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Julie Schwartz, Emilie Réalis-Doyelle, Lorane Le Franc, Pascal Favrel. A Novel Dop2/Invertebrate-Type Dopamine Signaling System Potentially Mediates Stress, Female Reproduction, and Early Development in the Pacific Oyster (*Crassostrea gigas*). *Marine Biotechnology*, 2021, 10.1007/s10126-021-10052-5. hal-03327466

**HAL Id: hal-03327466**

**<https://hal.science/hal-03327466>**

Submitted on 27 Aug 2021

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# A Novel Dop2/Invertebrate-Type Dopamine Signaling System Potentially Mediates Stress, Female Reproduction, and Early Development in the Pacific Oyster (*Crassostrea gigas*)

Julie Schwartz<sup>1</sup> · Emilie Réalis-Doyelle<sup>1</sup> · Lorane Le Franc<sup>1</sup> · Pascal Favrel<sup>1</sup>

Received: 29 April 2021 / Accepted: 20 July 2021

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## Abstract

The dopaminergic signaling pathway is involved in many physiological functions in vertebrates, but poorly documented in protostome species except arthropods. We functionally characterized a novel dopamine receptor in the Pacific oyster (*Crassostrea gigas*), activated by dopamine and tyramine with different efficacy and potency orders. This receptor — Cragi-DOP2R — belongs to the D<sub>1</sub>-like family of receptors and corresponds to the first representative of the Dop2/invertebrate-type dopamine receptor (Dop2/INDR) group ever identified in Lophotrochozoa. Cragi-DOP2R transcripts were expressed in various adult tissues, with higher expression levels in the visceral ganglia and the gills. Following an experiment under acute osmotic conditions, Cragi-DOP2R transcripts significantly increased in the visceral ganglia and decreased in the gills, suggesting a role of dopamine signaling in the mediation of osmotic stress. Furthermore, a role of the Cragi-DOP2R signaling pathway in female gametogenesis and in early oyster development was strongly suggested by the significantly higher levels of receptor transcripts in mature female gonads and in the early embryonic stages.

**Keywords** Dopamine receptor · Tyramine receptor · Dop2/INDR · Mollusk · Osmotic stress · Reproduction

## Introduction

The catecholamines dopamine, noradrenaline and adrenaline are involved in the regulation of a large panel of functions in vertebrates, such as the stress response, locomotion, motivation, attentional processes, and learning (Liu and Kaeser 2019; Tank and Lee Wong 2015). However, adrenaline and noradrenaline are present only in negligible quantities in protostomes; tyramine and octopamine are admittedly their respective counterparts because of their highly similar chemical structures, signaling pathways, and physiological functions (Roeder 2005, 2020). However, recent studies have extended the understanding of the catecholaminergic signaling pathways by establishing that adrenergic, octopaminergic, and tyramineric signaling pathways coexisted in the last common ancestor of Bilateria because the corresponding

receptors were found in representatives of protostomes and deuterostomes (Bauknecht and Jékely 2017). Both noradrenaline and adrenaline activate the specific receptors  $\alpha_{1,2}$  and  $\beta_{1,2,3}$ -adrenergic receptors that are distributed in a wide variety of organs like the heart, the kidneys, and the eyes. These receptors have protostome counterparts preferentially activated by tyramine and octopamine, i.e., TyrR (TyrR1, TyrR2) and OctR (Oct $\alpha$ R, Oct $\beta$ R), respectively. Regarding dopamine, five dopamine receptors are found in deuterostomes, divided into two families: the D<sub>1</sub>-like family of receptors including D<sub>1</sub>R (D1 dopamine receptor) and D<sub>5</sub>R, and the D<sub>2</sub>-like family of receptors including D<sub>2</sub>R, D<sub>3</sub>R, and D<sub>4</sub>R (Missale et al. 1998). These families are discriminated according to the G protein recruited after activation of the receptor: the G $\alpha_s$  protein for D<sub>1</sub>-like receptors leading to an increased level of intracellular cAMP and the G $\alpha_i$  protein for D<sub>2</sub>-like receptors leading to a decreased level of intracellular cAMP.

In protostomes, four families of dopamine receptors are currently identified in arthropods, named Dop1 (Gotzes et al. 1994; Sugamori et al. 1995; Blenau et al. 1998; Suo et al. 2002; Mustard et al. 2003; Ohta et al. 2009; Meyer et al. 2011, 2012; Xu et al. 2017), Dop2/INDR (for

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invertebrate-type dopamine receptor) (Feng et al. 1996; Ebert et al. 1998; Mitsumasu et al. 2008; Meyer et al. 2011, 2012; Troppmann et al. 2014; Nuss et al. 2015; Wu et al. 2015; Hill et al. 2016; Xu et al. 2017), Dop3 (Hearn et al. 2002; Beggs et al. 2005; Verlinden et al. 2015; Xu et al. 2017), and Dop/Ecd (for ecdysteroid) (Srivastava 2005; Abrieux et al. 2013; Kang et al. 2019). Among these receptors, Dop1, Dop2/INDR, and Dop3 exhibit functional and structural homologies with the dopamine receptors of vertebrates. The receptors of the Dop1 and Dop2/INDR families are indeed closer to the receptors of the D<sub>1</sub>-like family than to those of the D<sub>2</sub>-like family of vertebrate receptors since their activation leads to an increased level of intracellular cAMP (Mustard et al. 2005). In addition, the Dop2/INDR receptors can also couple to a G $\alpha$ q-type protein that triggers an increase of intracellular calcium (Verlinden 2018). However, the Dop2/INDR receptors appear to be phylogenetically closer to the  $\alpha$ -adrenergic-like octopamine receptors of arthropods also called Oct $\alpha$ Rs than to the D<sub>1</sub>-like receptors of vertebrates. Dop3 receptors are phylogenetically closer to D<sub>2</sub>-like receptors, and their activation by agonists leads to a decreased concentration of intracellular cAMP, as the activation of D<sub>2</sub>-like receptors does. However, activation of the Trica-Dop3 receptor in *Tribolium castaneum* led to an increased level of cAMP and intracellular Ca<sup>2+</sup> (Verlinden et al. 2015; Verlinden 2018). Receptors of the Dop/Ecd family are homologs of the  $\gamma$ -type adrenergic receptors of vertebrates and have only been characterized in *Drosophila melanogaster* (Srivastava 2005), *Agrotis ipsilon* (Abrieux et al. 2013), and *Helicoverpa armigera* (Kang et al. 2019). These receptors can be activated by dopamine and steroid hormones such as ecdysone and 20-hydroxyecdysone (Srivastava 2005; Abrieux et al. 2013; Kang et al. 2019).

Receptors phylogenetically close to the D<sub>1</sub>-like receptors of vertebrates have been identified in oyster and are involved in different physiological functions. Thus, in *Crassostrea angulata*, Ca-DAR1 is potentially involved in the proliferation stage of oocytes and displays sequence homologies with the D<sub>1</sub>-like receptors of vertebrates and arthropods (Yang et al. 2013). Furthermore, a homologous receptor of Ca-DAR1 corresponding to the receptor named CgD1DR-1 (Liu et al. 2018) has also been identified in the Pacific oyster (*C. gigas*) (Yang et al. 2013). The activation of CgD1DR-1 by dopamine led to increased intracellular cAMP, confirming it as a D<sub>1</sub>-like receptor. Interestingly, CgD1DR-1 is also involved in shell formation during oyster larval development, from the trochophore stage to the D-shaped larval stage. To our knowledge, no Dop2/INDR receptor had been identified in Lophotrochozoa as yet. The present study investigates a Dop2/INDR-type signaling in the Pacific oyster (*C. gigas*).

## Material and Methods

### Animal and Tissue Sampling

Two-year old adult oysters *C. gigas*, purchased from a local farm (Normandie, France), were used for transcription analyses. Stages of reproduction (stage (0), resting undifferentiated stage, stage (1), gonial multiplication stage; stage (2), maturation stage; stage (3), sexual maturity) were determined by histological analysis of gonad sections as described previously (Rodet et al. 2005). Adult tissues (mantle, mantle edges, gills, labial palps, digestive gland, gonad (mix of male and female gonads at all stages), the heart, and adductor muscle were sampled, and the visceral ganglia (VG) were carefully dissected out, thus limiting any contamination from the adjacent adductor muscles. All the samples were either placed in TriReagent (Sigma) or stored at  $-80^{\circ}\text{C}$  until use. To study the influence of osmotic conditions, oysters were transferred from seawater (33‰) to brackish water (8‰) at  $17^{\circ}\text{C}$ . 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 ( $0.5\text{ L}\cdot\text{h}^{-1}$ ) of distilled water over a period of 3 days. Tissues (gills and visceral ganglia) were sampled after 12 h of incubation of the oysters in brackish water at the final salinity. To prevent the closure of the shell, a wedge was inserted between the valves.

### Catecholamines

L-dopa, dopamine, noradrenaline, adrenaline, tyramine, and octopamine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### In Silico Analyses

Multiple sequence alignment was performed with dopamine, adrenergic, tyramine, and octopamine receptors from various species (Supplementary Table 1) using MUSCLE. The alignment was manually trimmed that spans from the first to the seventh transmembrane domains. To determine the relationship between Cragi-DOP2R and receptors from other species, phylogenetic and molecular evolutionary analyses were conducted using MEGA X (Kumar et al. 2018) based on the maximum likelihood method. The reliability of the inferred trees was estimated by applying the bootstrap procedure with 1000 replications. Receptor gene

organization was obtained by screening the new Refseq *C. gigas* genome assembly (BioProject: PRJEB35351) ([https://www.ncbi.nlm.nih.gov/assembly/GCF\\_902806645.1](https://www.ncbi.nlm.nih.gov/assembly/GCF_902806645.1)) (Peñaloza et al. 2021).

## Pharmacological Characterization of a *Crassostrea gigas* Dopamine Receptor

**Molecular Cloning of the Cragi-DOP2R and Transfection of Mammalian Cells** BLAST analysis of *C. gigas* transcriptomic database “GigaTON” using dopamine receptors of *Drosophila melanogaster* as query resulted in the identification of one full length cDNAs encoding Cragi-DOP2R (CHOYP\_DOPR2.1.1). The CDS of the Cragi-DOP2R gene was amplified by PCR (GoTaq® DNA polymerase, Promega) using gene-specific sense primer (5'-CACCATGTCGTATTCAGACACCG-3') harboring a Kozak consensus sequence and anti-sense primer (5'-TCAGCTTCGTTCGGCTTGAGC-3'). Ten nanograms of plasmid DNA (Pal 17.3 vector, Evrogen) from a *C. gigas* “all developmental stages and adult central nervous system” directional and normalized cDNA library was used as template. The resulting PCR products were directionally cloned into the pTarget expression vector (Invitrogen). The correct insertion of the PCR products was confirmed by sequencing. Human embryonic kidney (HEK293T) cells were transiently transfected with the Cragi-DOP2R/pTarget construct 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  $\text{G}\alpha_{16}$  subunit, a promiscuous G protein that can direct intracellular signaling of GPCRs to the release of calcium via the phospholipase C $\beta$  pathway, regardless of the endogenous G protein coupling of the receptor. To assess receptor activity independent of  $\text{G}\alpha_{16}$ , calcium responses were measured in cells expressing only the Cragi-DOP2R. Cells for negative control experiments were transfected with empty pTarget and  $\text{G}\alpha_{16}$ /pTarget constructs.

**Calcium Fluorescence Assay** Activation of the Cragi-DOP2R by candidate catecholamines ligands was monitored using a fluorescence-based calcium mobilization assay. Briefly, transfected HEK293T cells were loaded with Fluo-4 Direct (Invitrogen) plus probenecid (2.5 mM final concentration) (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 analyzed using SoftMax Pro (Molecular Devices). Candidate ligands were first tested at a final concentration of  $10^{-3}$  M. Concentration–response measurements of activating ligands were conducted in quadruplicate and for at least three independent experiments. Half maximal effective concentrations (EC50 values) were calculated from

concentration–response curves that were constructed using nonlinear regression analysis with a sigmoidal dose–response equation using Prism 5.0 (GraphPad Software, USA).

**cAMP Luminescence Assay** Cragi-DOP2R 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 of the candidate compounds using a FlexStation 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  $10^{-3}$  M.

## Expression Analyses

**Reverse Transcription and Quantitative PCR** RT-qPCR analysis was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad). 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  $\mu\text{g}$  of total RNA was reverse transcribed using 1  $\mu\text{g}$  of random hexanucleotidic primers (Promega), 0.5 mM 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/15 s, 60 °C/15 s) with gene-specific primers Qs-Cragi-DOP2R (5'-GGCAATCACGGACCCCAT-3') and Qas-Cragi-DOP2R (5'-AGCAGCCACACTAACGCA-3'). Using Qs-Cg-EF (5'-ACCACCCTGGTGAGATCAAG-3') and Qa-Cg-EF (5'-ACGACGATCGCATTCTCTT-3'), a parallel amplification of *C. gigas* elongation factor 1  $\alpha$  (EF1  $\alpha$ ) transcript (BAD15289) was carried out to normalize the expression data of the studied transcripts. EF1  $\alpha$  was found as a reliable normalization gene as no significant difference ( $p < 0.05$ ) of Ct values was observed between the different samples compared. Thus, the relative level of target gene expression was calculated by using the following formula:  $N = 2^{(\text{Ct}_{\text{Cg-EF1}\alpha} - \text{Ct}_{\text{target cDNA}})}$  (Badariotti et al. 2006). 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.

**Transcript Expression Analysis During Development** The transcriptome data of the different development stages are available in the GigaTON database (Riviere et al. 2015;

Zhang et al. 2012). Expression data were expressed in TPM (transcripts per kilobase per million reads) (Li et al. 2009) to provide a normalized comparison of Cragi-DOP2R expression between all samples.

## Statistical Analyses

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 differentially conditioned animals were compared using an unpaired Student's *t* test. Significance was set at  $p < 0.05$ .

## Results

### Characterization of Cragi-DOP2R

A cDNA sequence (CHOYP\_DOPR2.1.1) named Cragi-DOP2R, displaying sequence similarity with the arthropod dopaminergic and vertebrate adrenergic receptors, was retrieved from GigaTON, an oyster comprehensive transcriptomic database (Riviere et al. 2015). To investigate the relationship between Cragi-DOP2R and dopamine receptor family members, a tree was constructed with the receptor sequences of both vertebrates and protostomes including: D<sub>1</sub>-like family receptors, D<sub>2</sub>-like family receptors, Dop/Ecd, Oct $\alpha$ R, Oct $\beta$ R, and TyrR (Fig. 1). The tree shows that the Cragi-DOP2R receptor appears to be a dopaminergic receptor belonging to the Dop2/INDR family and appears closer to receptors of the Oct $\alpha$ R family than to the Dop1/D<sub>1</sub>-like family of receptors. Cragi-DOP2R also displays the domain architecture typical of the Dop2/INDR receptor subgroup with a rather long third cytoplasmic loop comprising 117 amino acids and a carboxy-terminal domain of 48 amino acid residues (Supplementary Fig. 1). In contrast to the Cragi-Dop3 gene situated on chromosome 7, Cragi-DOP2R, CgD1DR-1, Tyr1, and CgGPR1 genes are all located on chromosome 4, and a single exon harbors their coding DNA sequence (CDS) (Supplementary Fig. 2).

### Functional Characterization of Cragi-DOP2R

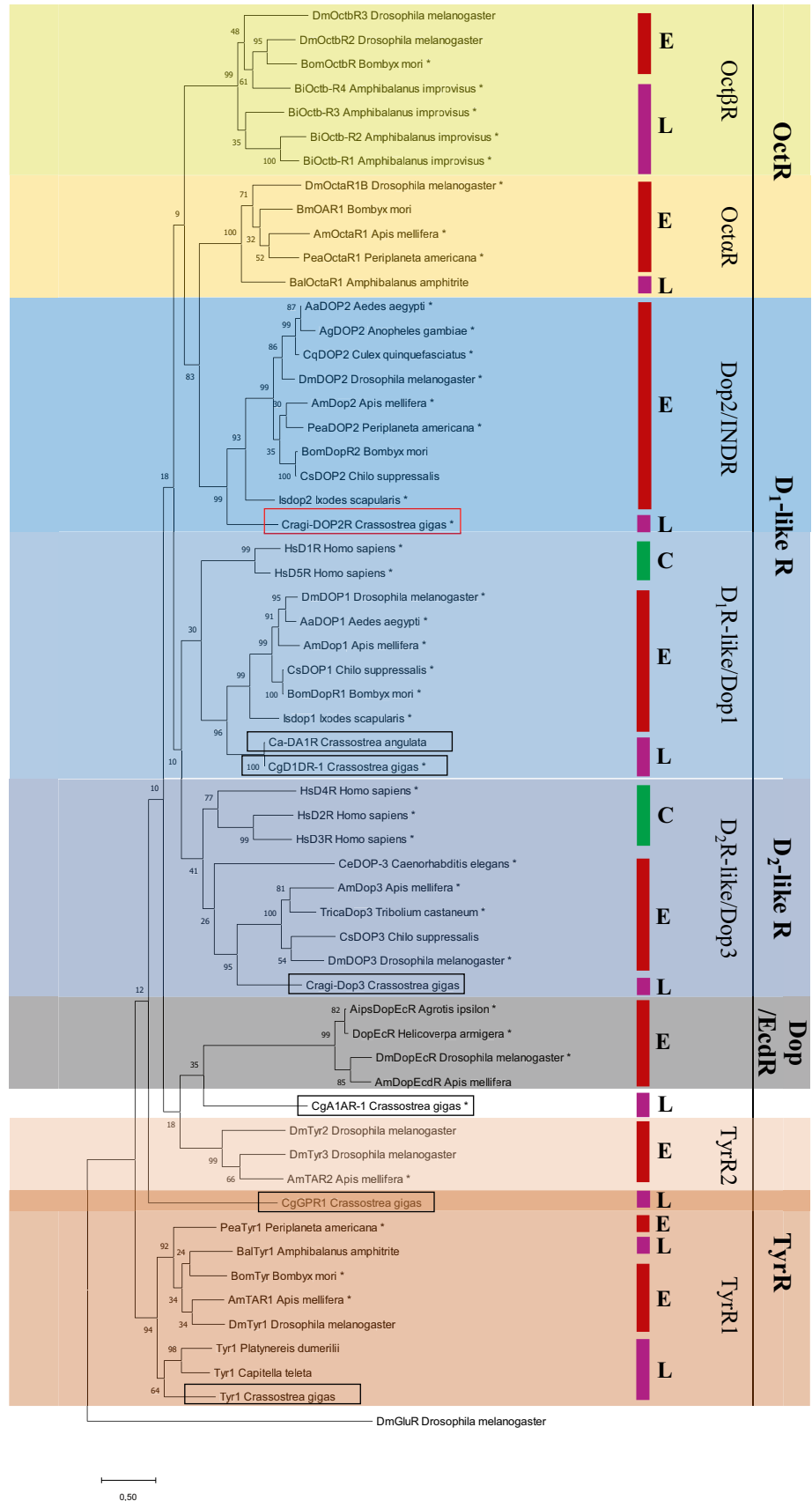
As a first step, transiently transfected HEK293T cells expressing Cragi-DOP2R with or without the promiscuous G $\alpha_{16}$  protein were challenged with rather high concentrations ( $10^{-3}$  M) of several catecholamines: L-dopa, dopamine, tyramine, octopamine, noradrenaline, and adrenaline (Supplementary Fig. 3). While L-DOPA and octopamine did not affect the cytosolic calcium concentration, dopamine, tyramine, noradrenaline, and adrenaline led to an increase of Ca<sup>2+</sup> suggesting that these catecholamines

activate Cragi-DOP2R. However, noradrenaline and adrenaline also led to an increase of intracellular Ca<sup>2+</sup> in the absence of Cragi-DOP2R expression suggesting that noradrenaline and adrenaline may activate endogenous receptors of HEK293T cells. Moreover, it should be noted that intensity signal induced by the administration of noradrenaline and adrenaline was identical in absence or presence of Cragi-DOP2R expression. Activation of Cragi-DOP2R with dopamine and tyramine appeared to be as effective both in the absence and in the presence of G $\alpha_{16}$  (Supplementary Fig. 4) and caused an increase in intracellular Ca<sup>2+</sup> involving the recruitment of a G $\alpha_q$  protein (Fig. 2). Dopamine was more efficient than tyramine to activate Cragi-DOP2R as the maximum intensity signal was higher with dopamine as compared to tyramine. Half maximal effective concentrations obtained by receptor activation is  $6.51 \pm 3.13 \cdot 10^{-9}$  mol l<sup>-1</sup> for dopamine and  $1460 \pm 970 \cdot 10^{-9}$  mol l<sup>-1</sup> for tyramine. Because the activation of Dop2/INDR family receptors can lead to an increase of intracellular cAMP, a possible transduction via G $\alpha_s$  was investigated using a cAMP luminescence assay. None of the catecholamines activated the cAMP signaling pathway even at concentrations as high as  $10^{-3}$  M.

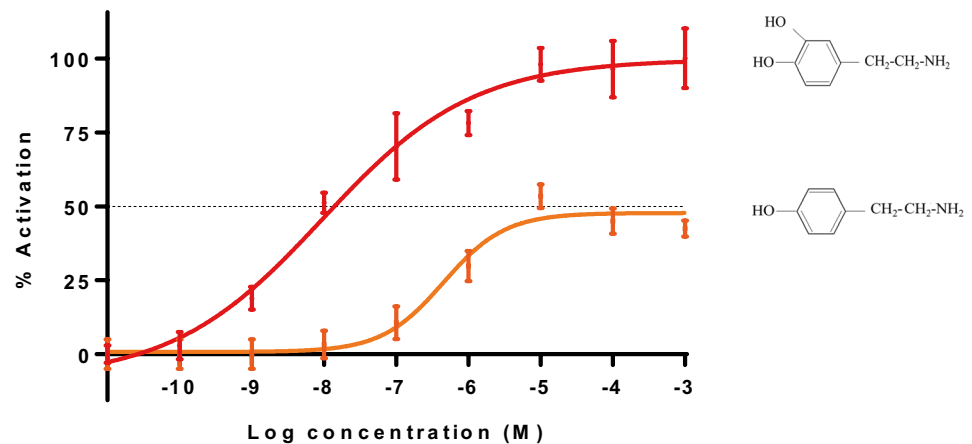
### Gene Expression of Cragi-DOP2R

The expression of Cragi-DOP2R gene was investigated by RT-qPCR in several adult tissues. Cragi-DOP2R gene is ubiquitously expressed in mantle, mantle edges, labial palps, digestive gland, gonad, heart, adductor muscle, visceral ganglia, and at significantly higher levels in the gills (Fig. 3). To investigate the possible involvement of Cragi-DOP2R in osmoregulation, we assessed the expressions of the gene encoding Cragi-DOP2R in gills and visceral ganglia of oysters transferred from seawater to brackish water following either an acute (AC) or mild (MC) osmotic conditioning protocol (see “Materials and Methods” section). A significant decrease of Cragi-DOP2R gene expression was found in the gills of oysters after the AC stress. In contrast, Cragi-DOP2R transcription levels were significantly increased in visceral ganglia after the AC stress. No significant difference was observed following MC conditioning in any of the tissues tested (Fig. 4). We investigated the expression of Cragi-DOP2R gene in female and male gonads over a reproductive cycle. Significantly higher levels of transcriptional expression were observed in female gonads at stage 3 (Fig. 5). Using the GigaTON database, the expression of Cragi-DOP2R was investigated during the development. Cragi-DOP2R gene was mainly expressed in oocytes and in the early developmental stages (two cells, four cells, and morula stages) (Fig. 6).

**Fig. 1** Phylogenetic representation of the relationship between Cragi-DOP2R and other catecholamine receptors. Phylogenetic and molecular evolutionary analyses were conducted using MEGA X based on the maximum likelihood method. The accession numbers of the sequences used to construct the tree are listed in Supplementary Table 1. Glutamate receptor of *Drosophila melanogaster* was chosen as outgroup. \*indicates functionally characterized receptors. *Crassostrea* receptors are framed in black, and Cragi-DOP2R is framed in red. C, Chordata; L, Lophotrochozoa; E, Ecdysozoa



**Fig. 2** Dose-dependent calcium responses induced by dopamine (red) and tyramine (orange) in HEK293T cells expressing Cragi-DOP2R. Data are shown as relative (%) to the highest value (100% activation) and represent the mean of an experiment ( $n=3$ ) performed in quadruplicate. Vertical bars represent the standard error of the mean (SEM)

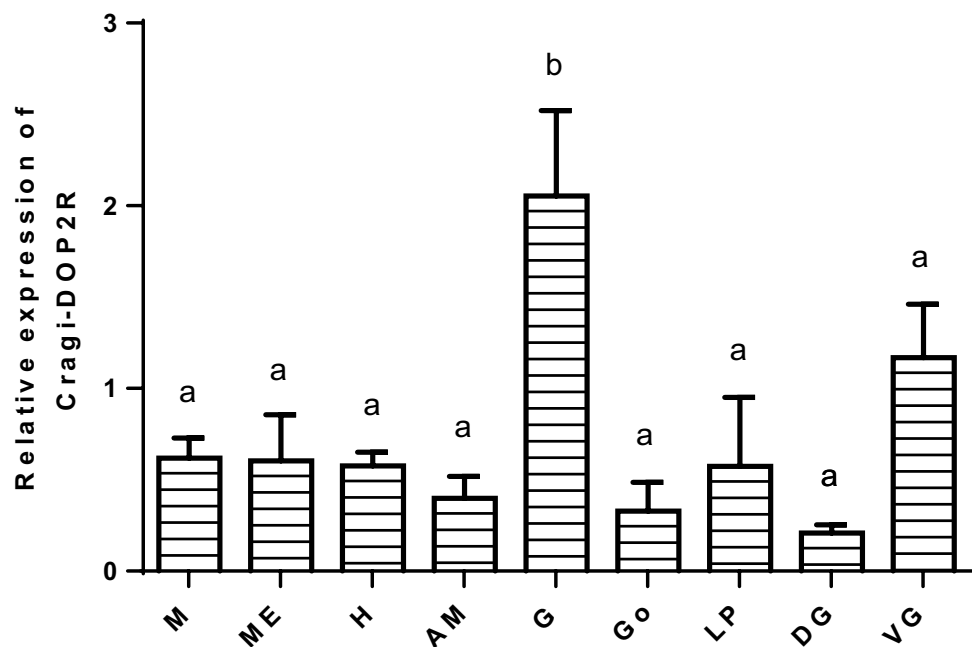


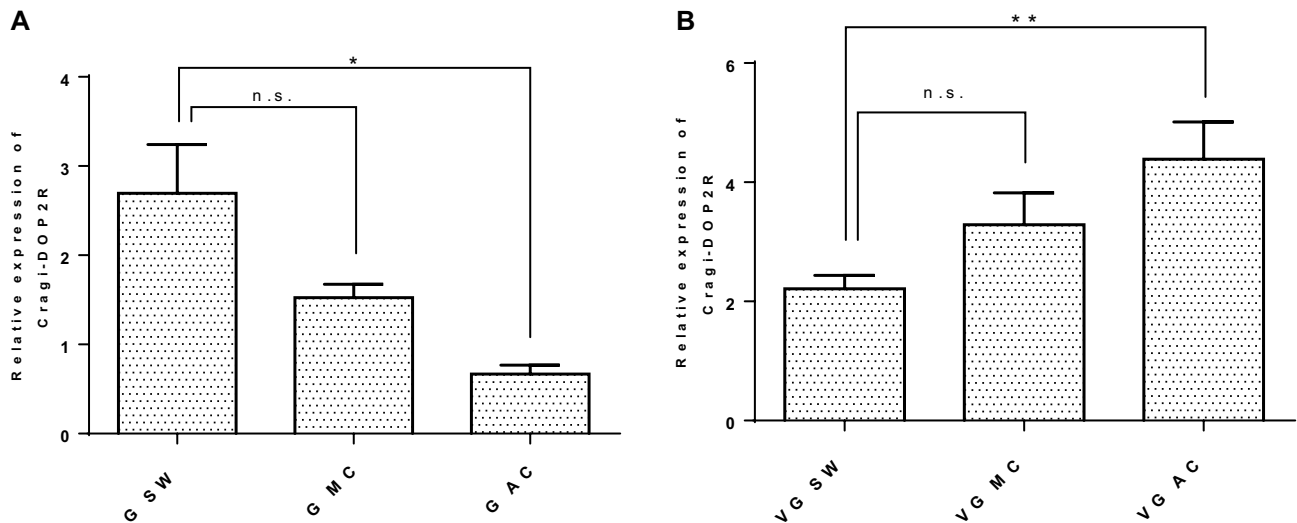
## Discussion

Dopaminergic signaling pathways are involved in a broad variety of physiological actions in Bilateria. Their involvement in processes such as the stress response, learning, or reproduction means that these pathways are widely studied in vertebrates and arthropods, especially because catecholamine receptors are the target of insecticides for pest control (Meyer et al. 2012; Alessi et al. 2014; Xu et al. 2017; Verlinden 2018; Kang et al. 2019). Experiments carried out in *Crassostrea* spp. have characterized receptor sequences displaying homologies with vertebrate D<sub>1</sub>-like receptors and arthropod Dop1 (Liu et al. 2018; Yang et al. 2013). The present report supplements current data on dopamine signaling pathways through the study of a novel dopamine receptor named Cragi-DOP2R in *C. gigas*.

Cragi-DOP2R was identified by investigating sequences displaying sequence similarity with arthropod dopamine receptors in the transcriptomic and genomic resources of the oyster *C. gigas* (Riviere et al. 2015). A broad-range phylogenetic analysis was performed, including dopamine receptors of Chordates, Ecdysozoa, and Lophotrochozoa and octopamine and tyramine receptors of Ecdysozoa and Lophotrochozoa. Cragi-DOP2R was clearly identified as an ortholog of vertebrate D<sub>1</sub>-like receptors and arthropod Dop2/INDR, making it the first Dop2/INDR identified in Lophotrochozoa. Further supporting this analysis, Cragi-DOP2R domain architecture displays a long third cytoplasmic loop and a rather long carboxy-terminal cytoplasmic domain as for members of the Dop2/INDR family (Mustard et al. 2005). In addition, Cragi-DOP2R CDS is encoded by a single exon, a feature of the D<sub>1</sub>-likeR family (Sunahara et al.

**Fig. 3** Relative expression levels of mRNAs encoding Cragi-DOP2R in adult tissues measured by real-time PCR. Each value is the mean + SEM of 5 pools each comprising 6 animals. Expression levels were normalized using Elongation factor 1  $\alpha$  (EF1 $\alpha$ ) transcripts as a reference





**Fig. 4** Relative expression levels of Cragi-DOP2R mRNAs in **A** the gills and **B** the visceral ganglia of oysters acclimated to brackish water (8‰). SW, seawater (33‰); MC, mild osmotic conditioning; AC, acute osmotic conditioning. Each value is the mean + SEM of 5 pools each comprising 5 animals. Expression levels were normalized

using Elongation factor 1  $\alpha$  (EF1 $\alpha$ ) transcripts as a reference. Results were statistically tested using a Student's *t* test. Significantly different means of samples from control and conditioned animals are indicated by \*\* ( $p < 0.001$ ), \* ( $p < 0.01$ ). n.s.: not significant

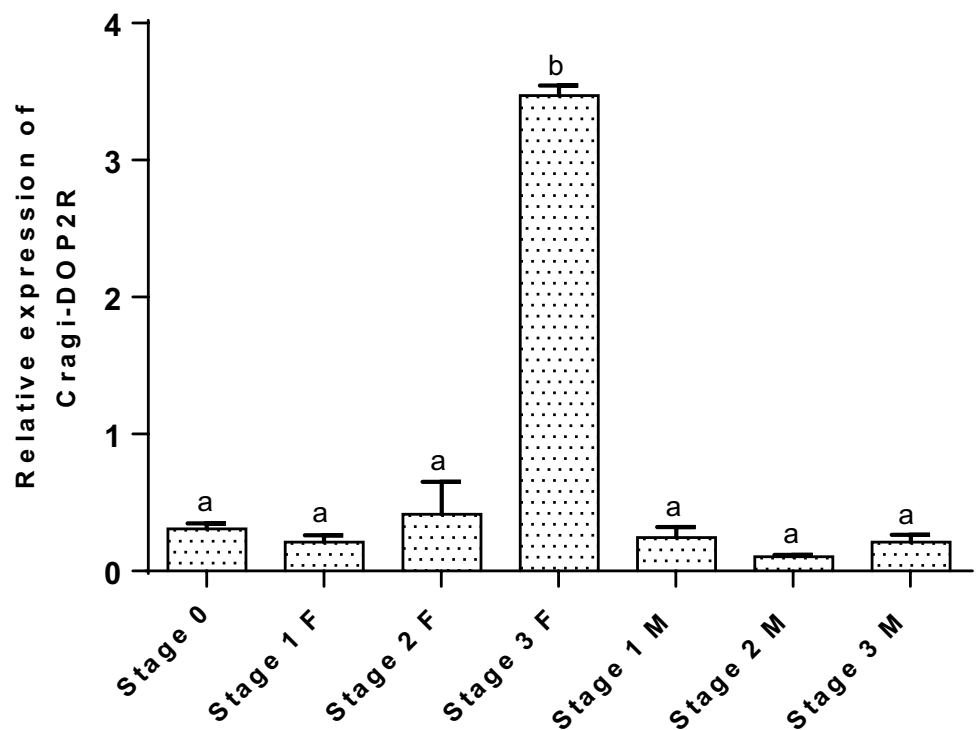
1990). Since Cragi-DOP2R, CgD1DR-1, Tyr1, and CgGPR1 are physically distantly located on chromosome 4, we cannot exclude that they emerged from multiple duplication steps of a common ancestral gene.

Further analysis confirmed that Dop2/INDRs were clearly more related to the Oct $\alpha$ R and the  $\beta$ -adrenergic-like octopamine receptor (Oct $\beta$ R) families (Troppmann et al. 2014)

than to the arthropod Dop1 and vertebrate D1-like receptors that include the functionally characterized receptors CaDAR1 of *C. angulata* (Yang et al. 2013) and CgD1DR-1 of *C. gigas* (Liu et al. 2018).

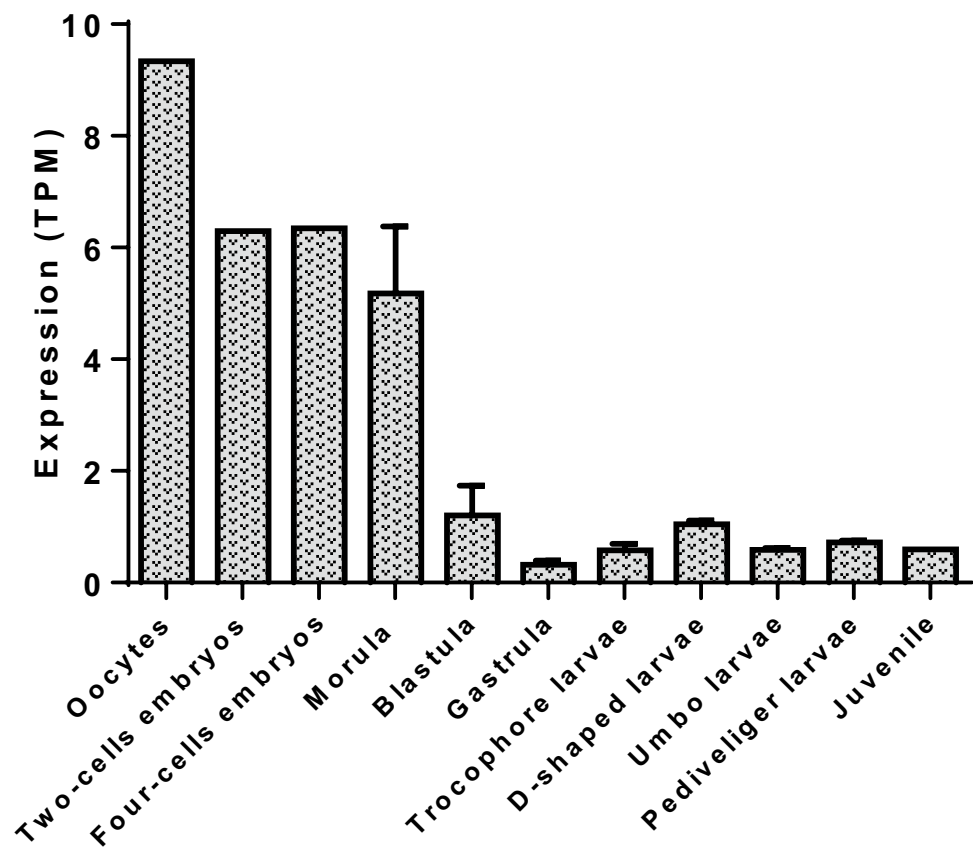
Different dopamine receptor families can be discriminated in vertebrates or arthropods based on their signaling properties. Activation of D<sub>1</sub>-like receptors and Dop2/INDR leads

**Fig. 5** Relative expression levels of Cragi-DOP2R mRNAs in the gonads measured by real-time PCR. Each value is the mean + SEM of 5 pools each comprising 6 animals. Expression levels were normalized using Elongation factor 1  $\alpha$  (EF1 $\alpha$ ) transcripts as a reference. Results were statistically tested with a one-way ANOVA,  $p < 0.05$ . Samples with significant statistical difference are marked with distinct letters. F, female; M, male; 0, stage 0 (sexual resting stage); 1, stage 1 (gonial multiplication stage); 2, stage 2 (tubule development and maturation stage); 3, stage 3 (sexual maturity stage)





**Fig. 6** Expression (TPM) of Cragi-DOP2R during developmental stages: oocytes, two-cell embryos, four-cell embryos, morula, blastula, gastrula, trochophore larvae, D-shaped larvae, umbo larvae, pediveliger larvae, and juvenile



to an increased level of intracellular cAMP (Kebabian and Caine 1979; Feng et al. 1996; Reale et al. 1997; Mustard et al. 2003; Meyer et al. 2012; Troppmann et al. 2014), while activation of  $D_2$ -like and Dop3 receptors leads to a decreased level of intracellular cAMP (Hearn et al. 2002; Beggs et al. 2005; Verlinden et al. 2015; Xu et al. 2017). In *Tribolium castaneum*, activation of TricaDop3 by dopamine leads to increased intracellular  $Ca^{2+}$  together with decreased cAMP (Verlinden et al. 2015). Surprisingly, although Cragi-DOP2R is phylogenetically related with the Dop2/INDR family, its activation by dopamine did not induce the intracellular cAMP signaling pathway but led to an increased  $Ca^{2+}$  level. This suggests the specific recruitment of a  $G\alpha_q$  protein, as in the AgDOP2-induced pathway in *Anopheles gambiae* (Hill et al. 2016). However, the activation of Dop2/INDR DmDOP2 (formerly Dop99B) in *Drosophila melanogaster* induces an increase of intracellular cAMP and  $Ca^{2+}$  (Feng et al. 1996; Han et al. 1996; Reale et al. 1997; Himmelreich et al. 2017). Cragi-DOP2R was activated by dopamine and tyramine, but  $EC_{50}$  values were in the nanomolar range for dopamine, thus fully compatible with its circulating concentration in *C. gigas* hemolymph (Lacoste et al. 2001b), and in the micromolar range for tyramine. Furthermore, dopamine was about twice as efficient as tyramine, suggesting that dopamine and tyramine are full and partial agonists of Cragi-DOP2R, respectively. Activation of Cragi-DOP2R by tyramine also

induced the  $G\alpha_q/Ca^{2+}$  signaling pathway, as shown for tyramine-specific TyrR2 receptors (Cazzamali et al. 2005; Huang et al. 2009). Although the tyramine concentrations required to activate Cragi-DOP2R were relatively high, they appeared congruent with a biological role of tyramine as a neurotransmitter (Lange 2009). Indeed, in the context of local release from nerve terminals, a pretty high concentration of catecholamines can be expected in vivo in the vicinity of the receptors. Receptor activation by distinct catecholamines is common because receptors are generally preferentially activated by a specific catecholamine but can bind other agonists due to their very close molecular structure. For instance, DmDOP2 was stimulated in order of potency by dopamine, noradrenaline, adrenaline and tyramine (Feng et al. 1996). Similarly, the adrenergic  $\alpha_1$  and  $\alpha_2$  receptors of *Platynereis dumerilii*, *Priapulul caudatus*, and *Saccoglossus kowalevskii* were activated by noradrenaline and adrenaline at concentrations in the nanomolar range and by tyramine, octopamine, and dopamine at concentrations approximately two orders of magnitude higher (Bauknecht and Jékely 2017). In the current state of our knowledge, no receptor specifically activated by tyramine had previously been characterized in *Crassostrea* spp. A putative octopamine/tyramine receptor named CgGPR1 (Ji et al. 2016) closely related to the *Lymnaea stagnalis* octopamine receptor OAR2 (Gerhardt et al. 1997) was identified in *C. gigas*, but neither tyramine nor

octopamine were efficient to activate it. Our phylogenetic analysis confirmed the presence of tyramine receptors in *C. gigas*, as already shown in various protostome species (Bauknecht and Jékely 2017). However, none of them had been functionally characterized as yet.

In order to better understand the involvement of Cragi-DOP2R in oyster physiology, the expression of the receptor was investigated in a wide variety of organs. The broad expression of Cragi-DOP2R confirmed the pleiotropic regulatory role of catecholaminergic signaling pathways found in other animal groups (Martínez-Ramírez et al. 1992; Selcho et al. 2009; Barron et al. 2010; Osinga et al. 2017). Although not investigated, we cannot rule out the expression of Cragi-DOP2R in hemocytes as reported for Cg-D1DR-1 (Liu et al. 2018). The expression of Cragi-DOP2R in the gills is particularly interesting because it demonstrates the existence of D1-like receptors in this organ and completes previous immunohistochemical and pharmacological assays that only identified D<sub>2</sub>-like receptors in the lateral cilia cells of the gills (Anador et al. 2011). In this latter study, only D<sub>2</sub>-like receptor-specific pharmacological agonists and antagonists mimicked and blocked the inhibitory action of dopamine on lateral cilia beating, respectively (Anador et al. 2011), knowing that the inhibitory action considerably reduces the water pumping rates (Jones and Richards 1993). In oyster (Carroll and Catapane 2007) as in the mussel *Mytilus edulis* (Catapane et al. 1978, 1979), the lateral ciliary activity of the gills is controlled by a reciprocal serotonergic-dopaminergic innervation from the ganglia. Furthermore, dopamine and other biogenic amines have been found in the gills and the nervous system of *C. virginica* (King et al. 2008). Therefore, it was relevant to investigate the role of Cragi-DOP2R in the gills and visceral ganglia of oyster. In their natural estuarine environment, oysters have to adapt to salinity fluctuations in order to maintain a relative homeostasis. To cope with these fluctuations, they have to integrate these changes in salinity and provide adapted responses probably mediated by hormones and neurotransmitters. The main organs involved in this regulation are the gills. The respective significant decrease and increase of Cragi-DOP2R expression in the gills and visceral ganglia of oysters under acute osmotic conditions suggests that the dopamine pathway is specifically involved in the regulation of the hyposaline stress response, similarly to the CCAP signaling pathway (Réalis-Doyelle et al. 2021). This contrasts with the calcitonin signaling pathway that appears to be involved in acclimation to mild hyposaline conditions (Schwartz et al. 2019). The high level of circulating dopamine in the hemolymph following a mechanical stress (Lacoste et al. 2001a, b) nicely correlates with the increase of Cragi-DOP2R gene expression induced by hyposaline stress in *C. gigas* ganglia that may induce adapted physiological responses of the organism. Interestingly, tyramine modulates the stress response in *Caenorhabditis elegans* via the TYRA-3 receptor (De Rosa

et al. 2019), a predicted Gq-coupled receptor (Wragg et al. 2007). However, the gills of oysters also fulfill several other biological roles via their ciliary activity, such as respiration, spawning, and feeding (Galtsoff 1964). Therefore, the dopamine signaling pathway may also control these functions.

Cragi-DOP2R expression in the gonads showed that levels were maximal in sexually mature (stage 3) females ready to spawn and characterized by gonad tubules filled with fully grown oocytes. These data are in line with observations in *C. angulata* showing significantly higher levels of Ca-DAR1 expression during the proliferation stage of the ovarian cycle (Yang et al. 2013). As Ca-DAR1 (Yang et al. 2013) is orthologous to CgD1DR-1 (Liu et al. 2016), CgD1DR-1 may also be involved in oocyte proliferation in *C. gigas*. Thus, oogenesis regulation would require the involvement of several dopamine receptors by assuming the contribution of CgD1DR-1 and Cragi-DOP2R receptors at the beginning and at the end of the ovarian cycle, respectively. The dopaminergic signaling pathway is well known for inhibiting reproductive functions in the animal kingdom (Dufour et al. 2020). In the sea urchin (*Strongylocentrotus nudus*), dopamine inhibits oocyte growth and maturation (Khotimchenko 1982). In the giant freshwater prawn (*Macrobrachium rosenbergii*), dopamine inhibits the gonad-stimulating-hormone, hence inhibited oocyte maturation (Tinikul et al. 2008, 2009). In *C. gigas* gonads, low expression of Cragi-DOP2R has been observed at the maturation stage (stage 2), and a significantly higher expression has been observed at the sexual maturity stage (stage 3) when oocyte maturation is presumed to be complete, suggesting a possible involvement of dopamine/Cragi-DOP2R signaling in the direct inhibition of oocyte maturation. Nevertheless, the existence of a maturation-stimulating hormone centrally inhibited by dopamine in oyster cannot be overruled. The significantly higher expression level of Cragi-DOP2R in stage 3 females also suggests a possible role of dopamine in spawning via Cragi-DOP2R. Supporting this view, dopamine has been suggested to be involved in the spawning of scallop (*Patinopecten yessoensis*) (Osada et al. 1987; Osada and Nomura 1989). Yet, the significantly higher expression of Cragi-DOP2R in the gonads of sexually mature females might simply reflect Cragi-DOP2R mRNA accumulation in the oocytes since these occupy most of the gonadic area at stage 3. This hypothesis is confirmed by the high expression level of the receptor in the oocytes, which persists during the early stages of oyster development. This expression profile reveals that Cragi-DOP2R mRNAs are maternally transmitted to embryos to support protein synthesis prior to the activation of the zygotic genome that occurs in the blastula stage (McLean and Whiteley 1974). This emphasizes a key role of dopamine signaling in early oyster development. This is also reminiscent of the impairment of the early embryonic

development of sea urchins when dopamine receptors were inactivated (Carginale et al. 1995). Dopamine influences the proliferation and differentiation of progenitor cells in mice (Popolo et al. 2004). Dopamine signaling could also regulate these activities in the early development stages of *C. gigas*. In early oyster embryos, cell differentiation leads to the development of locomotor cilia during the blastula stage. Interestingly, in the sea urchin (*Hemicentrotus pulcherrimus*), both dopamine receptors and a D<sub>1</sub>-like receptor could be involved in the formation of locomotor cilia (Katow et al. 2010). Moreover, inhibition of the dopamine synthesis pathway resulted in notably decreased motility of the embryos (Katow et al. 2010). A similar important role of dopamine signaling in the formation of locomotor cilia and in the motility of the blastula can be assumed in oyster: besides early development, dopamine signaling could be involved in the formation of the shell via the regulation of tyrosinase and chitinase in trochophore and D-shaped larvae (Liu et al. 2018, 2020). Cragi-DOP2R is also expressed in the different larval stages in *C. gigas*, possibly reflecting the emergence of the nervous system (Yurchenko et al. 2018).

We characterized a receptor activated by dopamine and tyramine in the oyster *C. gigas*. Activation of Cragi-DOP2R by these catecholamines led to increased intracellular Ca<sup>2+</sup> levels characteristic of the activation of Dop2/INDR receptors, further confirming the membership of Cragi-DOP2R to the D<sub>1</sub>-like family of receptors, and more specifically to the Dop2/INDR group. The Cragi-DOP2R signaling system seems to be involved in the regulation of a large variety of physiological processes such as osmoregulation, early development, and the regulation of female gametogenesis.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s10126-021-10052-5>.

**Acknowledgements** We are grateful to Marie-Pierre Dubos for her technical assistance.

**Author Contribution** Conceptualization: P.F. Methodology: J.S., E.R-D, P.F. Software: J.S., L.LF. Validation: J.S., P.F. Formal analysis: J.S., L.LF., E.R-D, P.F. Investigation: J.S., P.F. Writing — original draft: J.S., P.F. Writing — review and editing: J.S., P.F. Supervision, P.F. Project administration, P.F. Funding acquisition: P.F.

**Funding** This work was funded by the ANR project NEMO (Agence Nationale de la Recherche 14CE02 0020). J.S.'s PhD fellowship was co-financed by the NEMO project and by the European Union within the framework of the operational program FEDER/FSE 2014–2020.

## Declarations

**Conflict of Interest** The authors declare no competing interests.

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