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RESEARCH ARTICLE

Molecular and physiological characterization of a crustacean cardioactive signaling system in a lophotrochozoan – the Pacific oyster (*Crassostrea gigas*): a role in reproduction and salinity acclimation

Emilie Réalis-Doyelle*, Julie Schwartz, Marie-Pierre Dubos and Pascal Favrel‡

ABSTRACT

The crustacean cardioactive peptide (CCAP) is an important neuropeptide involved in the regulation of a variety of physiological processes in arthropods. Although this family of peptides has an ancestral origin, its function remains poorly understood among protostome species – apart from arthropods. We functionally characterized three G protein-coupled receptors (GPCRs) in the oyster *Crassostrea gigas*, phylogenetically related to ecdysozoan CCAP receptors (CCAPRs) and to chordate neuropeptide S receptors (NPSRs). Cragi-CCAPR1 and Cragi-CCAPR2 were specifically activated by the Cragi-CCAP1 and Cragi-CCAP2 peptides, respectively, both derived from the same CCAP precursor. In contrast, Cragi-CCAPR3 was only partially activated by CCAP1 and CCAP2 at high concentrations. The *Cragi-CCAPR1* and *Cragi-CCAPR2* genes were expressed in various adult tissues. They are both most expressed in the gills, while *Cragi-CCAPR3* is mainly expressed in the visceral ganglia (VG). Cragi-CCAP precursor transcripts are higher in the VG, the labial palps and the gills. Receptor and ligand-encoding transcripts are more abundantly expressed in the gonads in the first stages of gametogenesis, while the Cragi-CCAP precursor is upregulated in the VG in the last stages of gametogenesis. This suggests a role of the CCAP signaling system in the regulation of reproductive processes. A role in water and ionic regulation is also supported considering the differential expression of the CCAP signaling components in oysters exposed to brackish water.

KEY WORDS: Neuropeptides, CCAPs, Mollusk, Hyposaline stress, Reproduction

INTRODUCTION

The cyclic nonapeptide crustacean cardioactive peptide (CCAP) was first isolated from the pericardial organs of the shore crab *Carcinus maenas*, where it was first described to regulate heartbeat (Stangier et al., 1987). This effect was subsequently confirmed in several crustaceans (Chen et al., 2016; Fort et al., 2007). CCAP also affects cardiac activity in insects (da Silva et al., 2011; Nichols et al., 1999). In arthropods, CCAP regulates a variety of biological activities such as neuronal modulation in the stomatogastric ganglion in the crab (Weimann et al., 1997), hindgut contraction in the Vietnamese stick insect *Baculum extradentatum* (Lange and Patel, 2005) and midgut activity in the cockroach *Periplaneta americana* (Matsui et al., 2013; Sakai et al., 2004). CCAP has regulatory roles in the feeding behavior of the fruit fly *Drosophila melanogaster* (Williams et al., 2020), osmoregulation in the Pacific white shrimp *Litopenaeus vannamei* (Chen et al., 2016) and immunity in the mud crab *Scylla paramamosain* (Wei et al., 2020). CCAP is also involved in reproduction and development: it modulates oviduct activity and egg emission in the African migratory locust *Locusta migratoria* (Donini et al., 2001) and increases spermatheca contraction (da Silva and Lange, 2006); and it plays a role in larval and pupal development in the mosquito *Anopheles gambiae* (Estévez-ao et al., 2013), in hatching behavior in the red flour beetle *Tribolium castaneum* (Li et al., 2011), and in ecdysis behavior in the tobacco hornworm *Manduca sexta* and the fruit fly *D. melanogaster* (Park et al., 2003).

Although transcripts encoding CCAP precursors have been characterized in the main lophotrochozoan phyla including mollusks (Endress et al., 2018; Stewart et al., 2014; Veenstra, 2010), annelids (Conzelmann et al., 2013) and platyhelminths (Koziol et al., 2016), the biological role of the mature peptides has only been investigated in a few mollusks. In the pond snail *Lymnaea stagnalis*, CCAP modulates the activity of neurons of the buccal feeding network (Vehovszky et al., 2005). It is involved in reproduction by triggering spawning in the Sydney rock oyster *Saccostrea glomerata* (In et al., 2016) and by controlling oocyte transport and egg-capsule secretion in the cuttlefish *Sepia officinalis* (Endress et al., 2018). In contrast to arthropods, where CCAP precursors harbor only one copy of the mature peptide (Toullec et al., 2013), mollusk precursors comprise two or three copies of slightly distinct sequences (Endress et al., 2018; Stewart et al., 2014; Veenstra, 2010). Interestingly, the CCAP precursors from the Pacific oyster *Crassostrea gigas*, the owl limpet *Lottia gigantea* and the sea hare *Aplysia californica* contain two CCAP-type peptides with a distinctive spacing (4 or 5 amino acids) of the cysteine residues. This spacing probably implies specific structural constraints on the cyclic peptides generated by the formation of a disulfide bond. This raises the question of whether these two peptide types bind to separate receptors and regulate different physiological processes. CCAP receptors (CCAPRs) have been characterized functionally in a number of arthropods (Bao et al., 2018; Cazzamali et al., 2003; Lee et al., 2013a; Li et al., 2011), but not in other protostomian phyla. CCAPRs are unexpected orthologs of vertebrate neuropeptide S receptors (NPSRs) (Jékely, 2013; Mirabeau and Joly, 2013) and of echinoderm receptors that are activated by the NG peptide family of neuropeptides (NGR) (Semmens et al., 2015),

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although the respective cognate ligands share very little sequence similarity. Among protostomes, the CCAP signaling system remains poorly explored in Lophotrochozoa. This is why the present study focused on the functional characterization of CCAP receptors, and experimentally investigated the involvement of CCAP signaling in the regulation of physiological activities in the oyster *C. gigas*.

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 (M), mantle edges (ME), gills (G), labial palps (LP), digestive gland (DG), gonad (mix of all stages) (GO), heart (H), adductor muscle (MA) and the visceral ganglia (VG) were carefully dissected out.

A 1-year sampling of animals was undertaken to collect gonads and VG at different stages of reproduction. Oysters were collected and their gonads and VG were immediately dissected. VG and gonad tissues were sampled for each individual, frozen in liquid nitrogen and stored at −80°C. A part of each gonad sample was also fixed for histological analysis. Gonadal development stage and sex were determined by histological methods according to the four stages previously described (Lubet, 1959; Rodet et al., 2005): stage 0, corresponding to the resting undifferentiated stage with very few germinal stem cells; stage 1, corresponding to the gonial multiplication stage, with poorly developed tubules surrounded by a large matrix of vesicular connective tissue (VCT); stage 2, corresponding to the maturation stage with tubule development and VCT starting to regress [vitellogenesis occurs in females and all the cell of the germline can be observed in males (from spermatogonia to spermatozoa)]; and stage 3, corresponding to sexual maturity with tubules full of mature germinal cells.

To study the influence of osmotic conditions, oysters were transferred from seawater (33‰) to brackish water (8‰) at 17°C by addition of distilled water once in the seawater tank. To prevent the closure of the shell, a wedge was inserted between the valves before applying the stress. Tissues (VG and gills) were sampled and frozen after 12 h of incubation of the oysters in brackish water at the final salinity.

Peptide synthesis

Protostome structures of CCAP-type peptides were previously determined using mass spectrometry and nuclear magnetic resonance (NMR) (Endress et al., 2018; Jackson et al., 2009), confirming the presence of an intramolecular disulfide bridge and a C-terminal amidation (Stangier et al., 1987). Mature *C. gigas* CCAP (Cragi-CCAP1: VFCNGFFGCSNamide, and Cragi-CCAP2: LFCNTGGCFamide) post-translational modifications were inferred from these previous studies. Cragi-CCAP1, Cragi-CCAP2 and oxytocin peptides (Cragi-OT1: CFIRNCPQG-amide, and Cragi-OT2: GCFIRNCPPG-amide) were custom synthesized by GeneCust (Luxembourg). The peptides were synthesized to incorporate a disulfide bridge between the two cysteine residues and a C-terminal amidation.

In silico analyses

Multiple sequence alignment was performed using Clustal Omega (Sievers et al., 2011). Seaview (Gouy et al., 2010) was used to select the conserved protein regions and for manual correction of the alignment. PhyML was used to generate 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 cloned *C. gigas* CCAP-type receptors

Molecular cloning of Cragi-CCAPR and transfection of mammalian cells

The coding sequence of Cragi-CCAPR1 (XM_034446507.1) was amplified by PCR using the transcript-specific sense primer (5′-CACCATGGAATTTGACAACTTTACCGTTTCTC-3′) harboring a Kozak consensus sequence and the antisense primer (5′-TCACGCATGATACACTGATGTTATAGGAGCTT-3′). Ten nanograms of plasmid DNA (Pal 17.3 vector, Evrogen) from a *C. gigas* 'all developmental stages and adult central nervous system' normalized cDNA library (Fleury et al., 2009) was used as template. PCR was carried out in a 50 µl reaction volume containing 1.5 mmol l^{-1} $MgCl_2$, 200 mmol l^{-1} dNTPs, 1 mmol l^{-1} each of the primer couples, 1.25 units of GoTaq® polymerase and the appropriate buffer (Promega) in nuclease-free water. Samples were subjected to the following cycling parameters: 95°C for 2 min; 30 cycles of 95°C for 45 s, 60°C for 30 s and 72°C for 1 min; followed by 5 min at 72°C.

The resulting PCR product was directionally cloned into the eukaryotic expression vector pTARGET (Promega, Madison, WI, USA) and the correct insertion was confirmed by sequencing.

Two cDNAs encoding Cragi-CCAPR2 (XM_011419148) and Cragi-CCAPR3 (XM_034446911.1) were custom synthesized including a Kozak sequence (Genscript, USA), inserted into pTARGET and the construct was checked by Sanger sequencing.

Human embryonic kidney (HEK293T) cells were transiently transfected with the Cragi-CCAPR constructs using Fugene HD (Promega) according to the manufacturer's instructions. As a first step, co-transfection was done with a pcDNA3.1 expression construct for the human Gα16 subunit, a promiscuous G protein that can direct intracellular signaling of GPCRs to the release of calcium via the phospholipase Cβ pathway, regardless of the endogenous G protein coupling of the receptor (Mertens et al., 2004). To assess receptor activity independent of Gα16, calcium responses were measured in cells expressing only Cragi-CCAPRs. Cells for negative control experiments were transfected with empty pcDNA3.1 and Gα16/pcDNA3.1 construct.

Calcium fluorescence assay

Activation of Cragi-CCAPRs, by oyster Cragi-CCAP1, Cragi-CCAP2, Cragi-OT1 and Cragi-OT 2 synthetic peptides was monitored using a fluorescence-based calcium mobilization assay (Bigot et al., 2014). Briefly, transfected HEK293 T cells were loaded with Fluo-4 Direct (Invitrogen, Carlsbad, CA, USA) plus probenecid (Molecular Probes) (2.5 mmol l^{-1} final concentration) for 1 h (45 min at 37°C and 15 min at room temperature). The peptides to be tested were diluted at 5 times their final concentration in 20 mmol l^{-1} HEPES in Hank's balance salt solution pH 7.3 and distributed in a 96-well plate. A volume of 25 µl of the different peptide solutions was injected in the wells containing the cells and the fluorescence signal was immediately recorded. Excitation of the fluorophore was done at 488 nm. The calcium response was measured for 2 min at 525 nm using the FLEXstation 3 (Molecular Devices) at 37°C. Data were analyzed using SoftMax Pro (Molecular Devices). Candidate peptide 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 a nonlinear regression analysis with a sigmoidal dose–response equation using Prism 5.0 (GraphPad Software, USA).

cAMP luminescence assay

Cragi-CCAPR transfected HEK293T cells were incubated with Glosensor cAMP reagent (4% final concentration in the medium) (Promega) for 2 h at room temperature prior to the injection of the candidate ligands. cAMP luminescence response was measured for 30 min after injection using a FLEX station 3 (Molecular Devices) at room temperature. Data were analyzed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} mol l^{-1} and then at different peptide concentrations.

Reverse transcription and quantitative PCR

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). After treatment for 20 min at 37°C with 1 U of DNase I (Sigma) to prevent genomic DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotidic primers (Promega), 0.5 mmol l^{-1} dNTPs and 200 U MMuLV Reverse Transcriptase (Promega) at 37°C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70°C for 10 min. The GoTaq® qPCR Master Mix (Promega) was used for real-time monitoring of amplification (5 ng of cDNA template, 40 cycles: 95°C for 15 s, 60°C for 15 s) with transcript-specific primers for Cragi-CCAPR1 QS (5′-ACCGCCACAAGTACTATGCT-3′) and QA (5′-TCGCTCTGTGGTTCCATACG-3′), Cragi-CCAPR2 QS (5′-CAGATCCGCCAACCACCA-3′) and QA (5′-TACTCAGCG-CCGTCTCCT-3′), Cragi-CCAPR3 QS (5′-CGGCACCGCTCAC-AGTAA-3′) and QA (5′-TCTCTCAGGTTCAGTGCGC-3′), and Cragi-CCAP QS (5′-TGCGGATGAATTGTTGCAAA-3′) and QA (5′-TGTCTTCCGATAGCAGCTCA-3′) as sense (QS) and antisense (QA) primers, respectively. Accurate amplification of the target amplicon was checked by performing a melting curve. A parallel amplification of *C. gigas* Elongation Factor 1α (EF1α) QS-Cg-EF1α (5′-ACCACCCTGGTGAGATCAAG-3′) and QA-Cg-EF1α (5′-ACGACGATCGCATTTCTCTT-3′) transcript (BAD15289) was carried out to normalize the expression data of the analyzed transcripts. EF1α was used as a reliable normalization gene as no significant difference ($P<0.05$) of C_t values was observed between the different samples compared. The coefficient of variation of EF1α was less than 5% for all the tissue samples and experimental conditions. Thus, the relative level of expression of the target gene was calculated using the following formula: $N=2^{[Ct(Cg\text{-}EF1a)-Ct(target\ cDNA)]}$. The PCR amplification efficiency [$E=10^{(-1/slope)}$] for each primer pair was determined by linear regression analysis of a dilution series to ensure that E ranged from 1.98 to 2.02.

RNA-seq and abundance estimation of transcripts in the VG over a reproduction cycle

Library construction and sequencing were conducted at the Genome Quebec Innovation Centre (McGill University, Montréal, Canada).

Preparation of cDNA libraries

RNA extracted from VG collected from six animals of the same stage of reproduction were mixed to generate 26 pools corresponding to: three pools of stage 0; four pools of female stages 1, 2 and 3; four pools of male stages 1 and 2; and three pools of male stage 3. Total RNA of these 24 pools was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated from 250 ng of total RNA as follows. mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining steps of library preparation were performed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. The libraries were normalized and pooled at 3 nmol l^{-1} and then denatured in 0.05 N NaOH and neutralized using HT1 buffer.

Library sequencing

The pool of libraries now at 360 pmol l^{-1} was loaded on an Illumina cBot and the flowcell was run on a HiSeq 4000 for 2×100 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 1% level. The Illumina control software was HCS HD 3.4.0.38, and the real-time analysis program was RTA v. 2.7.7. Program bcl2fastq2 v2.20 was then used to demultiplex samples and generate fastq reads. The recovered transcriptomic data were submitted to the Sequence Read Archive (SRA) database under the accession number (PRJNA662446).

Assessment of transcript expression

Reads of the VG samples were aligned to the *C. gigas* GigaTON reference transcriptome (Riviere et al., 2015) using Galaxy. Estimated read counts for each sample were calculated using the TPM (transcripts per kilobase per million reads) method to provide a normalized comparison of transcript expression between all samples (Li et al., 2010). In the GigaTON database, the following contig names (in parentheses) corresponded to Cragi-CCAPR1 (CHOYP_CCAPR1.1.1), Cragi-CCAPR2 (CHOYP_LOC100574733.1.1), Cragi-CCAPR3 (CHOYP_CCAPR.1.2) and Cragi-CCAP (CHOYP_contig_055789).

Statistical analysis

Gene expression levels between different tissues and between samples at different reproduction stages were compared using one-way ANOVA followed by a Tukey *post hoc* test. Expression levels between animals in different salinity conditions were compared using an unpaired Student's *t*-test. Significance was set at $P<0.05$.

RESULTS

Molecular characterization of oyster cardioactive peptide receptors (Cragi-CCAPRs)

Three sequences displaying sequence similarity with vertebrate NPSR and ecdysozoan CCAPR were retrieved from GigaTON, an oyster comprehensive transcriptomic database (Riviere et al., 2015). These sequences, named Cragi-CCAPR1, Cragi-CCAPR2 and Cragi-CCAPR3, shared 30.7% of amino acid sequence identity. Alignment of Cragi-CCAPRs with other receptors of the family displayed an overall identity of 33.1% with *T. castaneum* CCAPR (ABN79651.1), 26.6% with *D. melanogaster* CCAPR (NP_996297.3) and 27.2% with human NPSR (NP_997056.1)

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(Fig. 1). Alignment of the Cragi-CCAPR cDNAs with the *C. gigas* genome sequence (https://metazoa.ensembl.org/Crassostrea_gigas/Info/Index) identified *Cragi-CCAPR1* (CGI_10019872), *Cragi-CCAPR2* (CGI_10025592) and *Cragi-CCAPR3* (CGI_10002705) genes, and revealed the existence of five introns at positions that are conserved in vertebrate NPSR and ecdysozoa CCAPR genes (Fig. 1). The phylogenetic analysis showed that deuterostome NPSR and NGR receptors cluster apart from protostome CCAPRs, which also clearly split into ecdysozoan and lophotrochozoan receptor subgroups (Fig. 2).

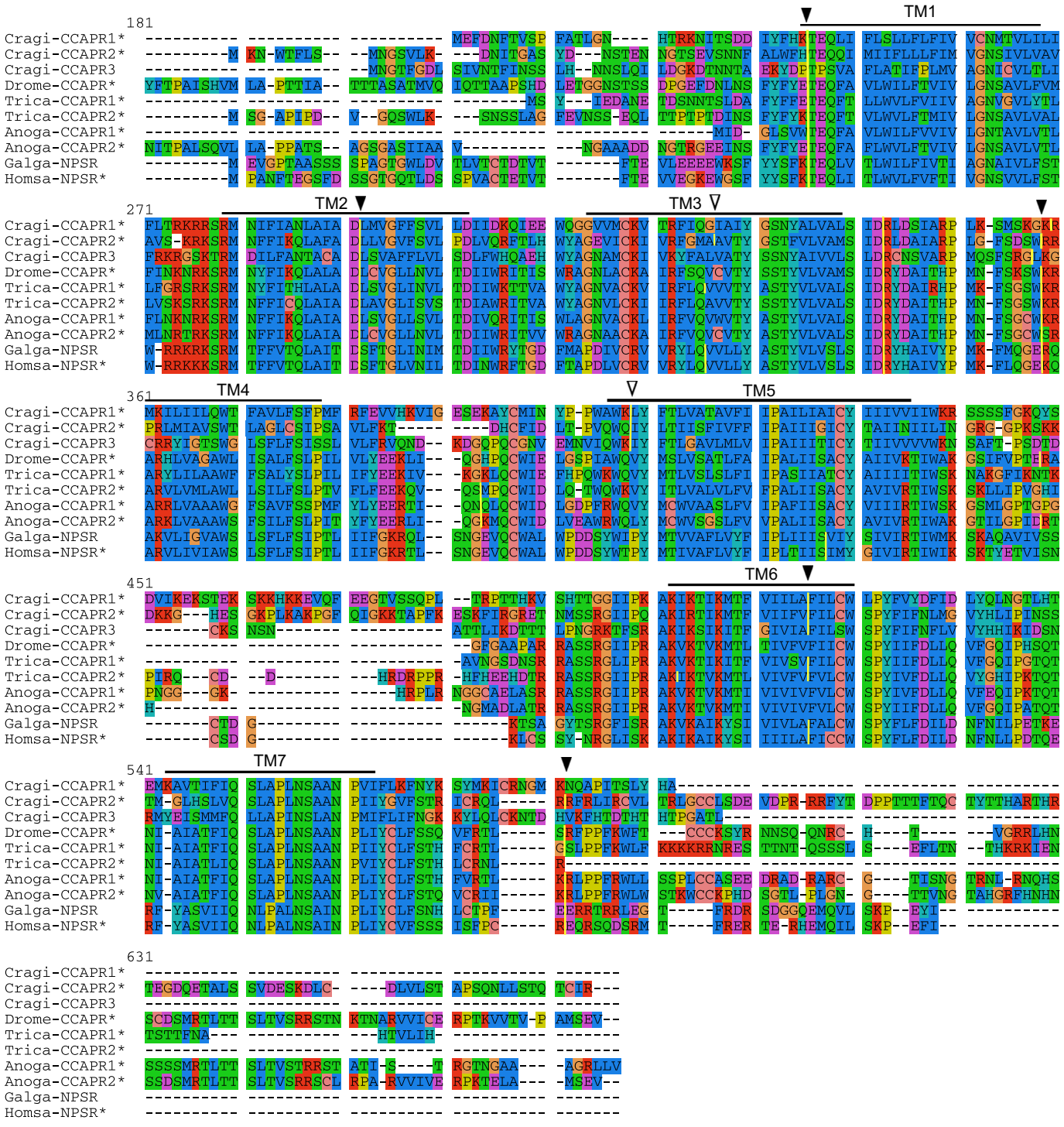

Fig. 1. Sequence alignment of the Cragi-CCAPRs and CCAPR family members. The amino acid sequence of *Crassostrea gigas* CCAPRs (Cragi-CCAPR1*_XP_011439299.1, Cragi-CCAPR2*_XP_011417450.2 and Cragi-CCAPR3_XP_034302802.1) was aligned with the sequences of *Anopheles gambiae* (Anoga-CCAPR1*_XP_321101.4 and Anoga-CCAPR2*_XP_321100.4), *Drosophila melanogaster* (Dro-CCAPR*_NP_996297.3), *Gallus gallus* (Galga-NPSR1*_A0A1D5PKZ0), *Homo sapiens* (Homsa-NPSR*_NP_997056.1) and *Tribolium castaneum* (Trica-CCAPR1*_ABN79651.1 and Trica-CCAPR2*_ABN79652.1) using CLUSTAL OMEGA. Asterisks indicate functionally characterized receptors. Solid black arrowheads indicate conserved introns and empty black arrowheads indicate non-conserved introns. TM1–TM7 correspond to the 7 transmembrane domains.

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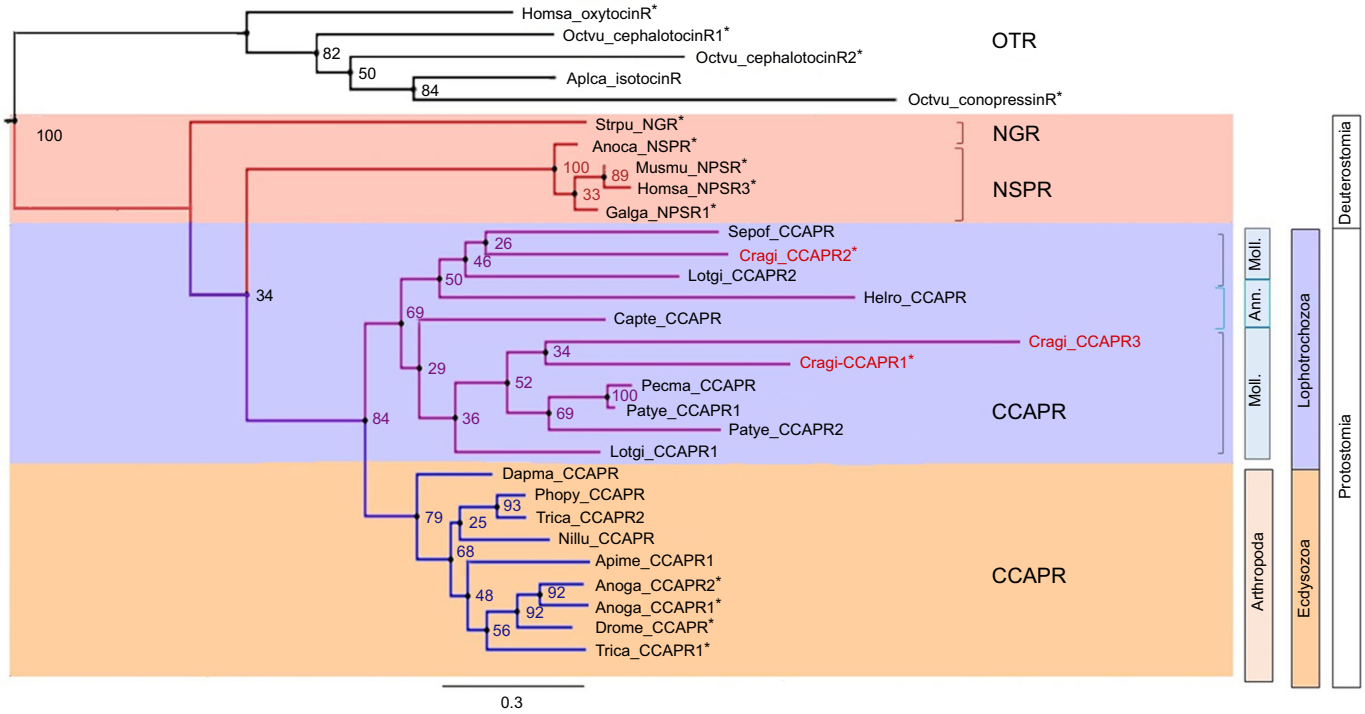

Fig. 2. Phylogenetic representation of the relationship between Cragi-CCAPRs and other CCAPR family members. Phylogenetic and molecular evolutionary analyses were conducted using Seaview based on the maximum likelihood method. The accession numbers and the sequences used to construct the tree are: *Crassostrea gigas*: Cragi-CCAPR1*_XP_011439299.1, Cragi-CCAPR2*_XP_011417450.2 and Cragi-CCAPR3_XP_034302802.1 (labelled in red); *Anolis carolinensis*: Anoca_NPSR*_UPI0002039B69; *Anopheles gambiae*: Anoga_CCAPR1*_XP_321101.4 and Anoga-CCAPR2*_XP_321100.4; *Apis mellifera*: Apime_CCAPR1_XP_001122652.2; *Aplysia californica*: Aplca_isotocinR_XP_012944426.1; *Capitela teleta*: Capte_CCAPR_ELU12393; *Daphnia magna*: Dapma-CCAPR_XP_032792060.1; *Drosophila melanogaster*: Drome-CCAPR*_NP_996297.3; *Gallus gallus*: Galga-NPSR1*_XP_025003312.1; *Helobdella robusta*: Helro_CCAPR_XP_009009991.1; *Homo sapiens*: Homsa_NSPR3*_Q6W5P4 and Homsa_oxytocinR_AAQ91333.1; *Lottia gigantea*: Lotgi_CCAPR1_XP_009052107.1 and Lotgi_CCAPR2_XP_009061331.1; *Mus musculus*: Musmu_NPSR*_NP_783609.1; *Nilaparvata lugens*: Nillu_CCAPR_XP_022200981.1; *Octopus vulgaris*: Octvu-CephalotocinR1*_XP_029640975.1, Octvu_CephalotocinR2* AB112347o and Octvu-OctopressinR* AB116233; *Patinopecten yessoenssis*: Patye_CCAPR1_XP_021367846.1 and Patye_CCAPR2_XP_021354155.1; *Pecten maximus*: Pecma_CCAPR_XP_033728648.1; *Photinus pyralis*: Phopy_CCAPR-XP_031344524.1; *Sepia officinalis*: Sepof_CCAPR (Endress et al., 2018); *Strongylocentrotus purpuratus*: Strpu_NGR* AJY59060.1; and *Tribolium castaneum*: Trica_CCAPR1*_ABN79651.1 and Trica_CCAPR2*_ABN79652.1. Asterisks indicate functionally characterized receptors. Branch node labels correspond to likelihood ratio test values. Moll., Mollusca; Ann., Annelida.

Conserved sequence patterns of Cragi-CCAPs with protostome and deuterostome orthologs

The *C. gigas* CCAP precursor (Cragi-CCAP) generating two CCAP mature peptides (Cragi-CCAP1 and Cragi-CCAP2) was previously characterized (Stewart et al., 2014). Cragi-CCAP1/2 display a clear homology with the CCAP peptides from other protostome species, in particular the two cysteine residues forming a disulfide bridge. However, each oyster peptide exhibits a specific spacing of the cysteines (Fig. 3). Cragi-CCAPs also align few amino acid residues with NPS, the vertebrate orthologous peptides. Cragi-CCAP1 also shares the characteristic NG motif of deuterostome NG peptides and NPS (Semmens et al., 2015). *Crassostrea gigas* oxytocin, Cragi-OT1 and Cragi-OT2 exhibit no sequence homology except for the two cysteine residues involved in the cyclic structure of the peptides.

Oyster CCAPs specifically activate distinct CCAPRs

A calcium mobilization assay was used to identify the cognate ligands of the three Cragi-CCAPRs. Transiently transfected HEK293T cells expressing Cragi-CCAPR1, Cragi-CCAPR2 and Cragi-CCAPR3 with or without the promiscuous Gα16 protein were challenged with high concentrations (10^{-5} mol l^{-1}) of synthetic Cragi-CCAP1, Cragi-CCAP2, Cragi-OT1 and Cragi-OT2. No signal was obtained with cells transfected with an empty vector or with a Gα16 expressing vector. Cragi-CCAPR1 showed a maximal activation by Cragi-CCAP1 and only 20% to 5% of the fluorescence signal was generated by the other peptides. Cragi-CCAPR2 was only activated by Cragi-CCAP2. All the peptides tested produced approximately 50% of the maximal fluorescence signal with Cragi-CCAPR3. Similar results were obtained in the presence or the absence of the promiscuous Gα16 protein. Thus, a dose-dependent activation of the oyster receptors was recorded by omitting the Gα16 protein (Fig. 4). Only Cragi-CCAP1 significantly activated Cragi-CCAPR1, with an EC_{50} of 1.84×10^{-8} mol l^{-1}, and Cragi-CCAP2 activated Cragi-CCAPR2 with an EC_{50} of 1.812×10^{-8} mol l^{-1}. Cragi-CCAPR3 was partially activated only with a pharmacological (10^{-5} mol l^{-1}) concentration of all the peptides tested. A possible transduction via Gαs was investigated using a cAMP luminescence assay, but none of the synthetic peptides activated the cAMP signaling pathway even at concentrations as high as 10^{-5} mol l^{-1}.

Gene expression of Cragi-CCAP signaling components

The expression of *Cragi-CCAPR1*, *Cragi-CCAPR2*, *Cragi-CCAPR3* and *Cragi-CCAP* genes was investigated by RT-qPCR in several adult tissues and following hyposaline stress. The genes encoding the oyster CCAP receptors were expressed at low levels in all adult

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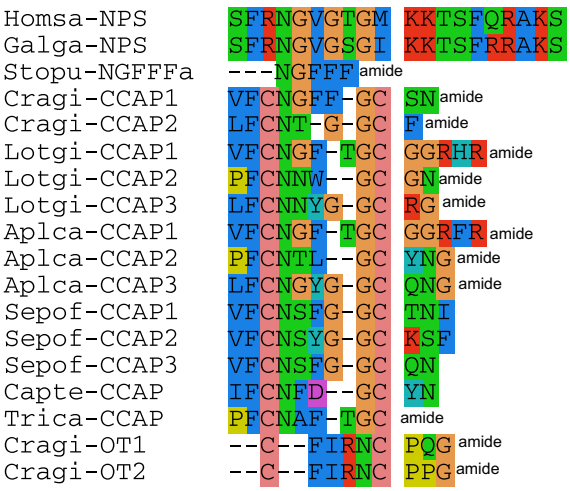

Fig. 3. Sequence alignment of the Cragi-CCAPs and CCAP family members. The amino acid sequences of *Crassostrea gigas* Cragi-CCAP1 and Cragi-CCAP2 (CU993625, AM869229) were aligned with the CCAP sequences from mollusks [*Aplysia californica*: Aplca-CCAP (GD209730); *Lottia gigantea*: Lotgi-CCAP (XP_009058491.1); *Sepia officinalis*: Sepof-CCAP (Endress et al., 2018)], annelids [*Capitella teleta*: Capte-CCAP (ELT88405.1)] and arthropods [*Tribolium castaneum*: Trica-CCAP (XP_008201233.2)]; the NPS sequences from *Gallus gallus* (Galga-NPS XP_025007694) and *Homo sapiens* [Homsa-NPS (NP_001025184.1)]; and the NGFFFamide sequence of *Strongylocentrotus purpuratus* [Strpu-NGFFFa (XP_030841418.1)]. The *C. gigas* sequences for oxytocin [Cragi-OT1 (AM853403) and Cragi-OT2 (AM854257)] were included.

tissues. *Cragi-CCAPR1* and *Cragi-CCAPR2* showed a significantly higher expression in the gills, whereas *Cragi-CCAPR3* was expressed at significantly higher levels in the VG. Cragi-CCAP transcripts were enriched in the VG, the labial palps and the gills (Fig. 5A). To investigate a potential role of the CCAP signaling system in reproduction, the expression of the receptor and the precursor transcripts was investigated in the gonad and the VG over a reproductive cycle. *Cragi-CCAPR3* excepted, *Cragi-CCAPR1*, *Cragi-CCAPR2* and *Cragi-CCAP* transcripts were expressed at significantly higher levels in stages 0 and 1 than in other stages in both female and male gonads (Fig. 5B). In the VG, no significant difference of expression was observed for *Cragi-CCAPR1* or *Cragi-CCAPR2*, though *Cragi-CCAPR3* showed significantly higher levels of transcripts in female stage 3 and also in the undifferentiated stage 0. In addition, the *Cragi-CCAP* precursor gene was expressed at significantly higher levels in male and female stage 3 (Fig. 5C).

Given the expression of *Cragi-CCAPR1/2* and *Cragi-CCAP* precursor genes in the gills, their expression was assessed in oysters transferred from seawater to brackish water in both the gills and the VG. *Cragi-CCAPR1*, *Cragi-CCAPR2* and *Cragi-CCAP* gene expression decreased following a transfer to brackish water in the gills (Fig. 6A), but not significantly in the VG (Fig. 6B). In contrast, *Cragi-CCAPR3* showed a trend for increase in the gills and a significant increase in the VG (Fig. 6A,B).

DISCUSSION

The present study reveals for the first time the existence of a functional CCAPR/CCAP signaling system in a lophotrochozoan species – the Pacific oyster *C. gigas*. Three GPCRs (Cragi-CCAPR1, Cragi-CCAPR2, Cragi-CCAPR3) displaying sequence homology with arthropod CCAPRs were characterized. Because of the ancestral common origin of the oxytocin/vasopressin (OT/VP) family of receptors with CCAPR/NPSR/NGR (Semmens et al., 2015) and given the sequence homology of Cragi-CCAPRs with OT receptors from other species, a phylogenetic analysis clearly demonstrated that they belonged to the CCAPR/NPSR/NGR group. Further strengthening this evolutionary link, oyster genes encoding Cragi-CCAPRs also shared orthologous introns with the other members of the CCAPR/NPSR-encoding gene family (Mirabeau and Joly, 2013; Valsalan and Manoj, 2014). Only Cragi-CCAPR1 and Cragi-CCAPR2 were selectively activated by Cragi-CCAP1 and Cragi-CCAP2, respectively, at nanomolar concentrations similar to those observed in insect species (Belmont et al., 2006; Lee et al., 2013b; Li et al., 2011). Such physiological concentrations are consistent with a role of mature CCAPs as circulating neurohormones in oyster. Yet, a neuromodulator role or, as suggested by the occurrence of Cragi-CCAP transcripts in a variety of tissues, a local paracrine effector role cannot be excluded. The two paralogous receptors Cragi-CCAPR1 and Cragi-CCAPR2 displayed distinct ligand selectivity; this was somewhat surprising because the two oyster CCAP peptides are encoded by a unique precursor and have similar primary sequences. Therefore, a certain degree of cross-reactivity of the two peptides with the two receptors was reasonably expected. Cragi-CCAP1 and Cragi-CCAP2 differ in their respective CX_5C and CX_4C spacing patterns implied in the formation of the conserved disulfide bond common to all mature CCAPs. Interestingly, species such as *Lottia gigantea* or *Patinopecten yessoenssis* harbor a CCAP precursor also comprising the two peptide types (Veenstra, 2010; Zhang et al., 2018) and also have two CCAP-type receptors. In contrast, *S. officinalis* harbors a precursor

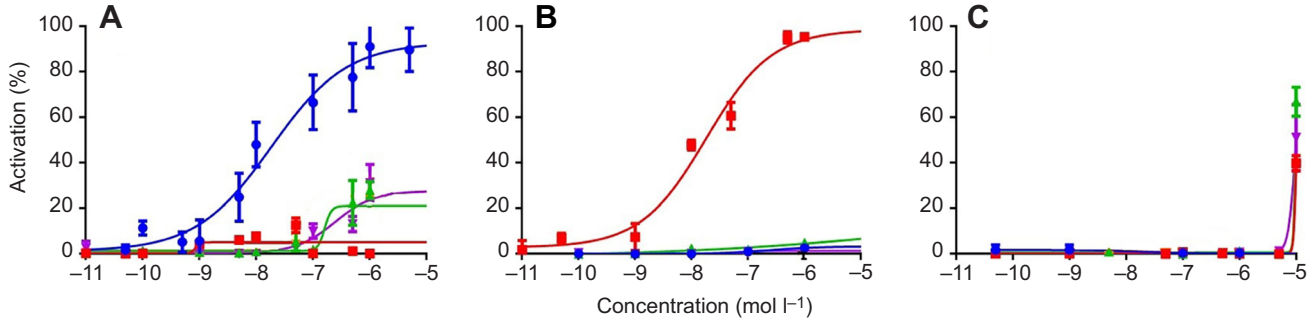

Fig. 4. Pharmacological characterization of Cragi-CCAPRs. Dose-dependent effects of Cragi-CCAP1 (blue circles), Cragi-CCAP2 (red squares), *C. gigas* oxytocins Cragi-OT1 (green triangles), Cragi-OT2 (purple triangles) on the activation of Cragi-CCAPR1 (A), Cragi-CCAPR2 (B) and Cragi-CCAPR3 (C) expressed in HEK293T cells. Data are shown as relative (%) to the highest value (100% activation) for a given peptide and represent the mean of an experiment (n=3) performed in triplicate. Vertical bars represent ±s.e.m.

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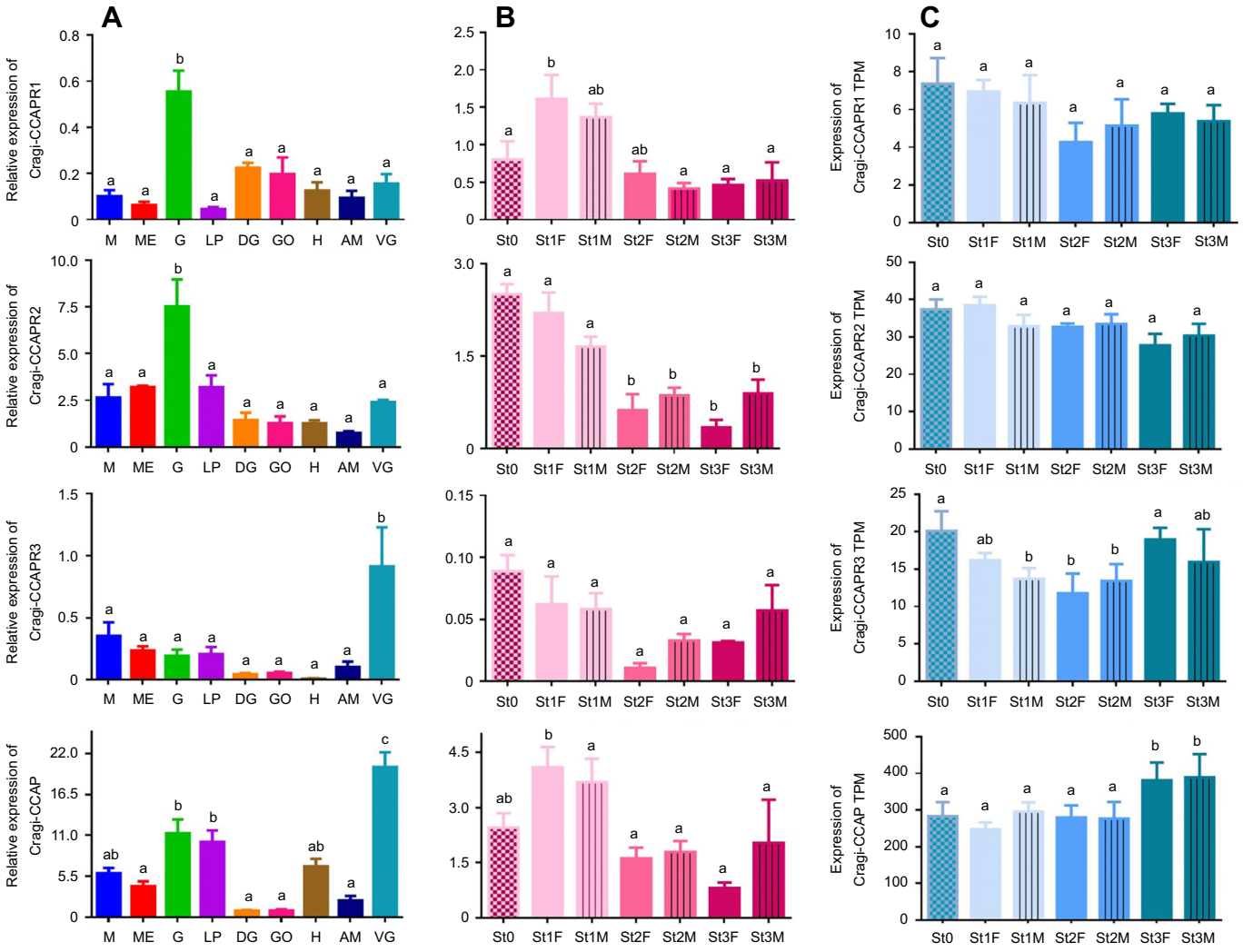

Fig. 5. Expression of mRNA encoding CCAP precursor and receptors in adult tissues. (A) Distribution of mRNAs encoding Cragi-CCAPR1, Cragi-CCAPR2, Cragi-CCAPR3 and Cragi-CCAP precursor in adult tissues: M, mantle; ME, mantle edge; G, gills; LP, labial palps; DG, digestive gland; Go, gonad (mix of all stages); H, heart; AM, adductor muscle; VG, visceral ganglia. (B) Distribution of mRNAs encoding Cragi-CCAPR1, Cragi-CCAPR2, Cragi-CCAPR3 and Cragi-CCAP precursor in the gonad during gametogenesis: stage 0, sexual resting stage; stage 1, gonial multiplication stage; stage 2, tubule development and maturation stage; and stage 3, sexual maturity stage. Each value is the mean+s.e.m. of five pools of six animals. Expression levels were normalized to Elongation Factor 1 α (EF1α) mRNA. (C) Expression of Cragi-CCAPR1, Cragi-CCAPR2, Cragi-CCAPR3 and Cragi-CCAP precursor encoding gene in the VG during the reproduction cycle expressed in transcripts per million (TPM) (data retrieved from https://www.ncbi.nlm.nih.gov/sra/PRJNA662446). Results were statistically tested with a one-way ANOVA (P<0.05). Vertical bars represent +s.e.m.

generating only one (CX_5C) peptide type and has only one CCAP-type receptor (Endress et al., 2018). A one amino acid residue difference in a short sequence probably entails specific physical constraints on the peptide structure, resulting in distinct binding properties. A possible evolutionary scheme could be that a receptor expressed in an ancestor of mollusks could bind and activate each peptide type with a distinctive affinity. Subsequent receptor gene duplication and coevolution between ligand and receptor would have resulted in the segregation of two independent signaling pathways in species harboring two CCAP-type peptides. In species with only one CCAP-type peptide, the receptor would have been lost owing to lower selective pressure. This hypothesis remains to be investigated experimentally in other mollusk species.

Concerning peptides encoded by distinct genes, the *C. gigas* calcitonin signaling system works in a similar manner: structurally related calcitonins strictly activate their own receptor without cross-reactivity (Schwartz et al., 2019). An analogous scenario was also proposed to explain the emergence of independent signaling systems for the structurally and evolutionarily related neuropeptides adipokinetic hormone (AKH), corazonin and AKH/corazonin-related peptide (ACP) in insects (Hansen et al., 2010).

The subfunctionalization of Cragi-CCAP1 and Cragi-CCAP2 with respect to receptor activation was seemingly not associated with specialization for biological functions, as both receptor genes displayed parallel tissue and hyposalinity-induced expression patterns, though with distinct levels. In contrast, Cragi-CCAPR3, a receptor that is phylogenetically more closely related to Cragi-CCAPR1 than to Cragi-CCAPR2, showed a distinctive expression pattern, with higher levels in the VG. This suggests a potential role in the modulation of neuronal circuits, as described for CCAP in the neural feeding circuit of the pond snail *L. stagnalis* (Vehovszky et al., 2005) or the ecdysis motor behavior of the moth *Manduca sexta* (Gammie and Truman, 1997). Cragi-CCAPR3 was only partially activated by micromolar concentrations of the two CCAP

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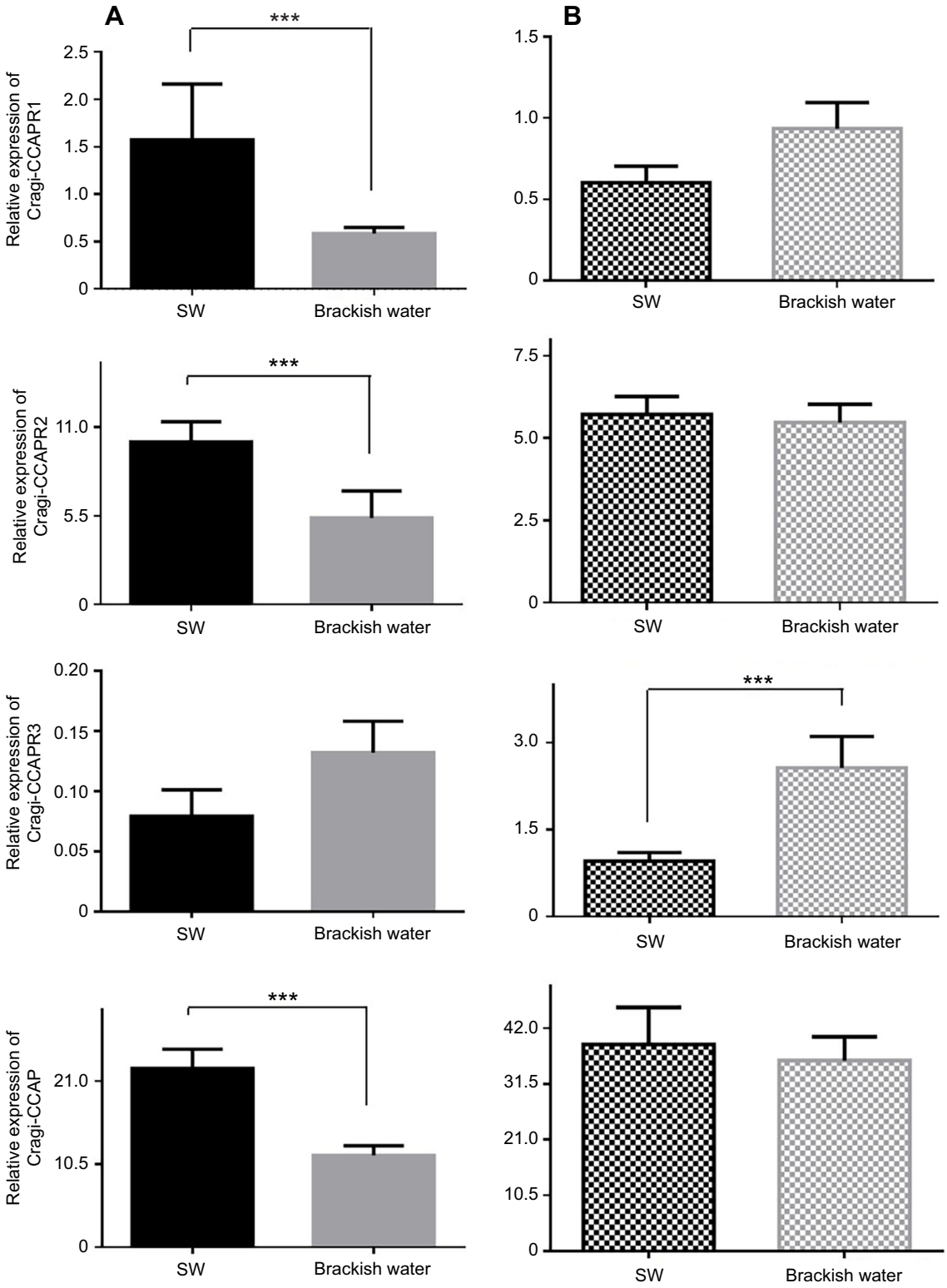

Fig. 6. Expression of mRNA encoding CCAP precursor and receptors in tissues of oysters after a hyposaline stress. Expression (normalized to EF1α mRNA) in the gills (A) and the VG (B). SW: seawater, BW: brackish water: Each value is the mean+s.e.m. of five pools of five animals. Results were statistically tested with a Student's *t*-test. Significantly different means of samples from control and conditioned animals are indicated by *** (P<0.001).

peptides and OT peptides. It is noteworthy that the third intracellular loop is shorter in Cragi-CCAPR3 than in Cragi-CCAPR1 and Cragi-CCAPR2. This may have some significance for the efficient coupling of the G-protein to the receptor and could explain the near-total absence of activation of Cragi-CCAPR3 by the peptides tested in our experiment. Although Cragi-CCAPR3 may also bind CCAP-unrelated ligands, these findings somehow question the actual functionality of the receptor alone. We cannot rule out that Cragi-

CCAPR3 serves as an agonist-independent co-receptor working via oligomerization with the other Cragi-CCAPRs (Rozenfeld and Devi, 2010).

The possible involvement of the CCAP signaling system in the regulation of physiological processes in *C. gigas* was mainly inferred from the tissue and hyposalinity-induced expression patterns of the genes encoding Cragi-CCAPRs and the Cragi-CCAP precursor. First, their expression levels in most adult tissues undoubtedly reflect the multifunctional activity of CCAP in oysters, as already mentioned for this family of neuropeptides in other species (Lee et al., 2013a; Möller et al., 2010; Tinoco et al., 2018). Intriguingly, Cragi-CCAPR1 and 2 were most highly expressed in the gills. In oysters, the gills create a steady current of water via the activity of their ciliated cells, which collect and sort food particles and serve for gamete dispersal during spawning (Galtsoff, 1964). They also play a major part in respiration and are implicated in acclimation to salinity (Meng et al., 2013). Interestingly, some of the physiological activities of oyster gills, especially feeding and reproduction, are regulated by the CCAP/NPS/NG family of peptides in other species (Tinoco et al., 2018). Indeed, CCAP signaling is involved in the regulation of food intake in mice (Peng et al., 2010), the starfish *Asterias rubens* (Tinoco et al., 2018), the pond snail *L. stagnalis* (Vehovszky et al., 2005) and the fruit fly *D. melanogaster* (Williams et al., 2020), and in the regulation of gamete release in the oyster *Saccostrea glomerata* (In et al., 2016), the sea cucumber *Apostichopus japonicus* (Kato et al., 2009), the cuttlefish *S. officinalis* (Endress et al., 2018) and the locust *L. migratoria* (Donini et al., 2001). CCAP signaling is also implicated in osmoregulation in the shrimp *L. vannamei* (Chen et al., 2016). Only this latter activity was investigated in the present study. As sessile organisms living in estuaries, oysters are particularly tolerant to fluctuations in salinity (Galtsoff, 1964; Guo et al., 2015). In *C. gigas* gills, hypoosmotic stress induces differential expression of a large number of transcripts related to immune responses, cell adhesion and communication, ion channels and signal transduction (Zhao et al., 2012). These cellular responses are probably mediated by extracellular signals, e.g. hormones and neuromodulators involved in the coordination of the activity of various tissues in the organism to contribute to a certain degree of homeostasis. The presence of CCAP signaling components in the gills clearly points to a role in the regulation of oyster gill functions. A hyposalinity stress indeed elicited a decrease in the expression of genes encoding Cragi-CCAP signaling components in the gills, with the exception of *Cragi-CCAPR3*. However, a decrease in CCAP signaling related gene expression was not observed in the VG. The decrease in expression of genes encoding CCAP signaling components in the gills may contribute to the decreased expression of voltage-gated Na^+/K^+ channel and aquaporin genes (Meng et al., 2013) or the downregulation of numerous transcripts (Ertl et al., 2019) observed in this organ following a hypo-osmotic challenge. Besides CCAP signaling, the oyster calcitonin signaling system is also involved in acclimation to salinity, but not in the context of an acute hyposaline stress (Schwartz et al., 2019). Furthermore, oysters subjected to hyposalinity treatments stop feeding (Heilmayer et al., 2008) and the ciliary activity of their gill tissues decreases (Paparo and Dean, 1984; Van Winkle, 1972). Consequently, in addition to a possible role in the regulation of the ion and water balance, the CCAP signaling system could also regulate gill cilia activity, resulting in changes in the water pumping rate and thus potentially modulating food intake and gas exchange. As gill cilia are also implicated in the spawning process, this may explain why CCAP triggers spawning in *S. glomerata* (In et al., 2016). The high levels of Cragi-CCAP precursor transcripts observed in the VG of sexually mature oysters is also consistent with this activity. It would be interesting to investigate whether CCAP modulates the ciliary activity of the gonoducts and the contractive activity of the adductor muscle in females, because they take part in the several consecutive steps leading to egg dispersal in the surrounding water. Regarding the possible involvement of CCAP signaling in the regulation of reproduction processes, it was interesting to observe a significantly higher expression of CCAP signaling components in the first stages of gametogenesis in male and female gonads. As the oyster gonad is a mixed tissue including germ cells, storage tissue and smooth muscle fibers, these first stages are characterized by the restart of gonial multiplication and the storage of reserves. Whether CCAP signaling affects one activity or the other cannot be established. The fine tuning of energy allocation in *C. gigas* gonads may be a player in the success of gametogenesis through neuroendocrine signaling pathways (Bigot et al., 2014; Jouaux et al., 2012). Thus, it is rational to speculate a role of CCAP signaling in the regulation of this biological activity.

Conclusions

We characterized a CCAP signaling system in the oyster *C. gigas*. The wide tissue distribution of the ligand and receptor-encoding transcripts suggests that oyster CCAP signaling regulates a variety of physiological activities. More specifically, we evidenced differential expression of CCAP signaling components over a whole reproductive cycle in the VG and gonads and a hyposalinity-induced decrease of the CCAP signaling components in the gills; this strongly suggests a role in the regulation of reproduction and gill activities, respectively. Although this study gives insights into the potential physiological roles of CCAP-type signaling in *C. gigas*, further *in vitro* or *in vivo* functional investigations are required to confirm these potential regulatory activities.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Data availability

Short read archives (SRA) corresponding to RNA expressed in the VG of males and females at different stages of reproduction can be retrieved from GenBank (accession number PRJNA662446): https://www.ncbi.nlm.nih.gov/sra/PRJNA662446

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