cragi-CT2 on Cragi-CTR2 (B) expressed in HEK293T cells. Concentration (mol l^{-1})–response data are shown relative to the highest value (100% activation) for a given peptide ligand and represent the mean of an experiment (*n*=3) performed in triplicate. Vertical bars represent the s.e.m.

Cg-CT-R was previously found to be downregulated in the mantle edges and the gills of oysters acclimatized to brackish water (Dubos et al., 2003), we assessed the expression of *Cg-CT-R*, *Cragi-CTR2*, *Cragi-CTP1* and *Cragi-CTP2* genes in oysters transferred from seawater to brackish water following either an acute (AC) or mild (MC) osmotic conditioning protocol (see Materials and Methods). Cragi-CTP1 gene expression decreased in both visceral ganglia and the mantle, although significantly in the latter tissue (Fig. 6A). This decrease paralleled the slight decrease in expression of the CTP1

Table 2. EC_{50} of Cragi-CT1/2 for respective receptor activation

		EC_{50} (mol l^{-1})		
Peptide	Assay	Cg-CT-R	Cragi-CTR2	Cragi-CTR3
Cragi-CT1a	Ca^{2+}	X	X	X
	cAMP	X	X	X
Cragi-CT1b	Ca^{2+}	$3.37(\pm3.12)\times10^{-7}$	X	X
	cAMP	$1.747(\pm4.03)\times10^{-7}$	X	X
Cragi-CTP2dp	Ca^{2+}	X	X	X
	cAMP	X	X	X
Cragi-CT2	Ca^{2+}	X	$1.70(\pm0.18)\times10^{-7}$	X
	cAMP	X	$0.18(\pm0.02)\times10^{-7}$	X

A cross indicates no activation.

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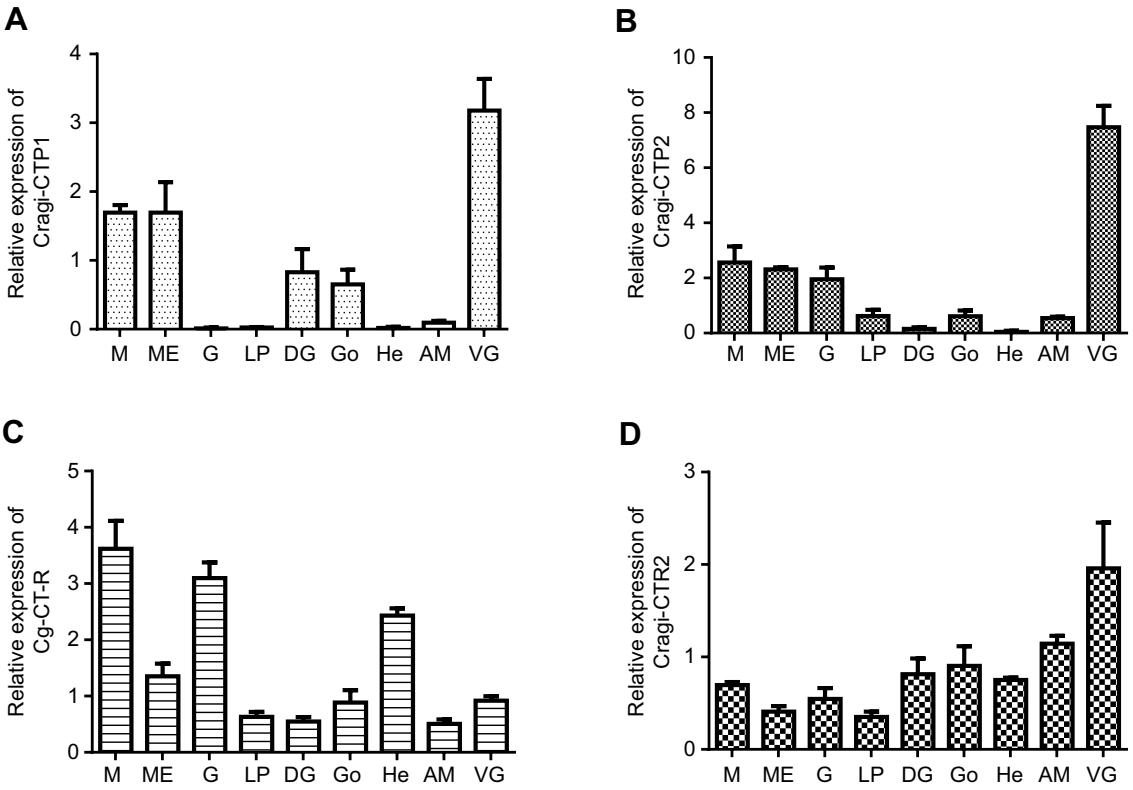

Fig. 5. Expression of mRNA encoding CT precursors and receptors in adult tissues. (A) Cragi-CTP1, (B) Cragi-CTP2, (C) Cg-CT-R and (D) Cragi-CTR2. Each value is the mean+s.e.m. of 5 pools of 6 animals. Expression levels were calculated as the number of copies of each specific transcript per 10^3 copies of elongation factor 1 α (EF1 α) mRNA. Results were statistically tested with a one-way ANOVA (P<0.05). M, mantle; ME, mantle edge; G, gills; LP, labial palps; DG, digestive gland; Go, gonad; He, heart; AM, adductor muscle; VG, visceral ganglia.

cognate receptor, *Cg-CT-R*, in the visceral ganglia, the mantle and the gills following hypo-osmotic conditioning (Fig. 6C) but unlike prior results (Dubos et al., 2003) this was not statistically significant. Expression of the *Cragi-CTP2* gene, encoding the precursor of the Cragi-CTR2-specific ligand, tended to be upregulated in oysters subjected to MC, although this was only significant in the visceral ganglia, not in the gills and the mantle (Fig. 6B). *Cragi-CTR2* gene expression was not affected by the transfer to brackish water in the tissues of oysters subjected to MC (Fig. 6D). Interestingly, except for a significant increase of *Cragi-CTR2* transcript in the gills, no response was observed after the AC stress in any tissue tested (Fig. 6).

DISCUSSION

This study supplements the small amount of existing data on the CT/DH31 signalling pathways in protostomes. Although CT-type peptides are notably absent in *Drosophila* as well as other flies and Hymenoptera (Veenstra, 2014), and DH31-type peptides presumably disappeared in molluscs (Conzelmann et al., 2013), both ecdysozoan and lophotrochozoan lineages exhibit the two evolutionarily related peptide forms (Cai et al., 2018; Conzelmann et al., 2013; Veenstra, 2014). They are believed to result from the duplication of an ancestral CT-type encoding gene present in the common ancestor of the protostomes. This gene generated the diverse vertebrate CT/CGRP family of peptides, and after duplication in an ancestral protostome it gave rise to the genes encoding the CT- and DH31-type peptides that were subsequently lost or retained in some lineages (Cai et al., 2018). Up to now, only DH31 signalling systems have been characterized in protostomes (Bauknecht and Jékely, 2015; Johnson et al., 2005; Zandawala et al., 2013). Using the Pacific oyster as an experimental species, the present report identifies for the first time the existence of a functional protostome CT-type signalling system.

Oyster CT precursors generate two distinct mature CT-type neuropeptides

Mining the oyster transcriptomic database GigaTON led to the identification of two transcripts (*Cragi-CTP1* and *Cragi-CTP2*), each encoding two peptides displaying the characteristics of CT-type neuropeptides. All the Cragi-CT peptides display the N-terminal distinctive cysteine residues, have a similar length of around 30 amino acids and, except for Cragi-CTP2dp, share the C-terminal proline-amide motif with their counterparts. Cragi-CTP2dp appeared as a singular evolutionary form deprived of a C-terminal amide. Besides these similarities, there were clearly two types of peptides based on the spacing of the cysteine residues: with respect to the cleavage site, the CTa-types displayed the C9/8XC motif, while the CTb-types displayed the C7XC motif. The arrangement of the oyster CTPs with two mature peptides is particularly intriguing as deuterostome CT-type peptide precursors contain either a single peptide (Muff et al., 1995; Sekiguchi et al., 2009) or two peptides, as observed in echinoderm CT-type precursors (Rowe et al., 2014; Suwansa-ard et al., 2018; Zandawala et al., 2017). In the protostomes, the annelid *P. dumerilii* CT and DH31 precursors (Conzelmann et al., 2013) and the DH31 and CT-A precursors in arthropods (Alexander et al., 2018; Christie, 2008; Christie et al., 2010; Coast et al., 2001; Gard et al., 2009; Li et al., 2007; Veenstra, 2014; Zandawala, 2012; Zandawala et al., 2011)

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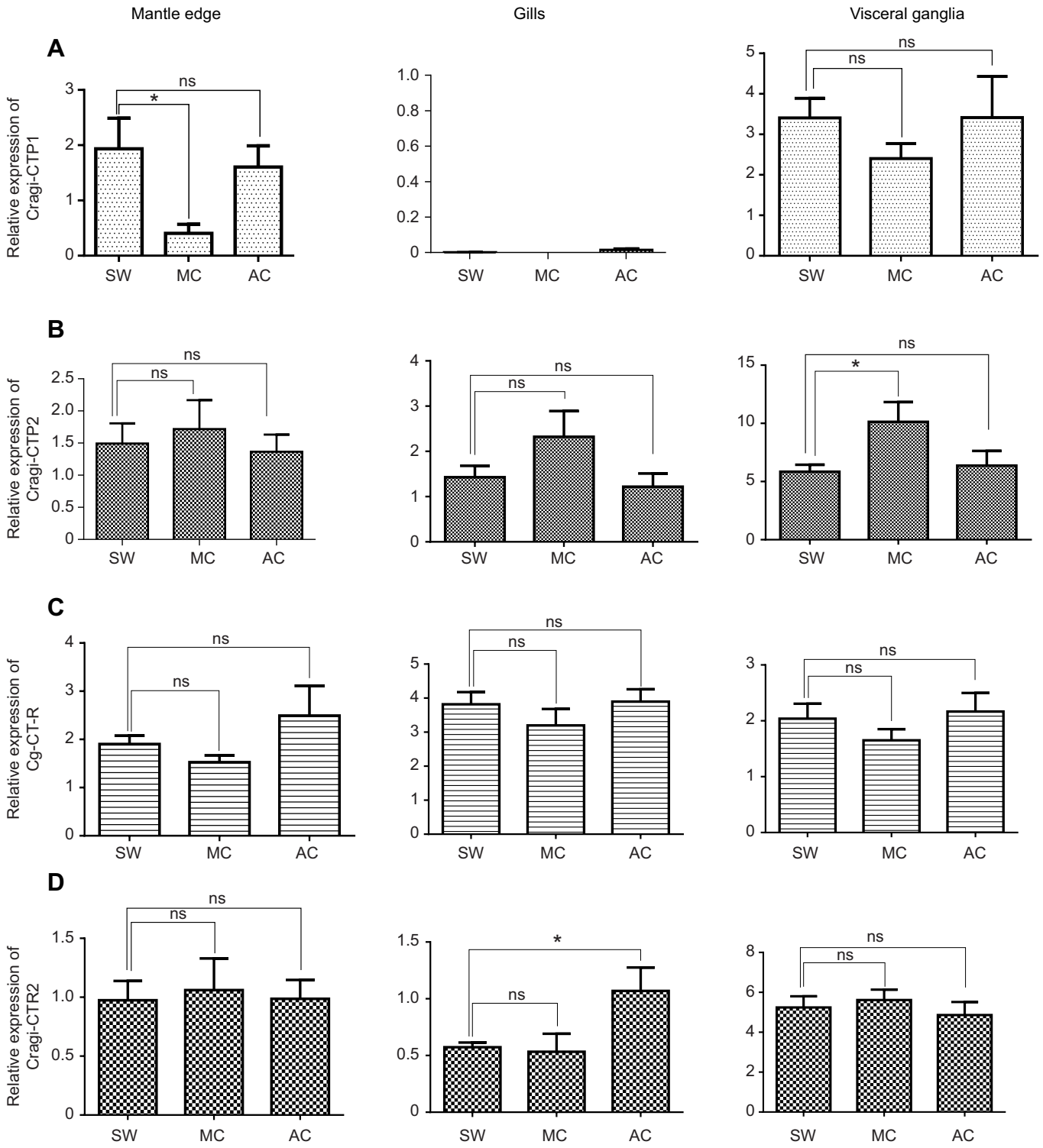

Fig. 6. Expression of mRNA encoding CT precursors and receptors in tissues of oysters acclimated to brackish water. (A) Cragi-CTP1, (B) Cragi-CTP2, (C) Cg-CT-R and (D) Cragi-CTR2. SW, seawater; MC, mild osmotic conditioning; AC, acute osmotic conditioning. Each value is the mean+s.e.m. of 5 pools of 5 animals. Expression levels were calculated as the number of copies of each specific transcript per 10^3 copies of elongation factor 1 α (EF1 α) mRNA. Results were statistically tested with Student's *t*-test. Significantly different means of samples from control and conditioned animals are indicated by an asterisk (P<0.05).

comprise only one peptide. In molluscs, this tandem peptide arrangement is observed in bivalves (Zhang et al., 2018) and also in gastropods (Fig. S2), although in cephalopods, the two precursors characterized in *Sepia officinalis* encode only one mature peptide (named SP-amide) (Zatylny-Gaudin et al., 2016); the same is true for the unique transcript detected in *Octopus bimaculoides* (XP_014768345.1). Thus, it is possible that this dual peptide precursor organization originated in the common ancestor of the bilateria and this feature was independently lost in certain lineages depending on their different selective pressures. Alternatively, the tandem peptide precursor structure may have evolved independently, probably by intragenic duplication, in protostomes and deuterostomes. The two

Cragi-CTPs are encoded by separate genes that show a remarkably conserved organization, with exons encoding similar parts of the mature peptides, suggesting that they probably arose through whole-gene duplication. Two distinct genes also exist in the genome of the cephalopod *O. bimaculoides*: in addition to XP_014768345.1, we detected another sequence encoding a mature CT-type peptide in the genome scaffold 69947 (Fig. S2).

Oyster CTR/CLR/DH31R-related receptors are specifically activated by oyster CT-type neuropeptides

By screening the GigaTON database (Riviere et al., 2015), we identified seven receptors, including the previously characterized oyster receptor Cg-CT-R (Dubos et al., 2003), revealing homologies with CTR/CLR/DH31R from other species. Only three of these receptors, namely Cragi-CTR2, Cragi-CTR3 and Cg-CT-R, were tested for their possible activation by Cragi-CTs. We found that Cragi-CT2 specifically and exclusively activated Cragi-CTR2, and Cragi-CT1b activated Cg-CT-R. None of the Cragi-CTs were effective in the activation of Cragi-CTR3 even at concentrations as high as 10^{-5} mol l^{-1}. Given the homologies between Cragi-CT1b and Cragi-CT2, one would expect a certain degree of cross-reactivity between the two ligands and the two receptors. Given the co-existence of four related CT neuropeptides in *C. gigas*, there seems to exist an evolutionary pressure on each receptor so that it can only be activated by its own ligand. A similar situation was observed in insects for the structurally related neuropeptides adipokinetic hormone (AKH), corazonin and AKH/corazonin-related peptide (ACP), which activate their cognate receptor without cross-reactivity (Belmont et al., 2006; Hansen et al., 2010).

Interestingly, none of the Cragi-CTa-types (C9/8XC motif) was found to activate the three tested receptors. We assume that among the phylogenetically more distantly related receptors identified in this study are potential receptors (Cragi-CTR7, Cragi-CTR5 or Cragi-CTR6) able to be activated by Cragi-CT1a, the mature form of which was detected in oyster visceral ganglia. Whether Cragi-CTP2dp represents a biologically active neuropeptide remains speculative as no corresponding molecular form was detected in visceral ganglia. As CT1a and CT1b represent end products of the same precursor, physiological connections probably exist between their respective signalling systems. Thus it would be interesting to characterize cognate receptors of CT1a. A lack of activation of Cragi-CTR3 by any of the Cragi-CTs can simply mean that there are additional, still uncharacterized, CT- or DH31-related neuropeptides expressed in *C. gigas*. The observation that Cragi-CTR3 and also Cragi-CTR5 and Cragi-CTR6 are phylogenetically close to functionally characterized DH31R from annelids and insects further supports the existence of DH31-related peptides in oyster. Another possibility would be that translocation to the membrane and/or activation of Cragi-CTR3 by its cognate ligand requires escort proteins such as RAMPs. Currently, no RAMP-like protein has been identified outside the chordate lineage (Sekiguchi et al., 2016). As RAMPs from different chordate species show very limited sequence identity, mining the protostome genomes for sequences homologous to vertebrate RAMPs proved fruitless. However, we cannot rule out the existence of functional though structurally distinct RAMP proteins in protostomes. Surprisingly, *Drosophila melanogaster* DH31R expressed in HEK-293 cells was only activated by Drome-DH31 provided that human RAMPs (1/2) were co-expressed. The signal was even higher and the receptor more sensitive to its ligand in the presence of *D. melanogaster* or human RCP (Johnson et al., 2005). This clearly points to a parallel with *D. melanogaster* DH31 and CGRP signalling in terms of molecular interactions and implies that the *D. melanogaster* receptor is functionally closer to vertebrate CLR than to CTR. This may evenly apply to Cragi-CTR3 and also to the other Cragi-CTRs so far not investigated. However, no accessory protein was required for the DH31 signalling systems of the kissing bug *Rhodnius prolixus* (Zandawala et al., 2013) and of the annelid *P. dumerilii* (Bauknecht and Jékely, 2015), or for the Cg-CT-R and Cragi-CTR2 signalling systems of *C. gigas*. Although we characterized the oyster RCP, its co-expression with Cg-CT-R or Cragi-CTR2 did not change the signal induced by the activation of the receptors by their specific ligands, thus reinforcing the hypothesis that Cg-CT-R and Cragi-CTR2 are functionally more closely related to vertebrate CTR than to CLR. RCP was initially associated with its ability to favour the coupling of CLR/RAMP complexes to Gαs, but recent findings suggest the involvement of this protein in alternative signalling pathways including transcriptional regulation (Dickerson, 2013). Cg-CT-R and Cragi-CTR2 both activate the adenylyl cyclase and phosphoinositide transduction pathways, though with distinct potencies for Cragi-CTR2/Cragi-CT2. This suggests the existence of multiple conformational states for the oyster receptors, with distinct pharmacological and signalling properties. Dual activation was also observed for vertebrate CT (Force et al., 1992), although cAMP signalling represents the most common pathway to mediate the activity of the CT/CGRP/DH31 peptide family (Gorn et al., 1992; Coast et al., 2001; Iga and Kataoka, 2015).

Biological implications of the oyster CT signalling systems

Both vertebrate CTs and insect DH31s are involved in ion and water balance (Hirsch et al., 2001; Zandawala, 2012). Interestingly, a role in ionic regulation was previously suggested for the *Cg-CT-R* gene given its main expression in the mantle edges and the gills, as well as its decreased expression in brackish water-acclimated oysters (Dubos et al., 2003). The present study also strongly points to a role of oyster CT signalling in ion or water regulation but with two opposite implications: Cg-CT-R/Cragi-CT1b expression was decreased while Cragi-CTR2/Cragi-CT2 was increased in lower saline concentration modalities. This dual command might be very efficient for the rapid adaptation of oysters to salinity fluctuations in their natural environment. Because the oyster Cg-CT-R/Cragi-CT1b- and Cragi-CTR2/Cragi-CT2-encoding genes do not seem to respond to an acute osmotic stress, these signalling systems might not mediate the stress response but more likely participate in osmotic control, as CGRP does in the eel gill (Lafont et al., 2006) and possibly in *Ciona intestinalis* (Kawada et al., 2010). Whether the oyster CT signalling systems specifically regulate calcium fluxes as their vertebrate counterparts do (Zaidi et al., 2002) remains speculative. The relatively high expression of oyster signalling components in the mantle edges (Dubos et al., 2003), a tissue involved in shell biomineralization (Geraerts, 1976), is strongly suggestive of such a role. In contrast to some oyster signalling systems (Bigot et al., 2012; Dubos et al., 2018; Schwartz et al., 2018), we found no involvement of the oyster CT signalling system in feeding regulation. This diverges from the role of DH31 in feeding regulation in arthropods (Nagata and Nagasawa, 2017). Apart from a role in ion or water balance in the oyster, its expression in a variety of tissues implicates CT signalling in a variety of biological functions. In other respects, the expression of Cragi-CTRs in visceral ganglia argues for a role in neurotransmission within the oyster central nervous system.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.F.; Methodology: J.S., M.-P.D., P.F.; Software: J.S., E.R.-D.; Validation: J.S., P.F.; Formal analysis: J.S., E.R.-D., P.F.; Investigation: J.S., E.R.-D., M.-P.D., B.L., P.F.; Resources: B.L., J.L.; Writing - original draft: J.S., P.F.; Writing - review & editing: J.L., P.F.; Supervision: P.F.; Project administration: P.F.; Funding acquisition: P.F.

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Supplementary information
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