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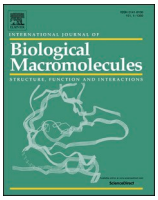

Structural and expression analysis of the dopamine receptors reveals their crucial roles in regulating the insulin signaling pathway in oysters

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ABSTRACT

Dopamine performs its critical role upon binding to receptors. Since dopamine receptors are numerous and versatile, understanding their protein structures and evolution status, and identifying the key receptors involved in the modulation of insulin signaling will provide essential clues to investigate the molecular mechanism of neuroendocrine regulating the growth in invertebrates. In this study, seven dopamine receptors were identified in the Pacific oysters (*Crassostrea gigas*) and were classified into four subtypes according to their protein secondary and tertiary structures, and ligand-binding activities. Of which, *DR2* (*dopamine receptor 2*) and *D(2)RA-like* (*D(2) dopamine receptor A-like*) were considered the invertebrate-specific type 1 and type 2 dopamine receptors, respectively. Expression analysis indicated that the *DR2* and *D(2)RA-like* were highly expressed in the fast-growing oyster "Haida No.1". After *in vitro* incubation of ganglia and adductor muscle with exogenous dopamine and dopamine receptor antagonists, the expression of these two dopamine receptors and ILPs (insulin-like peptides) was also significantly affected. Dual-fluorescence *in situ* hybridization results showed that *D(2)RA-like* and *DR2* were co-localized with *MIRP3* (*molluscan insulin-related peptide 3*) and *MIRP3-like* (*molluscan insulin-related peptide 3-like*) in the visceral ganglia, and were co-localized with *ILP* (*insulin-like peptide*) in the adductor muscle. Furthermore, the downstream components of dopamine signaling, including *PKA*, *ERK*, *CREB*, *CaMKK1*, *AKT*, and *GSK3β* were also significantly affected by the exogenous dopamine and dopamine receptor antagonists. These findings confirmed that dopamine might affect the secretion of ILPs through the invertebrate-specific dopamine receptors D(2)RA-like and DR2, and thus played crucial roles in the growth regulation of the Pacific oysters. Our study establishes the potential regulatory relationship between the dopaminergic system and insulin-like signaling pathway in marine invertebrates.

1. Introduction

Nervous systems, from simple nerve nets in primitive species to complex architectures in vertebrates, always transmit environmental stimuli and enable animals to generate body-wide responses [1]. In vertebrates, slight environmental stimulation, like temperature fluctuation, or food abundance change, may induce the anterior pituitary gland to secrete the growth hormone (GH). This hormone acts directly or indirectly through stimulate the production of insulin-like growth factors (IGFs) at their target tissue to regulate metabolic homeostasis and animal growth [2]. However, most invertebrates lack the classical GH-IGF aix, and only several neuropeptides, especially the ILPs, had been reported to function in the growth and metabolism regulation [3–5]. Besides, the monoamines such as dopamine and serotonin play indispensable roles in regulating the secretion of ILPs [6], but the mechanism of mutual regulation between the ILPs and monoamines is largely unknown in marine invertebrates.

Dopamine, one of the most critical monoamines, plays a fundamental role in hormonal regulation through their receptors [7]. The enzymes responsible for dopamine synthesis, metabolization, and storage, including TH (tyrosine hydroxylase), MAO (monoamine oxidase), and VMAT-2 (vesicular monoamine transporter 2), are found in the pancreatic beta cells [8,9]. Furthermore, under the exogenous dopamine incubation of the isolated islets, the secretion of insulin was inhibited, and the proliferation rate of insulin-positive cells was significantly decreased, while the apoptosis in pancreatic islets and beta cells was increased [10]. All these suggested that dopamine could be synthesized from the beta cells, and exerted an auto-paracrine regulation of insulin

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secretion, along with modulating the proliferation and apoptosis of pancreatic beta cells. Dopamine D2 receptors in the brain could modulate the plasma glucose level through parasympathetic or sympathetic nerves in mice [11]. D2 receptor agonist foundation remodels adipose tissue dopaminergic signaling and upregulates catabolic pathways, improving the metabolic profile in type 2 diabetes [12], which suggests that the dopamine receptor is the main target for dopamine involved in insulin autocrine and paracrine secretion. The effect of dopamine on insulin release was in a dose- and time-dependent manner. Dopamine significantly stimulated insulin secretion at a concentration of 10^{-8} M, while the higher concentrations of dopamine (10^{-7} - 10^{-4} M) inhibited the insulin secretion [13]. Higher dopamine concentrations might inhibit the release of insulin through binding to α2A-adrenergic receptors in a lower-affinity [9]. Additionally, dopamine regulates insulin secretion by triggering the cyclic AMP (cAMP)/PKA signaling which can ultimately affect multiple targets such as cAMP-regulated phosphoprotein (DARPP-32), dopamine transporter DAT, cAMP response element-binding protein (CREB), ionotropic glutamate receptors, and ion channels [14–16]. In particular, agonism of the D2 dopamine receptor also results in the formation of a protein complex containing the receptor, β-arrestin, PP2A, and engages the insulin downstream AKT/ GSK3 signaling pathway [17]. However, the regulatory relationship between dopamine and ILPs in marine invertebrates was largely unknown. Our previous study evinced that the recombinant ILPs protein could significantly affect the expression of the rate-limiting enzyme of dopamine synthesis, and accordingly affect the synthesis of dopamine [18], but the critical dopamine receptors participating in the crosstalk between the dopaminergic system and insulin signaling was still far away from clear.

Dopamine receptors belong to the rhodopsin-like seven-transmembrane receptors subfamily which is a group of the G protein-coupled receptors superfamily [7]. In vertebrates, the classification and evolution of dopamine receptors have been well studied. In humans, based on their structures, expression patterns, pharmacological properties, and coupled G proteins, five dopamine receptors were classified into the D1-like receptor subtype, which included D_1 and D_5, and the D2-like receptor subtype, which included D_2, D_3, and D_4 [19]. The structure analysis denotes that the length of the c-terminal tail and third cytoplasmic loop is different between D1-like and D2-like dopamine receptors in humans. These structures are important for dopamine binding, G-protein coupling, and signal transmission [20], and may be used as a key reference for the classification of dopamine receptors. However, in marine invertebrates, the literature on evolutionary relationships, protein structure, and physiological function analysis of dopamine receptors is scarce. In this regard, the illustration of their evolutionary status and finding the crucial dopamine receptors that participate in energy metabolism and growth regulation are most important, which will provide the foundation for further study on the neuroendocrine regulation of growth in invertebrates.

In the present study, we aim to figure out the evolutionary status and physiological functions of dopamine receptors in *C. gigas*. On this basis, we identify the crucial dopamine receptor participating in the ILPs secretion and growth regulation of oysters. Our work will establish the potential regulatory relationship between dopamine and ILPs in the Pacific oyster and provide valuable references for the construction of the neuroendocrine regulatory network in mollusks.

2. Materials and methods

2.1. Sequence analysis of dopamine receptors

The amino acid sequences of dopamine receptors, muscarinic acetylcholine receptors (AchM2 and AchM5), alpha-2-adrenergic receptors (ADRA2A), and 5-hydroxytryptamine receptors (HTR2A) from *Homo sapiens* (*Hs*), the dopamine receptors from *Drosophila melanogaster* (*Dm*), *Caenorhabditis elegans* (*Ce*), *Apis mellifera* (*Am*), *Octopus bimaculoides* (*Ob*), *Mizuhopecten yessoensis* (*My*), *Crassostrea virginica* (*Cv*), and *Crassostrea gigas* (*Cg*) were all retrieved from the NCBI followed by further phylogenetic analyses. The phylogenetic tree was constructed based on their amino acid sequence and protein structure using Mega 7 [21] and T-COFFEE (https://www.ebi.ac.uk/Tools/msa/tcoffee/) for clarifying the evolutionary status of the dopamine receptors in *C. gigas*.

2.2. Ligand-binding assay of the seven dopamine receptors

To further analyze the difference between the seven dopamine receptors, the ligand-binding sites and binding energy of the dopamine receptors were analyzed by using AutoDock Tools. The 3D chemical structure of the dopamine ligand was downloaded from the ZINC database (http://zinc15.docking.org/). The protein structure models of the dopamine receptors were built by SWISS-MODEL (https://swissmodel.expasy.org/interactive), and the result with over 30 % identity to the template was chosen for further docking analysis. Finally, the binding sites of dopamine receptors with their ligand were visualized with PyMOL.

2.3. RNA isolation, cDNA synthesis, and real-time PCR

Total RNA extraction and cDNA synthesis from the visceral ganglia and adductor muscle were performed by using Trizol Reagent (Invitrogen) and HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, R323) according to the manufacturer's instructions. The primer sets were designed using Primer Express software (Applied Biosystems, USA), and primer sets with an efficiency of 90–110 % were used for real-time PCR analysis (Table S1). The reaction volume and PCR cycling parameters were as described in our previous study [4]. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [22]. Statistical significance was determined by the one-way ANOVA and Student's *t*-test for multiple groups and two groups comparisons, respectively, and $P < 0.05$ was defined as significant differences.

2.4. The expression of the dopamine receptors in "Haida No. 1" and wild oysters

The visceral ganglia and adductor muscle were dissected from twelve two-year-old *C. gigas* individuals and were pooled into four biological replicates (three individuals/replicate). Total RNA isolation, cDNA synthesis, and real-time PCR were carried out as described above to determine the expression levels of the dopamine receptors in "Haida No. 1" and wild oysters.

2.5. In vitro dopamine and dopamine receptor antagonist treatment

The method of primary tissue culture, the dose, and the time of exogenous dopamine incubation was conducted according to our previous study [18]. In detail, the ganglia and adductor muscle were dissected from healthy two-year-old oysters, the tissues then were cut into pieces with sterile scissors and cultured with the primary medium in the 12-well plate at 26 °C. Dopamine hydrochloride (Sigma, H8502) was added to the primary medium with the final concentration of 0, 1, 3, 5, 10, and 20 μg/mL and maintained for 12 h. For the dopamine receptors antagonist treatment, the selective dopamine D1-like receptor antagonist SCH-23390 hydrochloride (MCE, HY-19545A) and the dopamine (D_2, D_3, D_4) receptor antagonist asenapine hydrochloride (MCE, HY-16567) were used. The SCH-23390 hydrochloride was recognized as the only D1-like dopamine receptors selective antagonist and had been widely used to understand the role of the dopamine system [23,24]. Asenapine hydrochloride was the type 2 dopamine receptor antagonist with Ki values of 0.42–1.3 nM, and it had been used to detect the dose-related effects on dopamine receptors. The SCH-23390 hydrochloride and asenapine hydrochloride was added to the primary medium with the final concentration of 0.3 nM and 1 nM, respectively, according to their Ki values mentioned in the user manual and previous studies [25,26].

After 12 h, the culture medium was discarded, and the cells were collected for RNA or protein extraction after washing three times with sterile 1 × PBS.

2.6. Western blot

After dopamine treatment, samples were collected and homogenized in 0.1 M PBS buffer, then centrifuged at 13,000*g* for 10 min, and the supernatants were collected. Protein concentration was measured using the Nanodrop 2000, then 20 μg proteins from each tissue were separated by 12 % SDS-PAGE and transferred to the PVDF membrane (Millipore, USA). The membranes were blocked with 5 % skim milk dissolved in TBST buffer overnight at 4 °C and washed three times with TBST, thereafter incubated with primary antibody (anti-ILPs, 1:500) in TBST for 1 h at 37 °C. After washing with TBST five times, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Beyotime, A0216, diluted 1:1000) for 30 min at 37 °C. The β-actin mouse monoclonal antibody (diluted 1:1000 in TBST) (Beyotime, AF0003) was used as a control. Protein expression was detected using enhanced chemiluminescence detection reagents (Vazyme, E411) and visualized using the GE ImageQuant LAS 4000 Mini system.

2.7. Dual-fluorescence in situ hybridization of dopamine receptors and ILPs

To confirm the regulatory relationship between dopamine and ILPs directly, dual-fluorescence *in situ* hybridization was performed. The visceral ganglia and adductor muscle of two-year-old *C. gigas* were collected and fixed in 4 % paraformaldehyde at 4 °C. Probes of digoxigenin-labeled ILPs and biotin-labeled dopamine receptors were synthesized according to the manufacturer instructions of the RNA labeling kit. For dual-fluorescence *in situ* hybridization, we first used a mixture of the dopamine receptors and ILPs probes for the hybridization step, and then the sections were washed and blocked with a blocking reagent (Roche). Slides were subsequently incubated for 1 h with Alexa-488 conjugated anti-digoxigenin (Jackson ImmunoResearch) and Alexa-647 conjugated anti-biotin (Jackson ImmunoResearch) (diluted 1:1000 with blocking reagent) for ILPs and dopamine receptors detection, respectively. The obtained results were viewed under Leica TCS SP98 Confocal laser scanning microscopy (Leica, Germany). The fluorescence values and colocalization analysis were calculated by using the ImageJ plugin ScatterJ.

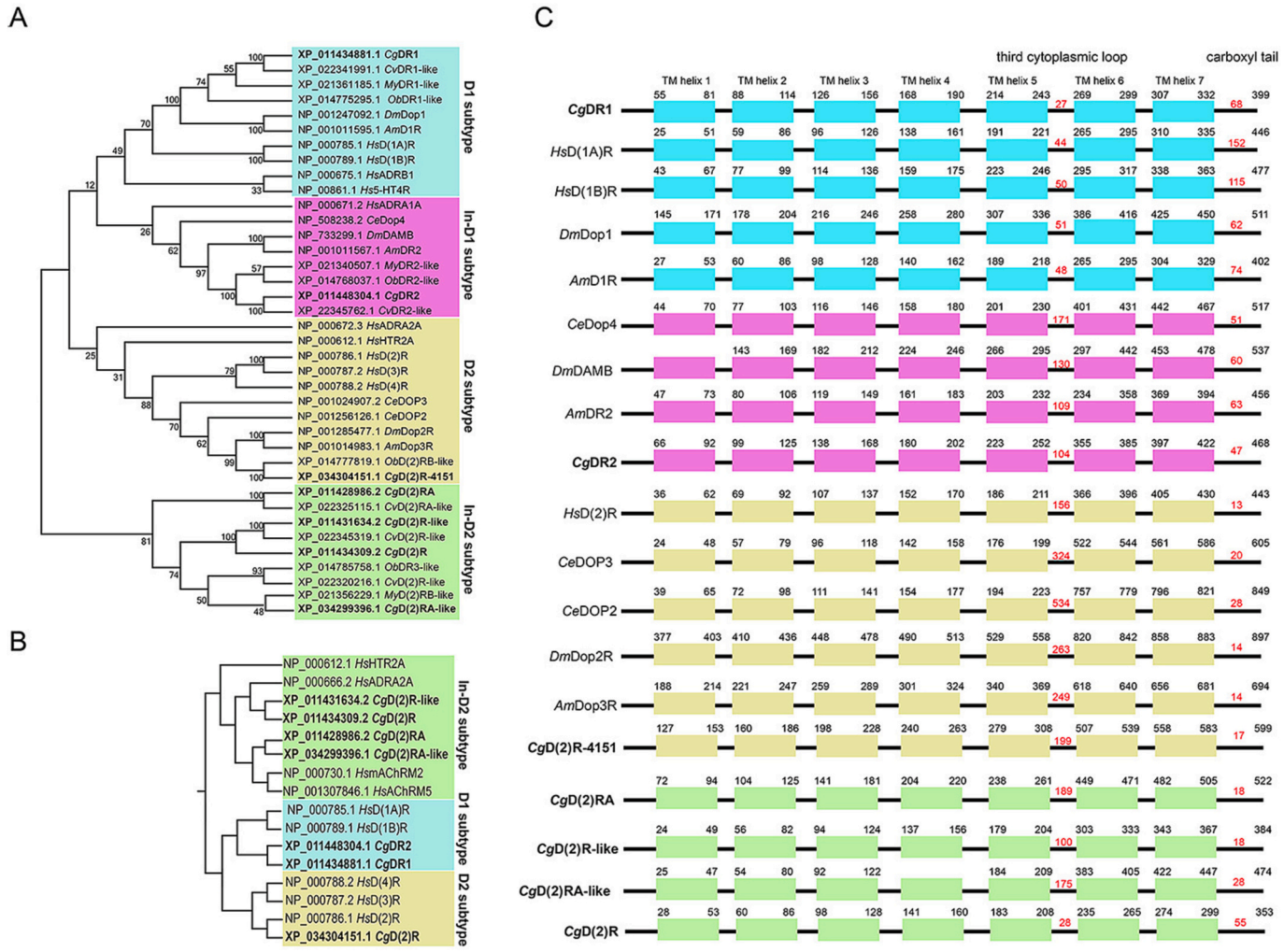

Fig. 1. Phylogenetic analysis of the dopamine receptors. Phylogenetic analysis of the dopamine receptors based on the amino acid sequence (A) and protein structure (B). (C) The schematic diagram of the secondary protein structure of the dopamine receptors.

3. Results

3.1. Phylogenetic and protein structure analysis of dopamine receptors

According to the recently published genome data (cgigas_uk_roslin_v1, Accession: GCA_902806645.1), seven dopamine receptor genes were identified in *C. gigas* (Table S2). As shown in the phylogenetic tree, *Cg*DR1 (dopamine receptor 1) was clustered into one clade with their counterparts from other invertebrates, including the *Cv*DR1-like, *My*DR1-like, *Ob*DR1-like, *Dm*Dop1, *Am*DR1, and further formed a large clade with the human type 1 dopamine receptors, including *Hs*D(1A)R and *Hs*D(1B)R. So we speculated that the *Cg*DR1 was a vertebrate homologous type 1 dopamine receptor (D1 subtype). The *Cg*DR2 (dopamine receptor 2), *Ce*Dop4, *Dm*DAMB, *Am*DR2, *Ob*DR2-like, *My*DR2-like, and *Cv*DR2 formed an invertebrate-specific branch which was close to the human type 1 dopamine receptors (Fig. 1A, B). Further protein secondary structure analysis revealed that the DR2 clade in invertebrates had a longer third cytoplasmic loop than that of the human D1 subtype receptors, but they had a similar length of the c-terminal tail, suggesting that the DR2 clade could be the invertebrate-specific type 1 dopamine receptor (In-D1 subtype) (Fig. 1C).

Phylogenetic and protein structure analysis illustrated that the *Cg*D(2)R-4151 (D(2) dopamine receptor) was closer to the type 2 dopamine receptors of humans, including the *Hs*D(2)R and *Hs*D(4)R (Fig. 1A-C), which suggested that the D(2)R-4151 of *C. gigas* might be homologous to the vertebrate type 2 dopamine receptor (D2 subtype). The other four dopamine receptors in *C. gigas*, including the D(2)R (D(2) dopamine receptor), D(2)R-like (D(2)-like dopamine receptor), D(2)RA (D(2) dopamine receptor A), and D(2)RA-like (D(2) dopamine receptor A-like) formed a branch that was specific in invertebrates. According to the T-coffee analysis, the D(2)R and D(2)R-like clustered into one clade with the ADRA2A of humans, the D(2)RA and D(2)RA-like clustered into one clade with the AchM2 and AchM5 of humans. These four receptors clustered into a larger clade with the humans HTR2A (Fig. 1B). Further protein secondary structure analysis found the D(2)R-like, D(2)RA, and D(2)RA-like of *C. gigas* all possessed similar length of the c-terminal tail with the vertebrate homology type 2 dopamine receptor (D2 subtype) (Fig. 1C).

3.2. Seven dopamine receptors have different ligand-binding sites and binding energy

As shown by the molecular docking analysis, the binding energy of the seven dopamine receptors was different (Fig. 2). The type 1 dopamine receptor (Fig. 2A, B) possessed higher binding energy than that of the type 2 dopamine receptor (Fig. 2C-G). In the type 1 dopamine receptor, the binding energy of the In-D1 subtype (DR2) (Fig. 2B) was higher than that of the D1-subtype (DR1) (Fig. 2A). Furthermore, the DR1 bound with dopamine ligands at GLU198, ASP212, and ASN196 with three pairs of hydrogen bonds (Fig. 2A), and DR2 bound with dopamine ligands at the GLU215, GLU217, ASP139 with three pairs of hydrogen bonds (Fig. 2B).

The binding energy of the type 2 dopamine receptors was similar, but the binding sites were slightly different (Fig. 2C-G). The D(2)R-4151 bound with the dopamine ligands at GLU186 and ASP205 with two pairs of hydrogen bonds (Fig. 2C). For the invertebrate-specific dopamine receptors, D(2)R and D(2)R-like bound with dopamine ligands at GLU and formed two pairs of hydrogen bonds (Fig. 2D, E), while the D(2)RA bound with the dopamine ligands at ASP328, ASP323, and SER324 with four pairs of hydrogen bonds (Fig. 2F), the D(2)RA-like bound with dopamine at ASP180, GLU414, TRY407, and SER409 with three pairs of hydrogen bonds (Fig. 2G).

3.3. Dopamine receptors were differentially expressed in "Haida No. 1" and wild oysters

Expression levels of the seven dopamine receptors in the ganglia and adductor muscle were different between "Haida No.1" and wild oysters. In the visceral ganglia, only *D(2)RA-like* was highly expressed in the fast-growing oyster "Haida No.1", while the other three dopamine receptors, including *DR1*, *D(2)R-4151*, and *D(2)R-like* were all highly expressed in wild oysters. The rest of the three dopamine receptors, including *DR2*, *D*

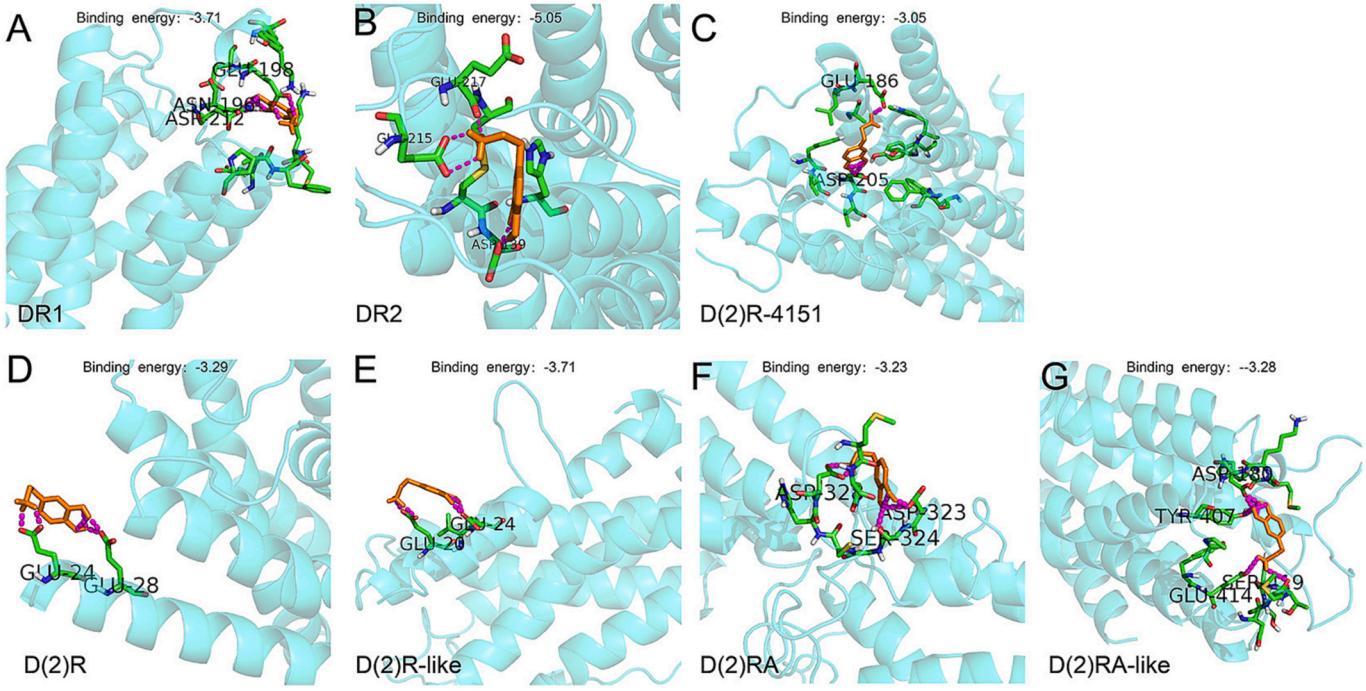

Fig. 2. Binding sites and binding energy of the dopamine receptors to the dopamine ligand. The light blue represents the protein structure of the dopamine receptors, the yellow represents dopamine ligands, the green represents the amino acid residues of dopamine binding sites, and the purple represents the hydrogen bond formed by binding dopamine to its receptors.

(2)R, and *D(2)RA*, showed no significant difference between the "Haida No.1" and wild oysters (Fig. 3A). In the adductor muscle, *DR2* and *D(2)RA-like* were highly expressed in "Haida No.1", while *DR1* and *D(2)R-like* were highly expressed in wild oysters, the expression of the other three dopamine receptors, including *D(2)R-4151*, *D(2)R*, and *D(2)RA*, showed no significant difference between "Haida No.1" and wild oysters (Fig. 3B).

3.4. Exogenous dopamine influenced the expression of D(2)RA-like and DR2

The exogenous dopamine affected the expression of the *D(2)RA-like* and *DR2* in a dose-dependent manner both in the ganglia and adductor muscle. In the ganglia, the expression of *DR2* peaked at 3 μg/mL or 5 μg/mL dopamine hydrochloride and was 1.8-fold than that of the control. At higher concentrations, including 10 μg/mL or 20 μg/mL dopamine hydrochloride, the expression of *DR2* was significantly inhibited. In contrast, the expression of *D(2)RA-like* reached 1.9-fold than that of control at 1 μg/mL dopamine hydrochloride, and showed a stepwise upregulated pattern with the increased concentration of the dopamine hydrochloride, finally reached up to 2.5-fold than that of control at 10 μg/mL (Fig. 4A). In the adductor muscle, the expression of *DR2* and *D(2)RA-like* peaked at 3 μg/mL and 1 μg/mL, which was 1.3-fold and 1.2-fold than that of the control, respectively. The relative expression levels showed a decreased trend against the increased concentration of dopamine hydrochloride (Fig. 4B).

3.5. Exogenous dopamine and dopamine receptor antagonists have a great impact on the expression of ILPs

The expression of ILPs in the ganglia and adductor muscle after dopamine hydrochloride treatment was also detected in order to further understand the effect of the dopaminergic system on the secretion of ILPs. As shown in Fig. 5, exogenous dopamine significantly affected the mRNA and protein expression of ILP in adductor muscle and the expressions of MIRP3, MIRP3-like, and ILP7 (insulin-like peptide 7) in ganglia. In detail, the expression of ILP in adductor muscle was upregulated with the increasing concentration of dopamine hydrochloride from 1 μg/mL to 10 μg/mL, while its expression in ganglia was slightly affected (Fig. 5A, F). The expression of ILP7 peaked at 10 μg/mL dopamine hydrochloride in ganglia and also showed a slight increase in adductor muscle at 5 μg/mL dopamine hydrochloride (Fig. 5B, E). Strikingly, the expression of the MIRP3 and MIRP3-like was more easily affected by the dopamine hydrochloride in ganglia, 1 μg/mL dopamine hydrochloride could dramatically induce the expression of MIRP3 and MIRP3-like, and a higher concentration would diminish their expression. On the contrary, the expression of MIRP3 and MIRP3-like in adductor muscles was slightly affected by the exogenous dopamine (Fig. 5C, D, E).

On the other hand, after *in vitro* incubation of ganglia and adductor muscle with dopamine receptor antagonists, the expression of the ILPs was all significantly down-regulated. In ganglia, the expression of *MIRP3*, *MIRP3-like*, and *ILP7* was all diminished with the treatment of SCH-23390 hydrochloride and asenapine hydrochloride, but the expression of *ILP* was not affected (Fig. 5G). In the adductor muscle, SCH-23390 hydrochloride significantly decreased the expression of *ILP*, *MIRP3*, and *ILP7*. Asenapine hydrochloride significantly reduced the expression of *MIRP3*, *MIRP3-like*, and *ILP7* (Fig. 5H).

3.6. Co-localization of dopamine receptors and ILPs

Two-color fluorescence *in situ* hybridization results showed that *D(2)RA-like* and *DR2* were all intensively expressed in the visceral ganglia and were colocalized with *MIRP3* and *MIRP3-like* (Fig. 6, A1-A4, B1-B4, D1-D4, E1-E4), while no positive signal of *ILP7* was detected (Fig. 6, C1-C4, F1-F4). The co-localization correlation coefficient of *D(2)RA-like* with *MIRP3* and *MIRP3-like* (Fig. 6, A5-B5) was greater than that of *DR2* (Fig. 6, D5-E5). It's worth noting that the positive signal of *D(2)RA-like* (Fig. 6, A5, B5, C5) was more extensive than *DR2* (Fig. 6, D5, E5, F5) in the visceral ganglia. In the adductor muscle, positive signals of *ILP* and *MIRP3-like* were detected in the *D(2)RA-like* expressing cells, and the co-localization correlation coefficient of *D(2)RA-like* with *ILP* and *MIRP3-like* (Fig. 7, A5, C5) was greater than *D(2)RA-like* with *MIRP3* and *ILP7* (Fig. 7, B5, D5). Also, the *DR2*, *ILP*, and *MIRP3-like* were also co-expressed in the adductor muscle (Fig. 8, A1-A5). The co-localization correlation coefficient of *DR2* with *ILP* and *MIRP3-like* was also larger than *DR2* with *MIRP3* and *ILP7* (Fig. 8, A5-D5). Furthermore, the positive signal of *DR2* (Fig. 8) was more extensive than *D(2)RA-like* (Fig. 7).

3.7. Dopamine regulates the secretion of ILPs through their downstream components

Real-time PCR results showed that the downstream components of the dopamine signaling, including the *PKA* (*cAMP-dependent protein kinase catalytic subunit 1*), *ERK* (*extracellular regulated protein kinases*), *CREB*, *CaMKK1* (*calcium/calmodulin-dependent protein kinase kinase 1*), *AKT* (*serine/threonine-protein kinase Akt*), and the *GSK3β* (*glycogen synthase kinase-3β*) were significantly influenced by the exogenous dopamine and the dopamine receptor antagonists. Expression of *PKA* was induced by 1 μg/mL dopamine hydrochloride, a higher concentration, 10 μg/mL dopamine hydrochloride, would inhibit its expression in ganglia. Similarly, dopamine receptor antagonists, SCH-23390

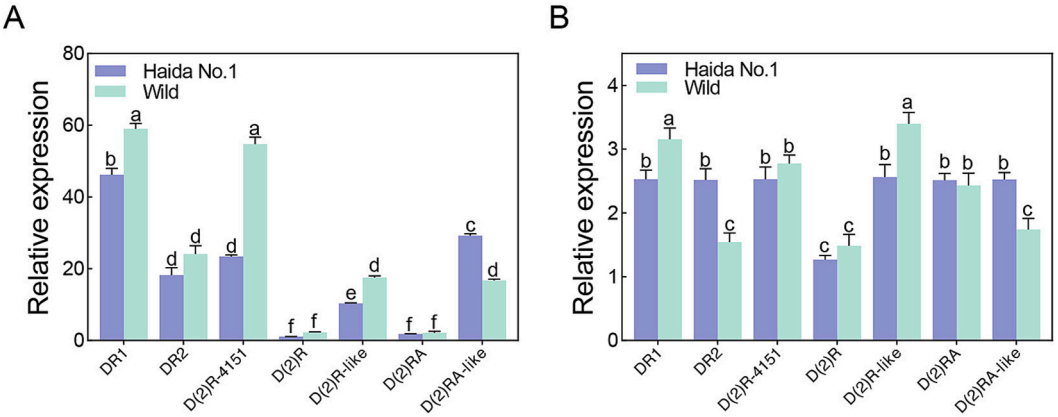

Fig. 3. Dopamine receptors showed different expression patterns between the "Haida No.1" and wild oysters. (A) The expression of the dopamine receptors in visceral ganglia (B) and in adductor muscles of "Haida No.1" and wild oysters. Data are expressed as mean ± SD ($n = 3$). The significant difference ($P < 0.05$) among groups is indicated by different lowercase letters.

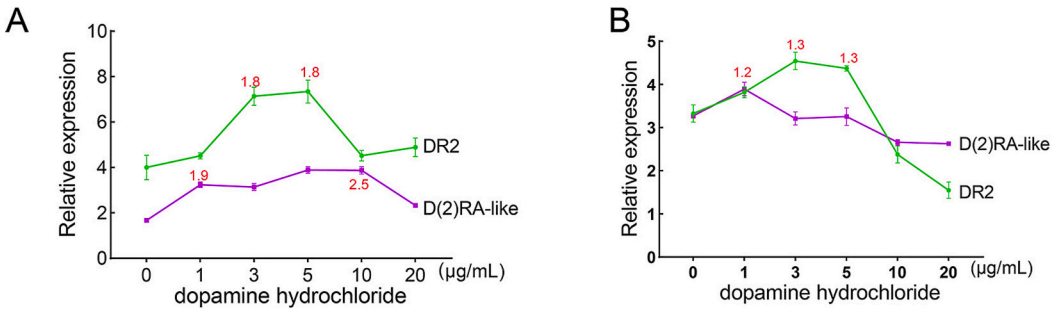

Fig. 4. Effect of dopamine hydrochloride on the expression of dopamine receptors. The expression patterns of *D(2)RA-like* and *DR2* in ganglia (A) and adductor muscles (B) at 12 h after treatment with dopamine hydrochloride at levels of 0, 1, 3, 5, 10, and 20 μg/mL. Data are expressed as mean ± SD (n = 3). The numbers on the broken line indicated the ratio of the highest expression level of the dopamine receptors to that of the control.

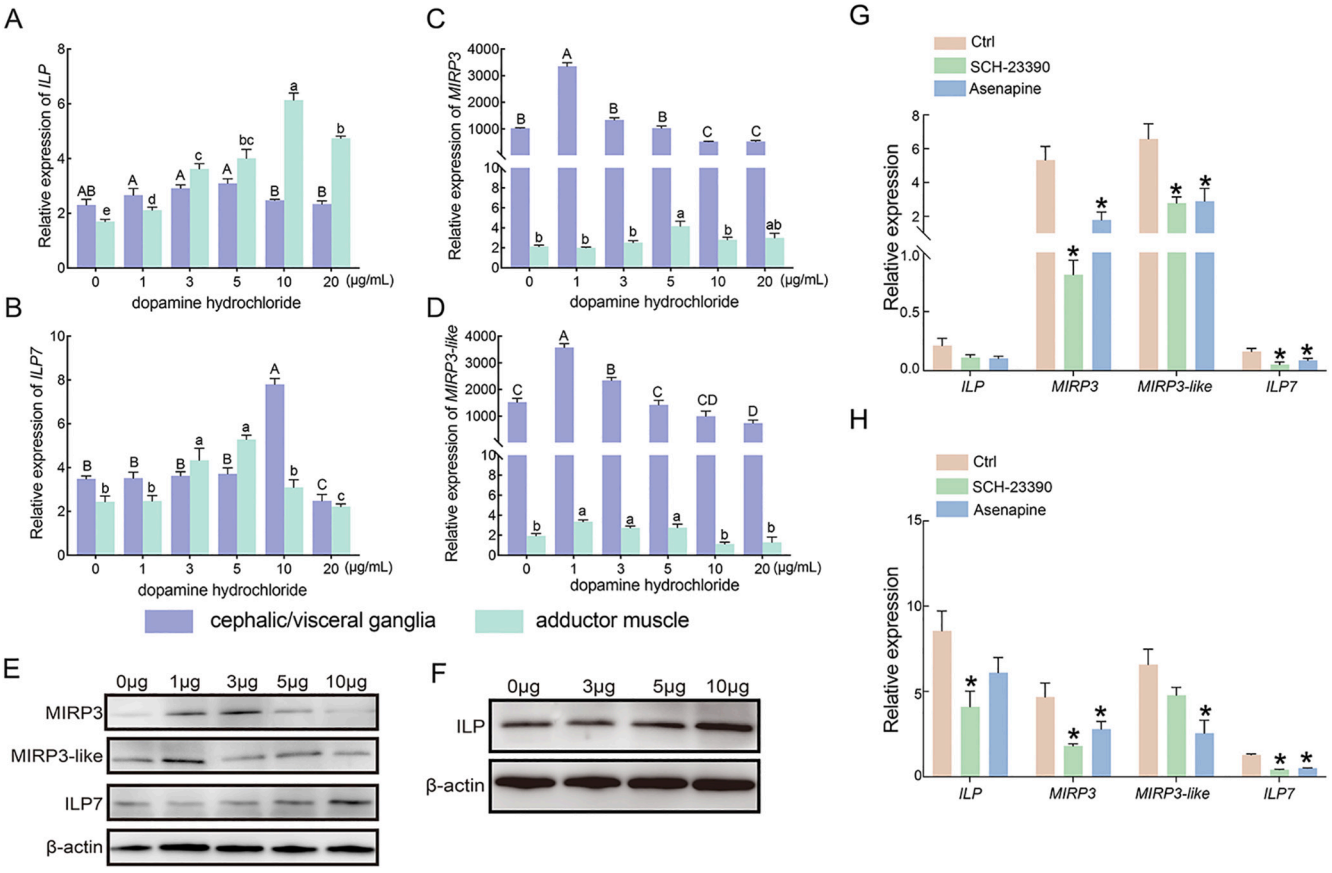

Fig. 5. Effect of dopamine hydrochloride and dopamine receptor antagonists on expression and secretion of the ILPs. The mRNA expression of *ILP* (A), *ILP7* (B), *MIRP3* (C), and *MIRP3-like* (D) in ganglia and adductor muscles at 12 h after treatment with dopamine hydrochloride at levels of 0, 1, 3, 5, 10, and 20 μg/mL. Data are expressed as mean ± SD (n = 3). The significant difference ($P < 0.05$) among groups is indicated by different lowercase letters. (E) Protein expression of the MIRP3, MIRP3-like, and ILP7 in ganglia at 12 h after treatment with dopamine hydrochloride at levels of 0, 1, 3, 5, and 10 μg/mL. (F) Protein expression of ILP in adductor muscles at 12 h after treatment with dopamine hydrochloride at levels of 0, 3, 5, and 10 μg/mL. Effects of dopamine receptor antagonist on the expression of ILPs in ganglia (G) and in adductor muscles (H). Data are expressed as mean ± SD (n = 3). The significant difference among groups is indicated by the "*".

hydrochloride and asenapine hydrochloride, also suppressed the expression of *PKA* both in ganglia and adductor muscles (Fig. 9A). In ganglia, the expression of *ERK* was not affected by the exogenous dopamine and the dopamine receptor antagonists. While in adductor muscles, the expression of *ERK* was increased with the treatment of 10 μg/mL dopamine hydrochloride, and decreased with the treatment of the D1-like receptor antagonist SCH-23390 hydrochloride (Fig. 9B). The expression of the *CREB* was also increased with the treatment of 1 μg/mL and 10 μg/mL exogenous dopamine, while decreased with the treatment of SCH-23390 hydrochloride and asenapine hydrochloride both in ganglia and in adductor muscle (Fig. 9C). In ganglia, the expression of *CaMKK1* only increased with the treatment of 1 μg/mL exogenous dopamine, and decreased with the treatment of D2-like receptor antagonist asenapine hydrochloride. In the adductor muscle, 1 μg/mL and 10 μg/mL exogenous dopamine induced the expression of *CaMKK1*. Both the D1-like receptor antagonist SCH-23390 hydrochloride and D2-like receptor antagonist asenapine hydrochloride treatment significantly inhibited the expression of *CaMKK1* (Fig. 9D). The expression of *AKT* and *GSK3β* was all activated with the treatment of 10 μg/mL exogenous dopamine and inhibited with the treatment of asenapine hydrochloride

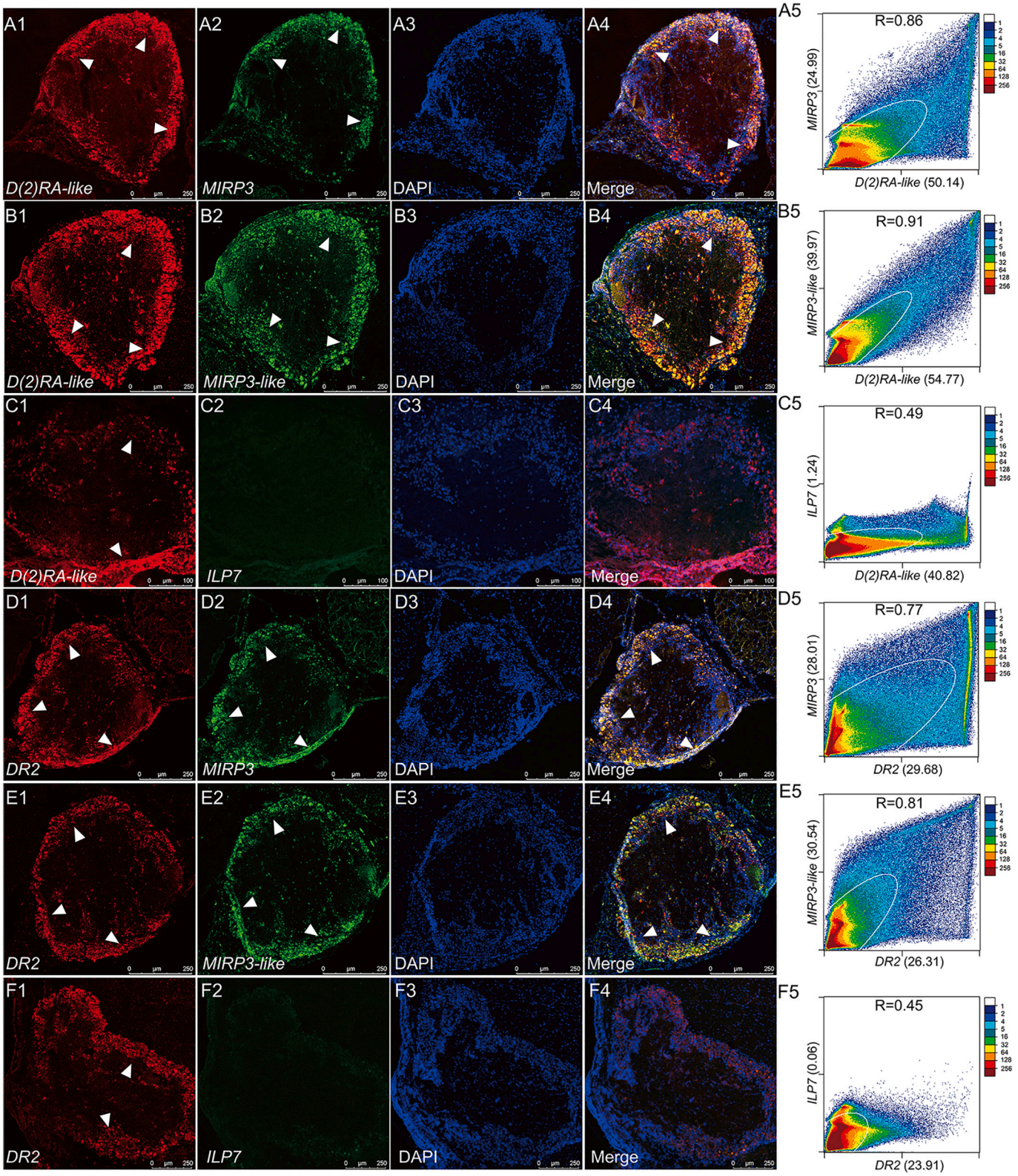

Fig. 6. Cellular co-localization of dopamine receptors and ILPs in the visceral ganglia of *C. gigas*. Cellular co-localization of *D(2)RA-like* with *MIRP3* (A1-A4), *MIRP3-like* (B1-B4) and *ILP7* (C1-C4) in visceral ganglia. Cellular co-localization of *DR2* with *MIRP3* (D1-D4), *MIRP3-like* (E1-E4), and *ILP7* (F1-F4). The arrowheads indicate the positive signals of *D(2)RA-like*, *DR2, MIRP3,* or *MIRP3-like*. The fluorescence value and colocalization coefficient of dopamine receptors and ILPs were shown in A5-F5.

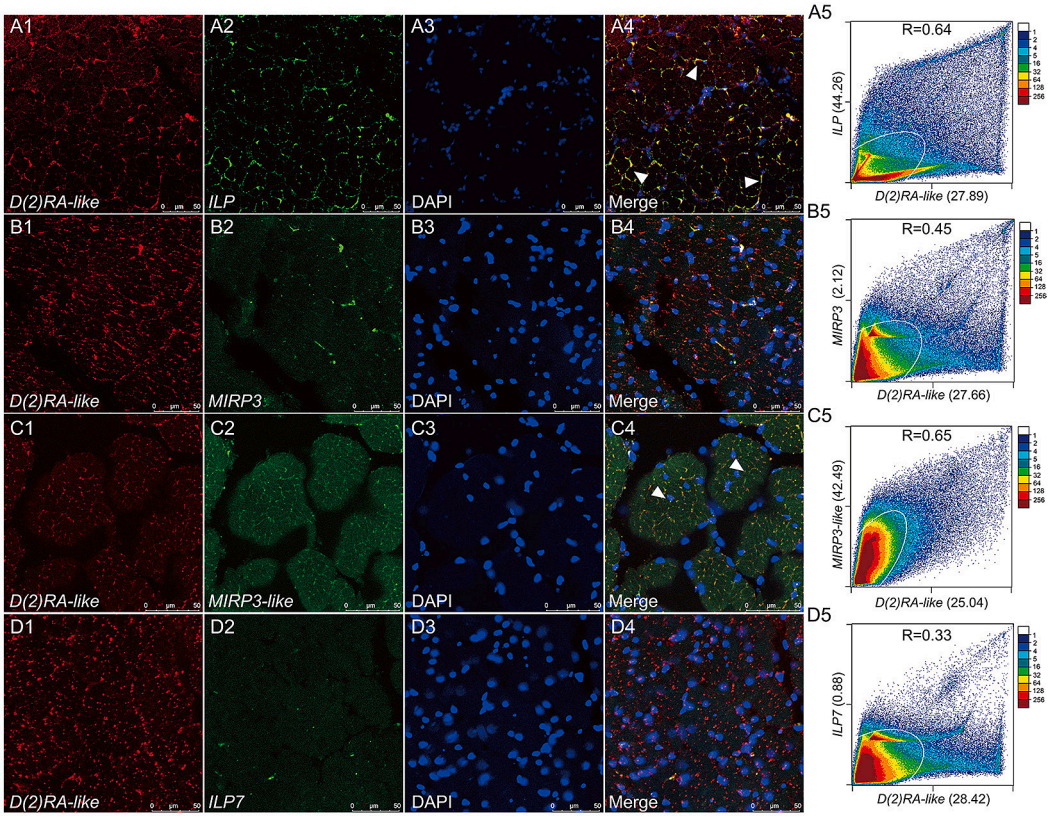

Fig. 7. Cellular co-localization of D(2)RA-like and ILPs in the adductor muscles of *C. gigas*. Cellular co-localization of *D(2)RA-like* with *ILP* (A1-A4), *MIRP3* (B1-B4), *MIRP3-like* (C1-C4), and *ILP7* (D1-D4). The arrowheads indicate the positive signals of *D(2)RA-like, ILP, MIRP3,* or *MIRP3-like*. The fluorescence value and colocalization coefficient of *D(2)RA-like* and ILPs were shown in A5-D5.

in ganglia and adductor muscle (Fig. 9E, F).

4. Discussion

Dopamine is involved in the regulation of complex physiological activities in CNS and the peripheral tissue through their G protein-coupled receptors [27,28]. The evolution and physiological functions of dopamine receptors are obscure in marine invertebrates. Our previous study found that insulin signaling and the dopaminergic system worked together to regulate the growth of oysters [18], but the receptors that participated in this process remained to be characterized. In this work, the phylogenetic analysis according to the protein's secondary and tertiary structures, and ligand-binding activities allowed us to classify the seven dopamine receptors into four subtypes. On this basis, the expression patterns, exogenous dopamine and dopamine receptors antagonist treatment, and the co-localization analyses all confirmed that dopamine regulated the secretion of insulin-like peptides through two important invertebrate-specific dopamine receptors, DR2 and D(2)RA-like.

The protein structures, ligand binding sites, and the mediated downstream pathways of the dopamine receptors had been used to classify their subtype in mammals, Osteichthyes, *D. melanogaster*, *A. mellifera*, and *C. elegans*. Studies found that the dopamine receptors in invertebrates were more numerous, functionally diverse, and do not have a one-to-one homologous counterpart with mammals with some dopamine receptors having been lost during the evolution of invertebrates to vertebrates [29–37]. In our present study, seven dopamine receptors were classified into four subtypes in our present work, including the D1 subtype (DR1), In-D1 1 suBtype (DR2), D2 subtype (D(2)R-4151), and In-D12 subtype (D(2)R, D(2)R-like, D(2)RA, and D(2)RA-like) according to their evolutionary status and protein structure, and ligand-binding site. D1-like subtypes and D2-like subtypes of dopamine receptors are not phylogenetically more related to each other than to other monoamines, such as the serotonin, adenosine, and histamine receptors [38,39]. In oysters, the In-D22 subtype dopamine receptor all clustered into one clade with other monoamines receptors such as the ADRA2A, AchM2, AchM5, and HTR2A of humans. Furthermore, the specificity of the receptor for natural ligands is also not very stringent among monoamine systems. For instance, the α2-adrenergic receptors and β-adrenergic-like receptors can also bind to dopamine [40,41], which confers some flexibility that is convenient for the monoamine receptor to bind to different ligands in different physiological systems [37]. The In-D22 subtype dopamine receptors which were close to other monoamine receptors may sometimes allow other monoamines to bind and activate the multiple monoamine signaling, thus enabling the oysters to deal with complex environmental fluctuations through the simple nervous system.

The predicted binding site for dopamine is located between transmembrane (TM) helices 3, 4, 5, and 6 [42]. Studies had found that ASP was the conserved residue for dopamine binding, the SER in TM5 was considered an important feature for dopaminergic binding and formed hydrogen bonds with the catechol hydroxyls of dopamine to increase the binding affinity [42,43]. In our results, except for D(2)R and D(2)R-like, all other five dopamine receptors had the conserved ASP binding site, and only D(2)RA and D(2)RA-like had the SER binding site, implying that these two dopamine receptors are important for dopamine's biological role in oysters.

Dopamine receptors are widely expressed in the central nervous system and the peripheral tissue and play crucial roles in regulating locomotion, cognition, emotion, and neuroendocrine activity [9]. In mice, dopamine D2 receptors knockout significantly reduced the activity of GH-IGF aix, the hypothalamic Ghrh expression, pituitary GH content,

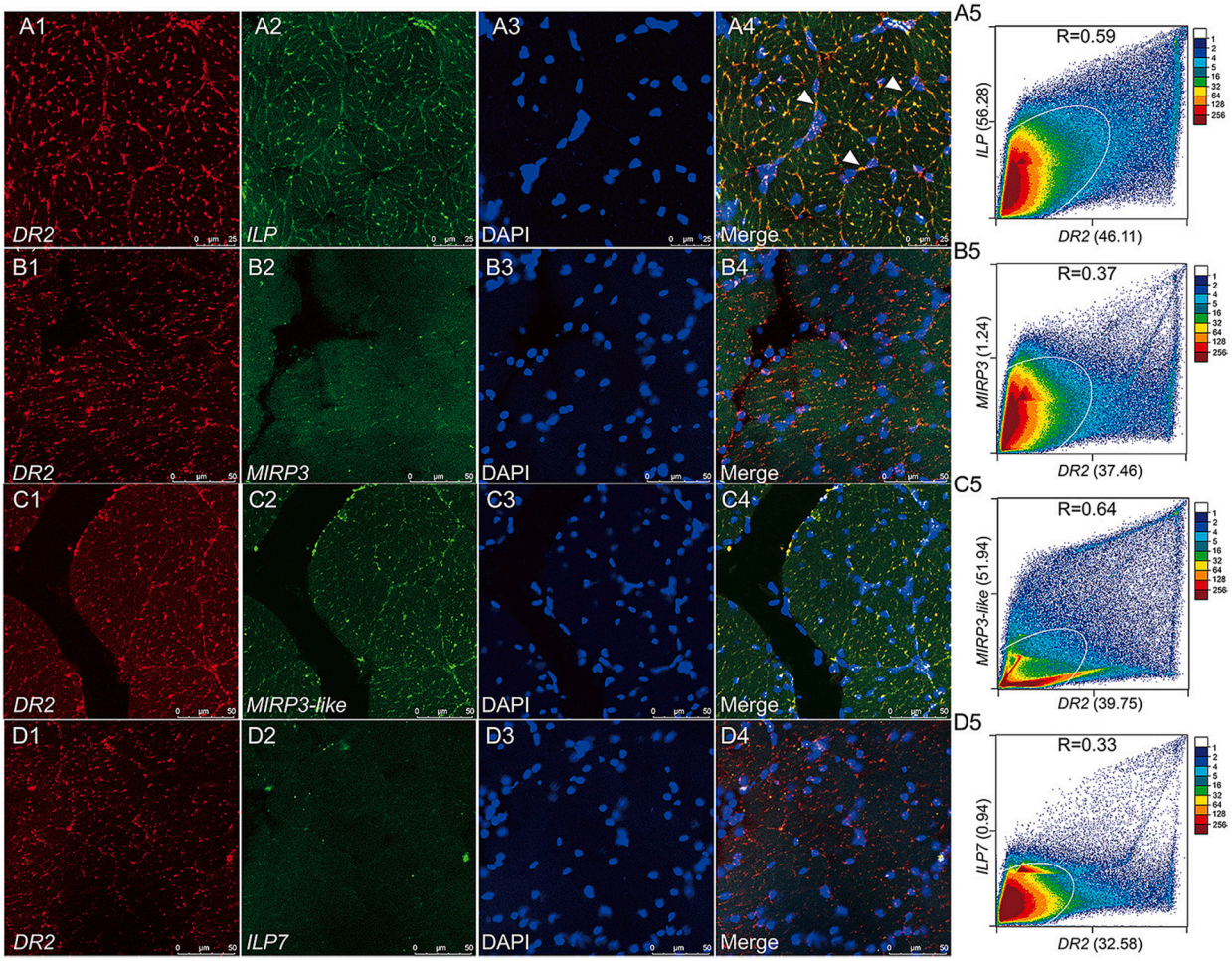

Fig. 8. Cellular co-localization of DR2 and ILPs in the adductor muscles of *C. gigas*.
Cellular co-localization of *DR2* with *ILP* (A1-A4), *MIRP3* (B1-B4), *MIRP3-like* (C1-C4), and *ILP7* (D1-D4). The arrowheads indicate the positive signals of *DR2, ILP* , *MIRP3,* or *MIRP3-like*. The fluorescence value and colocalization coefficient of *DR2* and ILPs were shown in A5-D5.

and serum IGF-I levels were all decreased, and finally inhibited the body growth [44]. Dopamine can also directly control movement by manipulating somatic motoneuron behavior and skeletal muscle, and this process is mainly mediated by D1-like dopamine receptors [45]. In mollusks, the crucial roles of visceral ganglia and adductor muscle in growth regulation have been widely reported. Our previous study confirmed that ILPs were dominantly expressed in visceral ganglia and adductor muscle. All four ILPs were expressed at higher levels in the fast-growing "Haida No. 1" than in wild oysters in adductor muscles [4]. Adductor muscles have been implicated in the storage and mobilization of nutrients in order to meet growth requirements. The activity of the adductor muscle is associated with feeding behavior and directly affects growth in the oyster [46,47]. The visceral ganglia contain various neurotransmitters, including the ILPs, dopamine, and serotonin, which function to regulate growth, development, and reproduction [48,49]. Especially for ILPs, they were generally produced from neural enrichment tissues such as the brain, clusters of neurons cell bodies, or the cerebral and visceral ganglia, then released into the hemolymph and transported to target cells, where they interact with receptors, triggering downstream signaling pathways to regulate the nutrition metabolism and growth of an organism [50]. Through single-cell sequencing of visceral ganglia, we also identified a cluster in which the MIRP3 and MIRP3-like specific expressed, confirmed the existence of IPCs in the visceral ganglia of oysters (data unpublished), which suggested that the ILPs might be synthesized from visceral ganglia and played indispensable roles in oyster growth. In addition, we confirmed that the expression of *TH*, the rate-limiting enzyme of dopamine, was positively associated with the growth rate in the oyster and was highly expressed in ganglia [18]. With the exact roles of the adductor muscle and ganglia in oyster growth, we just detected the expression of the seven dopamine receptors in these two tissues. Among all the seven dopamine receptors, only *D(2)RA-like* and *DR2* were highly expressed in visceral ganglia and adductor muscle. We speculated that dopamine might regulate the release of ILPs in the ganglia and adductor muscle through these two dopamine receptors in oysters.

The effect of local dopamine synthesis on insulin release has been widely studied. Accumulated evidence showed different doses of dopamine can induce opposite effects on insulin secretion [13], indicating the existence of the feedback loop in the dopaminergic system and insulin signaling regulatory network. In this study, with the treatment of exogenous dopamine, the expression of ILPs was significantly affected in the visceral ganglia and adductor muscles and showed a dose-dependent pattern that was consistent with other studies. In addition, the antagonist of dopamine receptors always induces the increase of glucose-stimulated insulin secretion [51]. Congruently, the knockout of the D2 dopamine receptor resulted in the reduction of pancreatic β-cell mass, suggesting that dopamine can modulate the cellular proliferation or apoptosis of the beta cells [52]. It's arguable that dopamine receptors are important for the physiological function and development of the pancreas in vertebrates. Once the activity of dopamine receptors was competitively inhibited by the antagonist, the expression of *ILPs* was significantly decreased in our study. Furthermore, the cellular co-

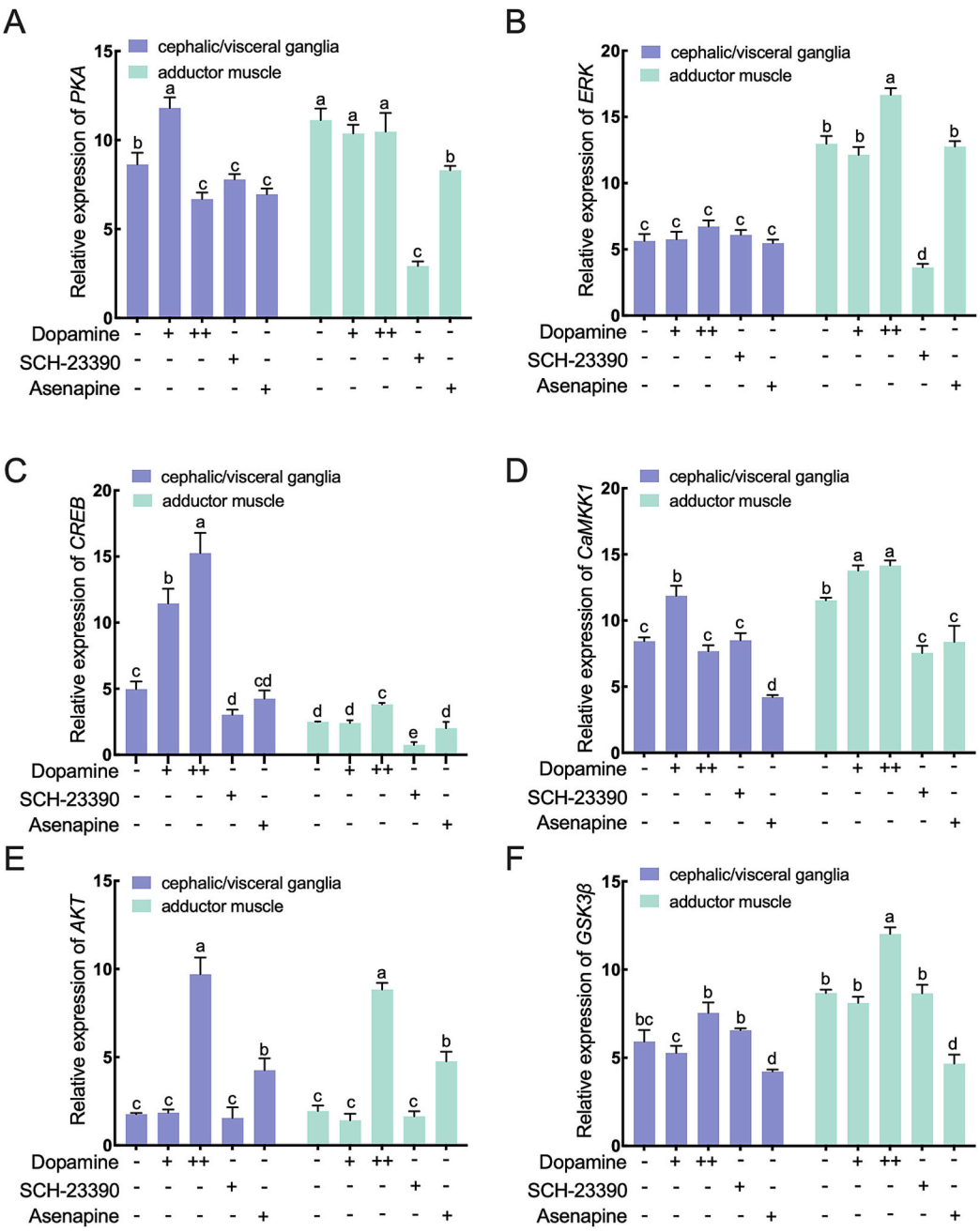

Fig. 9. Effect of dopamine hydrochloride and dopamine receptor antagonists on expression of the downstream components of dopamine signaling. The expression of *PKA* (A), *ERK* (B), *CREB* (C), *CaMKK1* (D), *AKT* (E), and *GSK3β* (F) in ganglia and adductor muscles at 12 h after treatment with 1 μg/mL dopamine hydrochloride (represented as "+"), 10 μg/mL (represented as "++") dopamine hydrochloride, the D1-like receptor antagonist SCH-23390 hydrochloride (represented as "SCH-23390"), and D2-like receptor antagonist asenapine hydrochloride (represented as "Asenapine"). Data are expressed as mean ± SD (n = 3).

localization indicated a co-expression of *D(2)RA-like* and *DR2* with *MIRP3* and *MIRP3-like* in the visceral ganglia and co-expressed with *ILP* and *MIRP3-like* in adductor muscles. Based on the aforementioned finding, we speculated that the invertebrate-specific type 1 and type 2 dopamine receptors, DR2 and D(2)RA-like, could play indispensable roles in the crosstalk between the dopaminergic system and insulin signaling. The positive signal of *D(2)RA-like* was more extensive than the *DR2* in visceral ganglia, while the positive signal of *DR2* was more extensive in adductor muscle, suggesting that the D(2)RA-like might play the most crucial role in ganglia, while the DR2 might most likely participate in the regulation of the insulin signaling in adductor muscle.

The activation of the dopamine receptor triggers several signaling cascades, including the inhibition of cAMP and decreased PKA activity which ultimately results in the activation of ERK and increased cell surface expression of DAT [7]. Upon the activation of type 2 dopamine receptors, the β-arrestin, PP2A, and Akt formed a complex that leads to the deactivation of Akt and release of the tonic inhibitory effects of Akt on GSK3 [53]. Studies found that the Akt was associated with cell growth and also functioned in the modulation of glucose homeostasis and dopamine transporter protein trafficking [54,55]. GSK3 was first identified as a downstream effector of insulin activation leading to glycogen synthesis. All these factors functioned to regulate the activity of the insulin downstream signaling and play indispensable roles in energy metabolism, which eventually influenced oyster growth. Furthermore, Gαi/o-dependent D2 regulation of gene expression dictated by the transcription factor CREB depended on an equilibrium between the binding of Par-4 and of calmodulin to the D2 receptor [56]. The elevation of cAMP levels induced by type 1 dopamine receptors could provide a second round of feedback by attenuating some of the effects of Ca^{2+} signaling [7]. The indispensable roles of CREB and Ca^{2+} metabolism in insulin secretion had been confirmed [57–60]. In our study, the expression of *PKA* and *CaMKK1* was increased at 1 μg/mL

dopamine hydrochloride and decreased at 10 μg/mL dopamine hydrochloride. The dopamine receptor antagonist treatment could also inhibit their expression, which was consistent with the expression patterns of *MIRP3* and *MIRP3-like*. The results suggested that dopamine regulated the secretion of *MIRP3* and *MIRP3-like* through the PKA signaling and Ca^{2+} metabolism. Furthermore, the expression of *ERK* was only induced by 10 μg/mL dopamine hydrochloride in the adductor muscle, the D1-like dopamine receptor antagonist SCH-23390 hydrochloride also significantly inhibited its expression, which was consistent with the expression of *ILP* in the adductor muscle. Thus we speculated that the D1-like dopamine receptor DR2 might participate in the secretion of ILP in adductor muscle through the PKA/ERK signaling in oysters. In addition, the expression of *AKT* and *GSK3β* was significantly increased at 10 μg/mL dopamine hydrochloride both in ganglia and adductor muscle which was consistent with the expression of *ILP* and *ILP7*. The D2-like dopamine receptor antagonist asenapine hydrochloride significantly induced the expression of *AKT*, which further inhibited the expression of its target gene *GSK3β* both in ganglia and adductor muscle, suggesting that the type 2 dopamine receptor D(2)RA-like might participate in the secretion of ILP through the AKT/GSK3β signaling. Furthermore, the expression of *CREB* was up-regulated with the treatment of exogenous dopamine and down-regulated with the treatment of dopamine receptor antagonists, which was consistent with the expression of ILPs. Our findings indicated that dopamine might regulate the secretion of ILPs through the downstream PKA and AKT/GSK3β signaling. The CREB may participate in regulating the expression of ILPs as a most important transcription factor, which deserves future investigation.

In this study, we classified dopamine receptors in *C. gigas* and analyzed the regulatory relationship between the dopaminergic system and insulin signaling. We found two important dopamine receptors, D(2)RA-like and DR2, which might play indispensable roles in the crosstalk between dopamine and insulin-like pathway both in the central nervous system and the peripheral tissue. Our work established the potential regulatory relationship between dopamine and insulin signaling pathways in the Pacific oyster, laid a foundation for studying the neuroendocrine regulation mechanism of oyster growth, and provided a valuable reference for constructing the neuroendocrine regulatory network in mollusks.

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CRediT authorship contribution statement

Yongjing Li: Investigation, formal analysis, Writing - Original Draft. **Ying Tan:** Assisted to complete the experiment, and search for literature. **Liting Ren:** Assisted to complete the experiment and data analysis. **Qi Li:** Supervision and Resources. **Jianxin Sui**: Assisted to prepare the insulin-like peptide antibodies. **Shikai Liu:** Modified the manuscript and provided financial support.

Declaration of competing interest

The authors declare that there are no financial or other potential conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.125703.

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