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Research paper

Crosstalk between dopamine and insulin signaling in growth control of the oyster

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ABSTRACT

Neuroendocrine hormones such as dopamine and insulin/insulin-like peptides play indispensable roles in growth regulation of animals, while the interplay between dopamine and insulin signaling pathways remains largely unknown in invertebrates. In the present study, we showed that tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis, was highly expressed in all tissues of the fast-growing oysters, and gradually increased with the development, which indicated the potential role of dopamine in growth regulation. Incubated with dopamine hydrochloride and insulin-like peptide recombinant proteins *in vitro* induced the expression of TH, suggesting a mutual regulatory relationship between insulin and dopamine signaling. Fasting and re-feeding experiments confirmed the role of TH in food intake regulation, also provide a clue about the potential regulatory relationship between the FoxO and TH. Further luciferase assay experiment confirmed that FoxO was involved in transcriptional regulation of TH gene through binding to its specific promoter region. This work provided insights into the crosstalk between dopamine and insulin signaling in growth control of mollusks.

1. Introduction

Growth of organisms is under control of multiple genetic and environmental factors. Intracellular signaling pathways, such as the insulin/insulin-like growth factor signaling (IIS) pathway and transforming growth factor β (TGF-β) pathway, play indispensable roles in growth regulation (Weiss and Attisano, 2013; Zhang and Liu, 2014). Environmental factors, such as temperature and food abundance, are also known to regulate growth of animals (Avery and Shtonda, 2003; So et al., 2012). In addition, neuroendocrine hormones such as growth hormone and insulin-like peptides, and bioamine such as dopamine and serotonin, all play important and diverse roles in growth regulation (Ashpole et al., 2015; Li et al., 2021a; Ling and Raikhel, 2018; Nagashima et al., 2016; Vélez et al., 2018).

Insulin-like peptides in *Drosophila* are produced from the insulin producing cells (IPCs) in the brain, while in mollusks without brain, insulin-like peptides are found in ganglia and other food intake related tissues, and play indispensable roles in the growth regulation and energy metabolism (Gomot et al., 1992; Kellner-Cousin et al., 1994; Li et al., 2021a; Nässel et al., 2013; Smit et al., 1988; Zhang and He, 2020). The neuromodulator dopamine, as the most important amine neurotransmitter, is produced from the dopaminergic neuron and plays a key role in motor control, motivated behaviors, food intake, and body size control (Hamasaka and Nässel, 2006; Nagashima et al., 2016; Xie et al., 2018). In addition, the interaction between dopamine and insulin signaling pathways had been reported in mammals (Kleinridders and Pothos, 2019). Insulin affects dopamine signaling through activation of insulin receptors and the downstream phosphoinositide 3-Kinase (PI3K)/AKT (also known as protein kinase B) signaling, and the activated AKT finally induces expression of the dopamine transporter (Nash,

Abbreviations: TH, tyrosine 3-monooxygenase; TH-like, tyrosine 3-monooxygenase-like; DAT, sodium-dependent dopamine transporter; MAO, dopamine-degrading enzyme monoamine oxidases; IIS, insulin/insulin-like growth factor signaling; PI3K, phosphoinositide 3-Kinase; AKT, known as protein kinase B (PKB); ILP, insulin like peptide; ILP7, insulin like peptide 7; MIRP3, molluscan insulin-related peptide 3; MIRP3-like, molluscan insulin-related peptide 3 like; ILPR, insulin like peptide receptor; IRS, insulin receptor substrate; Jak, Janus kinase; STAT3, signal transducer and activator of transcription 3.

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2017). In mammals, disturbed insulin signaling is always associated with alterations of tyrosine 3-monooxygenase or tyrosine hydroxylase (TH) activity, and the dopamine synthesis can be reverted through restoration of the insulin signaling (Fiory et al., 2019; Fordahl and Jones, 2017), suggesting the dopamine signaling was under the control of insulin signaling. However, in marine invertebrates, where dopamine is produced, and how dopamine and insulin signaling interplay to systematically regulate growth and metabolic homeostasis remain largely unknown.

TH, the rate-limiting enzyme of dopamine biosynthesis, is the marker to assess the level of dopamine (Hamasaka and Nässel, 2006). TH is expressed within central and peripheral catecholaminergic cells and is involved in a wide variety of physiological processes in both vertebrates and invertebrates (Rao et al., 2007). Previous studies revealed that TH was involved in regulation of neuroendocrine activity and food intake through controlling dopamine synthesis (Lindblom et al., 2006). Moreover, the receptors of insulin, leptin, ghrelin, and orexin are all ubiquitously expressed in dopaminergic neurons and colocalized with TH, indicting the potential relationship between the expression of TH and the activity of those hormones (Figlewicz and Sipols, 2010). Furthermore, TH promoter contains various regulatory elements binding with known growth-related transcription factors, including the hypoxia-inducible factor 1-alpha (HIF-1α) (Ban et al., 2017), activating protein-1 (AP1) and AP2 (Nakashima et al., 2003), forkhead transcription factor O (FoxO) and cAMP-response element binding protein (Gruntenko et al., 2016; Lewis-Tuffin et al., 2004). Therefore, we further investigate the role of dopamine signaling in growth regulation through analyzing the transcriptional regulation mechanism of TH.

In our previous study, we characterized the insulin-like peptide signaling and revealed its involvement in growth regulation in the Pacific oyster, *Crassostrea gigas* (Li et al., 2021a). In this work, we further investigated the interaction between insulin and dopamine signaling in growth control. The *C. gigas* is one of the most important aquaculture species around the world. We performed a selective breeding program of the *C. gigas* in China since 2007 and produced a fast-growing variety named as "Haida No. 1" (Li et al., 2011). The fast-growing oyster variety is now not only widely used for aquaculture in China but also provides an ideal model for growth study in the oyster. In the present study, we determined expression profiles of TH in various tissues between "Haida No. 1" and wild oysters, and during fasting and re-feeding treatment, to characterize the role of TH and dopamine in oyster growth. Furthermore, we performed experiments using *in vitro* tissue culture with incubation of dopamine to confirm the effect of exogenous dopamine on the TH, and insulin-like peptide recombinant proteins incubation was used to explore the interplay between dopamine and insulin signaling pathways. Dual-luciferase assay experiment was further carried out to reveal transcriptional regulation of TH via FoxO, a critical transcription factor in insulin signaling. The results suggested that dopamine played indispensable roles in growth regulation of *C. gigas* through interacting with insulin signaling, and the rate-limiting enzyme of dopamine synthesis, TH, was under control of the critical transcription factor of insulin signaling, FoxO. This work provided an insight into the crosstalk between dopamine and insulin signaling in growth control of oysters, which will be valuable for further investigation on the molecular mechanism of growth regulation in mollusks and could be also in other invertebrates.

2. Materials and methods

2.1. Animals

The healthy two-year-old "Haida No. 1" (95 ± 5 mm in shell height) and wild *C. gigas* (70 ± 8 mm in shell height) used in this study were cultured in same oyster farm and were maintained with same practice in Rongcheng, Weihai (Shandong, China). Twelve individuals were randomly selected from each of "Haida No. 1" and wild oyster populations. For tissue-specific expression profiling, nine tissues, including labial palp, gill, mantle, digestive gland, hematocyte, heart, visceral ganglia, cephalic ganglia, and adductor muscle were dissected, flash-frozen in liquid nitrogen, and stored at −80 °C freezer until RNA isolation. All animal experiments were conducted following the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China.

2.2. Real-time PCR and statistical analysis

The primer sets used for real-time PCR were designed using Primer Express software (Applied Biosystems, USA) and provided in Supplementary Table 1. Linear standard curves were generated with serial 2-fold dilutions of cDNA isolated from adductor muscle to determine the primer efficiency. Primer sets with an efficiency of 90–110% were used for real-time PCR analysis. All real-time PCRs were carried out in a LightCycler 480 real-time PCR machine (Roche, Switzerland) with a total reaction volume of 20 μL containing a mixture of 10 μL 2 × SYBR Premix ExTaq (TaKaRa, Japan), 2.0 μL of diluted cDNA templates, 6 μL of PCR-grade water, 1.0 μL of each forward and reverse primers. The PCR cycling parameters were set as follows: 95 °C for 3 min, followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 30 s. The melting curves at the end of each qPCR reaction were used to confirm that only one specific product was detected. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Significant differences ($P < 0.05$) were determined using the one-way ANOVA and Student's *t*-test as appropriate for single or multiple comparisons, respectively.

2.3. Tissue-specific expression profiles of CgTH gene determined by RT-PCR

For tissue-specific expression analysis, samples for each tissue collected from twelve individuals were pooled into four biological replicates (three individuals/replicate) to evaluate the expression of the *CgTH* gene between "Haida No. 1" and wild group. Briefly, total RNA was extracted from the pooled tissues using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1000 ng) was reversely transcribed into first-strand cDNA using PrimeScript RT Master Mix Perfect Real-Time Kit (TaKaRa, Japan), according to the manufacturer's instructions. Real-time PCR was carried out according to the method mentioned above.

2.4. Tissue-specific expression of CgTH determined by in situ hybridization

For *in situ* hybridization (*ISH*) analysis, we used 6-month-old juvenile oysters to exmaine the expression of *TH* in all tissues at a time. The whole soft body of 6-month-old *C. gigas* was collected and fixed in 4% paraformaldehyde at 4℃. Probes of sense and antisense digoxigenin-labeled RNA strands were transcribed *in vitro* with an RNA labeling kit (Roche, Germany) from the plasmid DNA containing *TH* from *C. gigas*. The sense probe of *TH* was also set up to guarantee the authenticity of the result. *ISH* was performed according to the protocol as previously described (Li et al., 2021a). All the *ISH* images were acquired with a Zeiss Axio Scope A1 microscope equipped with an AxioCam MRc5 digital camera.

2.5. Protein expression determined by Western blot

Protein expression was analyzed in eight tissues, including labial palp, gill, mantle, digestive gland, heart, cephalic ganglia, visceral ganglia, and adductor muscle, which were homogenized in 0.1 M PBS buffer. Samples were centrifuged at 13,000*g* for 10 min and the supernatants were collected. Protein concentration was measured using the Nanodrop 2000. The anti-Tyrosine hydroxylase was prepared using the

Table 1
Sequence characteristics of *TH* and *TH-like* genes in *Crassostrea gigas*.

Gene name	Gene full name	Amino acid (aa)	mRNA (bp)	ORF (bp)	NCBI Gene ID	Chromosome location
TH	*tyrosine 3-monooxygenase*	515	2260	1548	105,337,798	LG5
TH -like	*tyrosine 3-monooxygenase-like*	515	2275	1548	117,691,858	LG5

denatured tyrosine hydroxylase derived from pheochromocytoma in rats as an antigen, and the antibody could be used in the marine mollusks. The specificity of the antibody was confirmed by performing immunohistochemistry (Supplementary Fig. 1). For western blot experiment, proteins (20 μg) from each tissue were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (Millipore, America). The membranes were blocked with 5% skim milk dissolved in TBST buffer overnight at 4 ℃. After that, the membranes were washed three times with TBST, then incubated with primary antibody (anti-Tyrosine hydroxylase, Millipore, AB152, 1:1000) in TBST for one hour at 37 °C. After washing with TBST five times, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Beyotime, A0208) diluted 1:1000 in TBST for 30 min at 37 °C. The β-actin mouse monoclonal antibody (diluted 1:1000 in TBST) (Beyotime, AF0003) was used as control. Protein expression was detected using enhanced chemiluminescence detection reagents (Vazyme, E411) and visualized using the GE ImageQuant LAS4000mini system.

2.6. Expression of CgTH gene during larval development stages

Approximately 100 larvae samples from "Haida No.1" and wild oysters were collected in triplicate at four development stages, including D-shape larvae, early umbo larvae (17d), late umbo larvae (25d), and eye-spotted larvae, which were used to detect the *CgTH* expression. RNA extraction, cDNA synthesis, real-time PCR, and statistical analysis were carried out as described above.

2.7. In vitro culture of tissues and treatment with dopamine and insulin-like peptide recombinant proteins

For *in vitro* culture experiments, the cephalic/visceral ganglia (mixed) and adductor muscle were dissected from healthy two-year-old oysters, washed with PBS (pH 7.4) three times, treated with antibiotics containing penicillin (1000 IU/mL) and streptomycin (800 μg/mL) for 30 min, then washed with primary medium which was consisted of L15 medium and M199 (V: V = 1:1, pH 7.2–7.4) plus 5% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Finally, the tissues were cut into pieces with sterile scissors and cultured in a 12-well plate at 26 °C. For treatment with dopamine, the dopamine hydrochloride (Sigma, H8502) was added to the primary medium with the final concentration of 0, 1, 3, 5, 7, 10, 15, 20 μg/mL and maintained for 12 h. We determined the concentration of the dopamine hydrochloride based on our the preliminary experiment (Supplementary Fig. 2). For treatment with insulin-like peptide recombinant proteins, the recombinant proteins, which were purified from the BL21 using the prokaryotic expression plasmid pET32a containing the insulin-like peptide sequences of *C. gigas*, was added to the primary medium with final concentration of 0, 5, 10, 15, 20 μg/mL and maintained for 12 h. After that, the culture medium was discarded, and the cells were collected for RNA or protein extraction after washing with sterile PBS three times.

2.8. Plasmid constructs and site-directed mutagenesis

The promoter region of *CgTH* gene was retrieved from genome sequence in NCBI database. To identify the core element of the promoter, the sequence was truncated to different lengths and subcloned into the pGL3-basic vector (Promega, USA) according to the manufacturer's instruction of ClonExpress II One Step Cloning Kit (Vazyme, C112). In addition, primers containing the homologous sequence of the expression plasmids pcDNA3.1(+) vector (Invitrogen, UK) were used to amplify the ORF of FoxO and then subcloned into pcDNA3.1(+) vector. All plasmid DNA used in the present study was prepared using the EndoFree Max Plasmid Kit II (Tiangen, China). All the PCR parameters were set as follows: 95 °C for 3 min, and then 95 °C 30 s, 60 °C 30 s, 72 °C 1 min for 37 cycles, finally 72 °C for 5 min. Mutations of putative FoxO binding sites within the *CgTH* promoter region were carried out according to the manufacturer's instructions of Mut Express MultiS Fast Mutagenesis Kit V2 (Vazyme, C215). The primer sequences used for this study were provided in Supplementary Table 1.

2.9. Cell culture, transient transfections, and luciferase assays

The 293 T cells were cultured in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 2% Penicillin-Streptomycin solution (Sangon, Shanghai) with 5% CO_2 at 37℃. The 293 T cells were transfected using Xfect™ Transfection Reagent (Takara, Japan) with the following plasmids: 1) 0.5 μg of normal or deletion constructs of *CgTH* promoter cloned into pGL3-basic luciferase reporter plasmids; 2) 0 ~ 0.45 μg of pcDNA3.1 expression plasmid (Invitrogen, UK) containing the cDNA encoding FoxO; and 3) 0.1 μg/well of pRL-TK (Promega, USA). Renilla luciferase was employed as an internal control for transfection efficiency. The day before transfection, cells were seeded into 24-well plates with a concentration of $1x10^5$ cell/mL. At the time of transfection, 80% 293 T cells were confluent. The transfection solution was made of 75 μL Xfect reaction buffer containing precomplexed DNA and 1 μL Xfect Polymer Volume. At 48 h after transfection, cells were washed with PBS and lysed in 100 μL luciferase lysis buffer. Firefly luciferase and Renilla luciferase readings were obtained using the Dual-Luciferase Reporter Assay System (Promega) and SYNERGY H1 microplate reader (BioTek, USA).

2.10. Sequence analysis of CgTH

The amino acid sequences of TH from *Homo sapiens*, *Gallus gallus*, *Xenopus laevis*, *Anolis carolinensis*, *Danio rerio*, *Homarus americanus*, *Ctenocephalides felis*, *Tetranychus urticae*, *Drosophila melanogaster*, *Aplysia californica*, *Octopus bimaculoid*, *Mizuhopecten yessoensis*, *Limulus polyphemus*, *Lymnaea stagnalis*, *Crassostrea virginica*, and *Crassostrea gigas* were retrieved from the NCBI followed by further phylogenetic analysis. Multiple alignments of TH proteins were performed with BioEdit software, and the phylogenetic tree was constructed using the neighbor-joining (NJ) approach in MEGA7 (Kumar et al., 2016), the TH of *Amphimedon queenslandica* was used as an outgroup. The reliability of topological structure was tested using 1000 bootstrap replications.

3. Results

3.1. TH identification in C. gigas

Two *TH* genes including *tyrosine 3-monooxygenase* (*TH*) and *tyrosine 3-monooxygenase-like* (*TH-like*) were identified in *C. gigas*, according to the latest version of genome assembly in NCBI database (Assembly: cgigas_uk_roslin_v1, Accession: GCA_902806645.1, Release date: 14-Feb-2020). Their gene names, sequence characteristics, and accessions

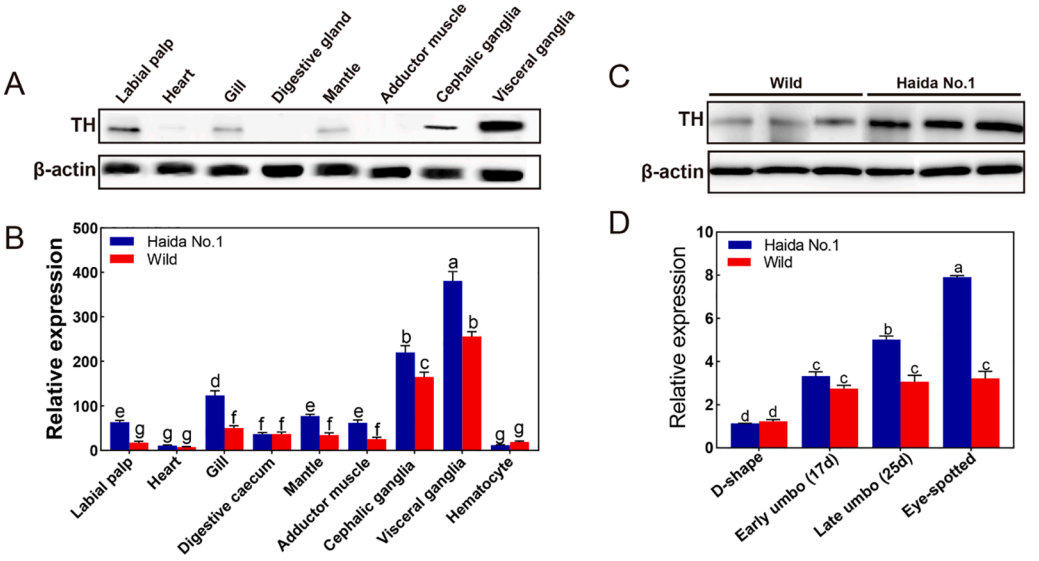

Fig. 1. Expression of *Cg*TH was associated with growth rate of *Crassostrea gigas*. (A) Expression of *Cg*TH in various tissues detected by Western blot. (B) mRNA expression of *CgTH* gene in various tissues detected by real-time PCR. Data are expressed as the mean ± SD (n = 4). The significant difference ($P < 0.05$) among various tissues is indicated by different lowercase letters. (C) Comparison of *Cg*TH levels between fast-growing "Haida No.1" and wild oysters (n = 3) as determined by Western blot. (D) Expression of *CgTH* was constantly increased during larval development. Data are expressed as mean ± SD (n = 3). The significant difference ($P < 0.05$) among different stages is indicated by different lowercase letters.

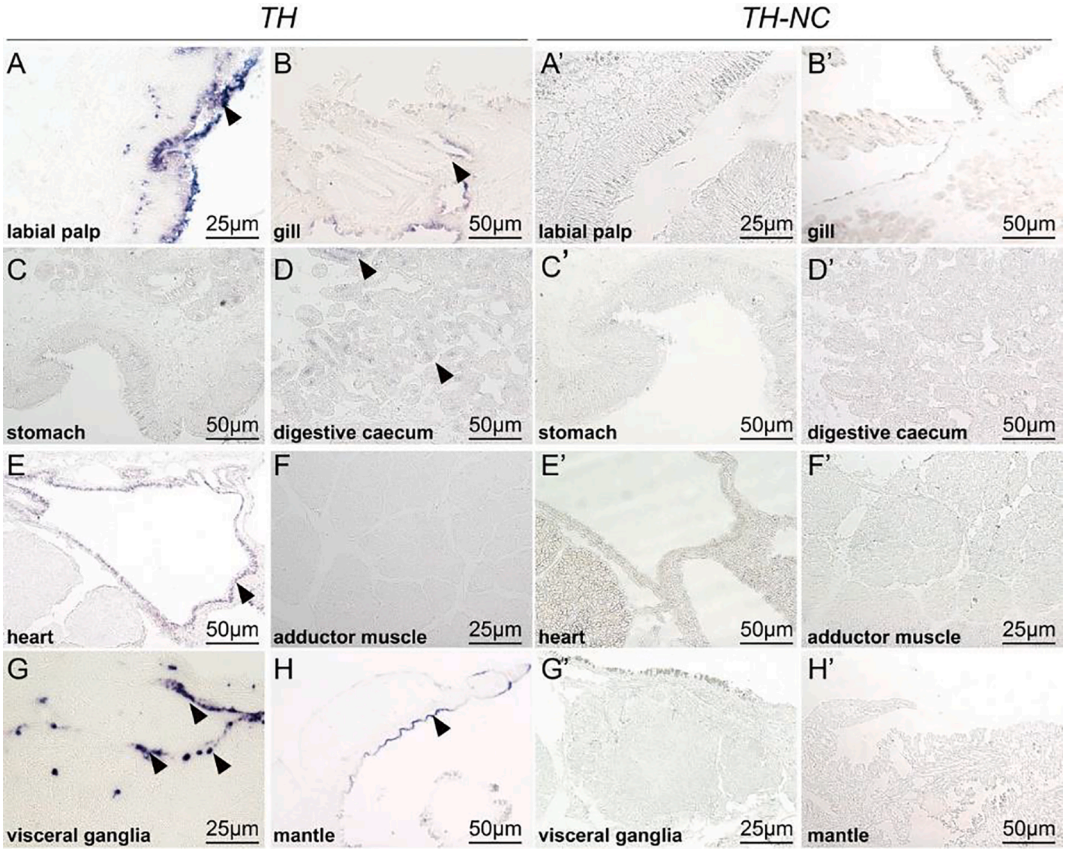

Fig. 2. Tissue-specific distribution of *CgTH* by *in situ* hybridization. Expression of *CgTH* was observed in the labial palp (A), gill (B), digestive caecum (D), visceral ganglia (G), and mantle (H), while no signal was detected in stomach (C) and adductor muscle (F), in 6-month-old oysters. Arrowheads indicated the positive signals of *CgTH*.

were provided in Table 1. The *TH* and *TH*-like genes were located at a same chromosome and coding identical amino acid sequences, we focused on one copy of *TH* gene (*Cg*TH, LOC105337798) for further analysis. Phylogenetic analysis suggested that the *Cg*TH was clustered into one clade with TH orthologs of other mollusk species, then clustered together with TH from vertebrates and arthropods (Supplementary Fig. S3). Further sequence analysis showed that *Cg*TH contained a potential catalytic domain, three conserved metal-binding sites, and seven cofactor binding sites which were similar to the TH of vertebrates and other invertebrates (Supplementary Fig. S4).

3.2. Expression of CgTH is associated with growth rate of C. gigas

Tissue-specific expression profiling in various tissues of *C. gigas* revealed that *CgTH* was highly expressed in visceral and cephalic ganglia, followed by gill, mantle, adductor muscle, digestive gland, and

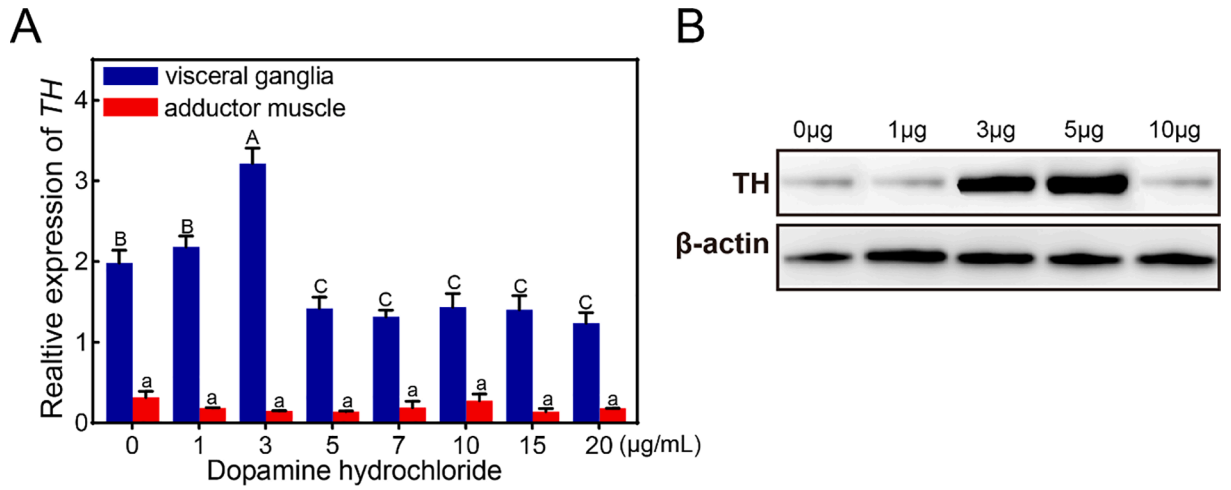

Fig. 3. Dopamine hydrochloride induced expression of *Cg*TH. (A) Expression of *CgTH* in tissues of adductor muscle (red bar) and cephalic/visceral ganglia (blue bar) at 12 h after treatment with dopamine hydrochloride at levels of 0, 1, 3, 5, 7, 10, 15, and 20 μg/mL detected by real-time PCR. Data are expressed as mean ± SD (n = 3). The significant difference ($P < 0.05$) among groups is indicated by different lowercase letters. (B) Expression of *Cg*TH in cephalic/visceral ganglia at 12 h after treatment with dopamine hydrochloride at levels of 0, 1, 3, 5, and 10 μg/ mL determined by Western blot.

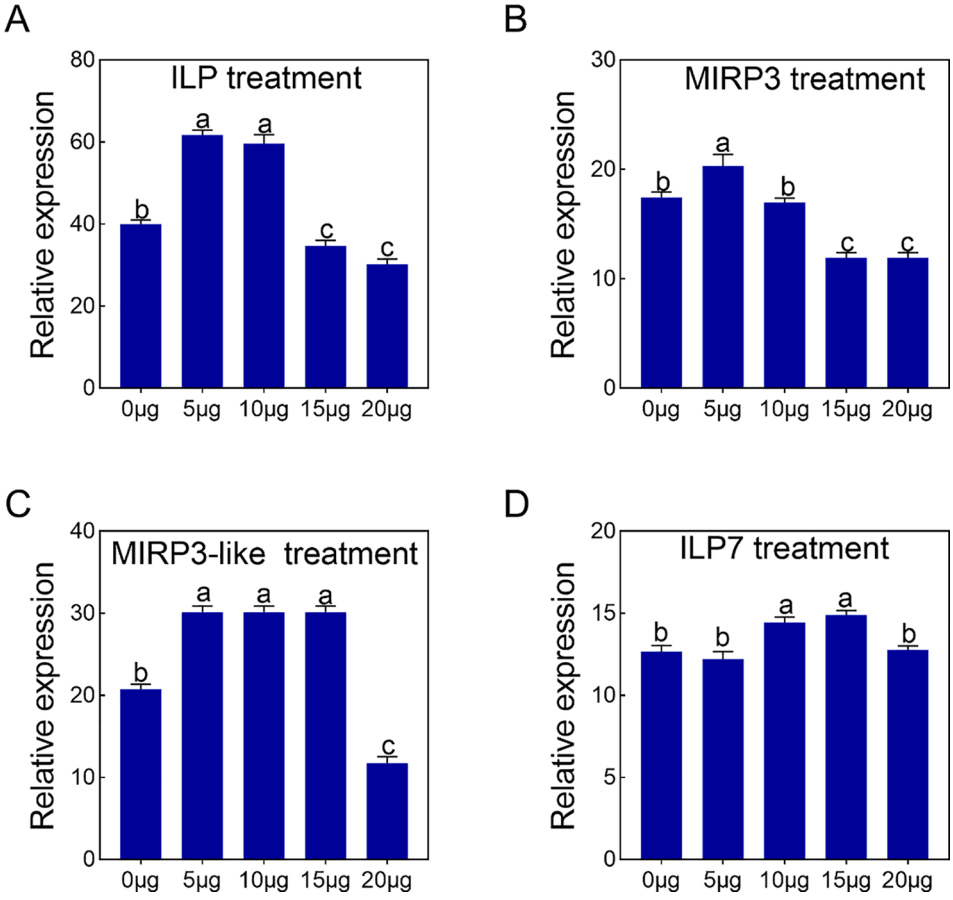

Fig. 4. Effect of insulin-like peptide recombinant proteins on the expression of *CgTH*. The expression of *CgTH* gene at 12 h after treatment with the ILP (A), MIRP3 (B), MIRP3-like (C), and ILP7 (D) recombinant protein at levels of 0, 5, 10, 15, and 20 μg/mL. Data are expressed as mean ± SD (n = 3). The significant difference ($P < 0.05$) among groups is indicated by different lowercase letters.

heart (Fig. 1A, B and supplementary Fig. S5), indicating that ganglia could be the main tissue for dopamine synthesis. The *Cg*TH was expressed at higher levels in fast-growing "Haida No. 1" than wild oysters in all tissues (Fig. 1C and supplementary Fig. S5), suggesting that expression of *Cg*TH is highly associated with growth rate of the oysters. Furthermore, the expression of *CgTH* was consistently increased during larval development and peaked at eye-spotted larvae stage in "Haida No.1", but in wild oysters, the expression of *CgTH* was just slightly increased at early umbo larvae (Fig. 1D). The *ISH* analysis further confirmed that *CgTH* was highly expressed in the visceral ganglia, with

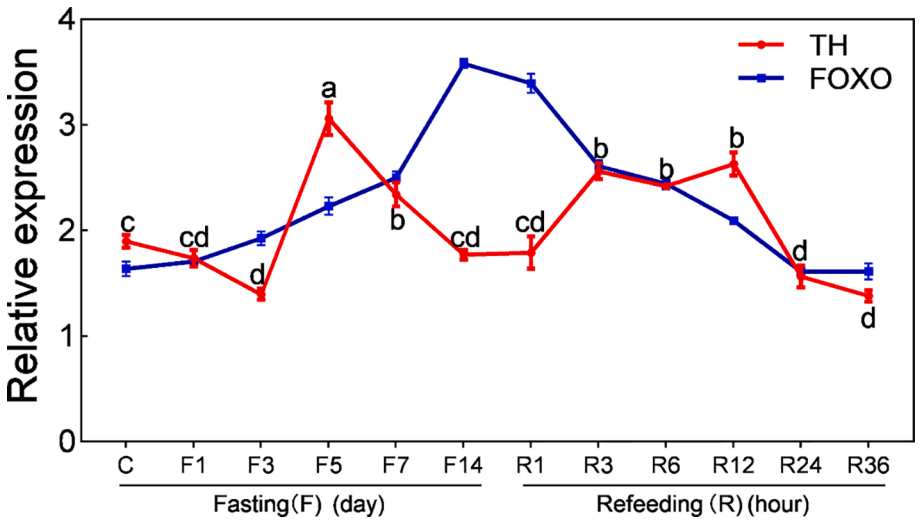

Fig. 5. Expression of *CgTH* was altered by levels of nutrients. The one-year-old oysters fasted for 14 days followed by refeeding for 36 h and were sampled at various time points for expression analysis. Expression levels of *CgTH* gene were determined at the beginning of experiment (control group, indicated by C), 1, 3, 5, 7, and 14 days after fasting (indicated by F1d, F3d, F5d, F7d, and F14d, respectively), and 1, 3, 6, 12, 24 and 36 h after re-feeding (indicated by R1h, R3h, R6h, R12h, R24h, and R36h, respectively). Data are expressed as the mean ± SD (n = 3). Significant difference ($P < 0.05$) among different time points is indicated by different lowercase letters. The expression data of the FOXO was retrieved from the previous study (Li et al., 2021b).

positive signals being also detected in labial palp, gill, digestive caecum, heart, and mantle, which were consistent with the real-time PCR results (Fig. 2).

3.3. Dopamine effectively induced the expression of CgTH

In vitro incubation with low concentration of dopamine hydrochloride significantly induced the expression of *CgTH* in cephalic/visceral

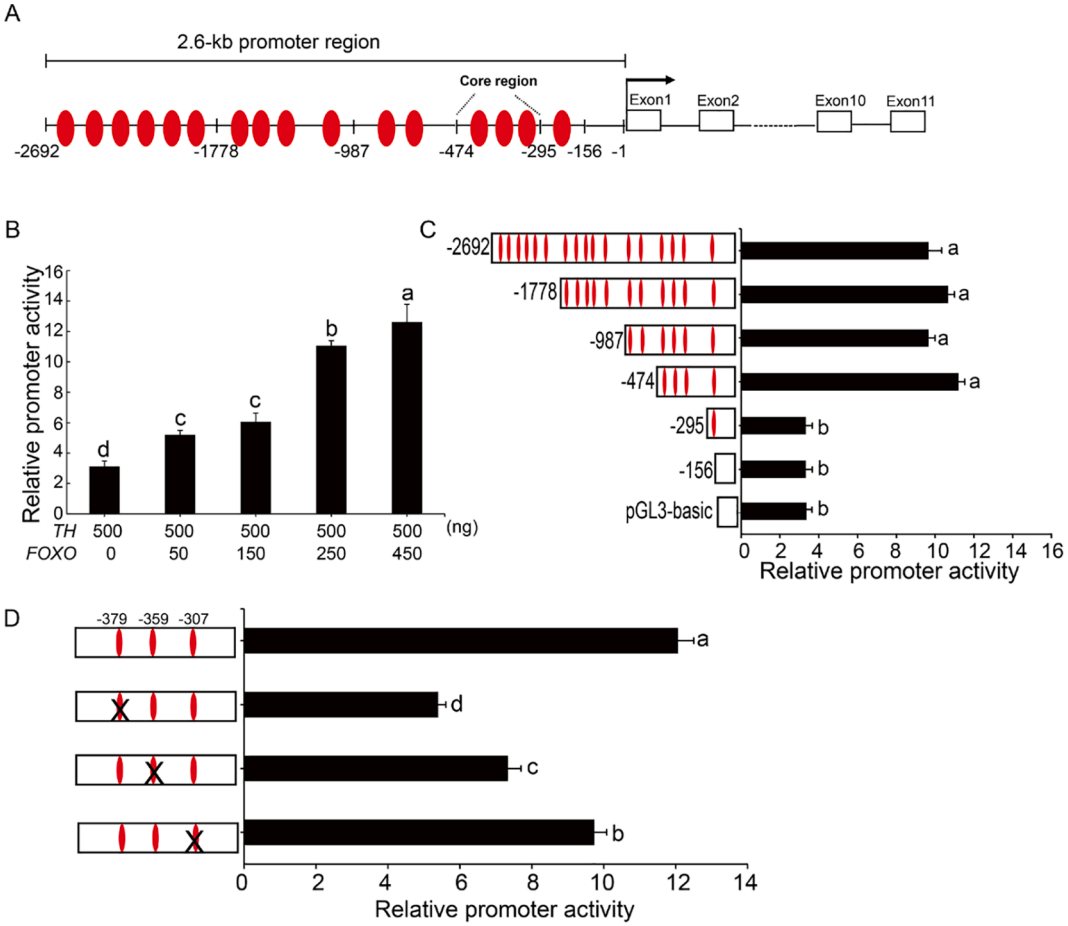

Fig. 6. FoxO binds to specific region within the promoter of *CgTH* gene. (A) Schematic presentation of the putative FoxO binding sites in the promoter region of *CgTH*. (B) FoxO activated the transcriptional activity of *CgTH* in a dosage-dependent pattern. (C) *CgTH* gene promoter deletion analysis. (D) *CgTH* gene promoter mutation analysis. Left panel: schematic presentation of the 5′-deletion constructs, the positions of the putative FoxO binding sites (C) and mutation sites (D) marked in red. Right panel: the relative promoter activities of *CgTH* which were determined by measurement of Firefly and Renilla luciferase activities and normalized with the empty pGL3-basic vector. Bars represent mean values ± SD (n = 3). The significant difference ($P < 0.05$) among groups is indicated by the different lowercase letters.

ganglia, while high concentrations of dopamine hydrochloride, including 7, 10, 15, and 20 μg/mL, significantly suppressed its expression (Fig. 3 and supplementary Fig. S5), indicating that dopamine synthesis was strictly controlled under the appropriate dopamine levels, and high levels of dopamine would pose negative feedback on its synthesis. Notably, the expression of *CgTH* in adductor muscle was not affected by the exogenous dopamine (Fig. 3), indicating that the major location for dopamine synthesis would be the ganglia.

3.4. Insulin-like peptides affect dopamine synthesis

Effects of insulin-like peptide recombinant proteins on the expression of *CgTH* were also investigated (Fig. 4). The results showed that all the four insulin-like peptide recombinant proteins could activate the expression of *CgTH* in a dosage-dependent pattern. The ILP recombinant protein activated the expression of *CgTH* under the concentration of 5 and 10 μg/mL, while suppressed its expression at high concentrations, including 15 and 20 μg/mL (Fig. 4A). Similarly, incubation with 5 μg/mL MIRP3 recombinant protein would activate the expression of *CgTH*, but suppress its expression with concentration of 15 and 20 μg/mL (Fig. 4B). The MIRP3-like recombinant protein activated the expression of *CgTH* under the concentration of 5, 10, and 15 μg/mL, and suppressed at 20 μg/mL (Fig. 4C). The ILP7 activated the expression of *CgTH* under the concentration of 10 and 15 μg/mL (Fig. 4D).

3.5. Nutrient abundance affects dopamine synthesis

The expression of *CgTH* was significantly suppressed on day 3 after fasting treatment, while was significantly increased after long-term fasting on day 5, then decreased with further fasting treatment. After refeeding, the expression of *CgTH* was acutely increased within 12 h once the nutrient was abundant (Fig. 5), but was decreased to a regular level shortly at 24 h. In addition, our previous study found the critical transcription factor of insulin signaling, FoxO, showed an opposite expression pattern with the TH (Fig. 5), indicating the potential regulatory relationship between the TH and FOXO.

3.6. Insulin signaling regulates dopamine signaling via transcriptional regulation of CgTH by FoxO

To further confirmed the regulatory relationship between the TH and FOXO, we analyzed the promoter region of the *CgTH* gene. We predicted 16 FoxO binding sites which possessed a core sequence of "TGTT(T/G)" in the forward strand or "(A/C)AACA" in the reverse strand (Fig. 6A and supplementary Fig. S6). Dual-luciferase reporter assay experiments suggested that FoxO could transcriptionally regulate *TH* through directly binding with the specific promoter element (Fig. 6B). Further analysis with truncated fragments of the *TH* promoter sequence revealed three core elements between position −474 and −295 because deletion of this region drastically reduced the promoter activity (Fig. 6C). Site-directed mutagenesis was further applied to the three key FoxO binding sites, respectively. The promoter activity was decreased more significantly once the −379 site was mutated than the other two binding sites (Fig. 6D). Therefore, we reasoned that the three FoxO binding sites were all critical, while the −379 site was the most important binding site for transcriptional regulation of Cg*TH* by FoxO in *C. gigas*. And the insulin signaling regulates dopamine signaling via transcriptional regulation of FoxO on the TH, the rate-limiting enzyme of dopamine synthesis.

4. Discussion

Neuroendocrine hormones such as dopamine and insulin/insulin-like peptides play indispensable roles in growth regulation of animals, while the interplay between dopamine and insulin signaling pathways remains largely unknown in invertebrates such as mollusks. In the present study, we showed that expression of TH, the rate-limiting enzyme of dopamine, was positively associated with growth rate in the oyster. The expression of *Cg*TH was significantly induced in tissue culture of cephalic/visceral ganglia and adductor muscle incubated with different concentrations of dopamine hydrochloride. Furthermore, incubation with four insulin-like peptide recombinant proteins could also affect dopamine synthesis, suggesting the mutual regulatory relationship between insulin and dopamine signaling. The expression of *CgTH* was altered accordingly to the levels of nutrients during fasting and refeeding experiment, indicating the potential role of dopamine in regulation of food intake and growth. Dual-luciferase assay experiment showed that FoxO was involved in transcriptional regulation of *TH* gene through interacting with DNA response elements within the promoter region.

In vertebrates, interactions between the dopamine pathway and appetite regulators have been proposed. The TH-positive neurons are always close to the orexin-positive axons, and the expressions of orexin receptors are ubiquitous within the dopamine neurons (López, 2009; Puskás et al., 2010; Vucetic and Reyes, 2010). TH plays indispensable role in growth control through the regulation of food intake and neuroendocrine activity. In our present study, we showed that *Cg*TH was expressed at higher levels in all tested tissues of the fast-growing variety "Haida No.1" than wild oysters, at both mRNA and protein levels. In addition, the expression of *CgTH* was being constantly increased during larval development and was altered with the levels of nutrient abundance. As the expression of TH reflects the level of synthesized dopamine, these results suggested the crucial role of dopamine in growth regulation of *C. gigas*.

Insulin and dopamine signaling interact to affect food intake, reward, and mood. In addition, receptors of metabolic hormones including insulin and leptin were expressed not only in peripheral tissues but also in multiple areas of the brain (Figlewicz and Sipols, 2010; Murray et al., 2014). Previous studies revealed that the insulin receptor substrate 2(IRS2)/PI3K/Akt and Janus kinase (Jak)/ signal transducer and activator of transcription 3 (STAT3) signaling pathways were involved in the dopamine system (Morton et al., 2009; Palmiter, 2007; Russo et al., 2007), suggesting that these metabolic hormones and signaling may function to regulate the dopamine signaling. In our present study, exogenous dopamine affected the expression of TH, the rate-limiting enzyme of dopamine synthesis. Under low concentration, exogenous dopamine could induce dopamine synthesis, while under high concentration, exogenous dopamine could obviously inhibit the expression of TH, and suppressed the dopamine synthesis activity. All those results suggested the expression of the TH was closely correlated with the dopamine content in *C. gigas*. The insulin signaling regulates the dopamine system through inducing the expressions of dopamine transporter (DAT) and dopamine-degrading enzyme monoamine oxidases (MAO) and then activating the uptake of released dopamine (Figlewicz et al., 1994; Cai et al., 2018). In our present study, incubation with four insulin-like peptide recombinant proteins significantly induced the expression of *CgTH*, which was consistent with the observation in mice (Fiory et al., 2019). Besides, the 5 μg/mL or 10 μg/mL insulin-like peptides induced the expression of *CgTH*, indicating that dopamine synthesis was increased. Our previous study revealed that the insulin-like peptides were dominantly expressed in the neural enrichment tissues especially in visceral ganglia (Li et al., 2021a), which was consistent with the observation of *CgTH*. Moreover, with the increased level of insulin-like peptides, the expression of *TH* was decreased, suggesting that high levels of insulin-like peptides may induce synthesis and accumulation of dopamine, and in turn affect the expression of *TH* through feedback regulation. All those results suggested the mutual regulatory relationship between insulin and dopamine signaling in the oyster.

FoxO is a key component of the insulin signaling pathway and is involved in diverse cellular and physiological processes including cellular homeostasis, metabolism, and the stress response (Menon and

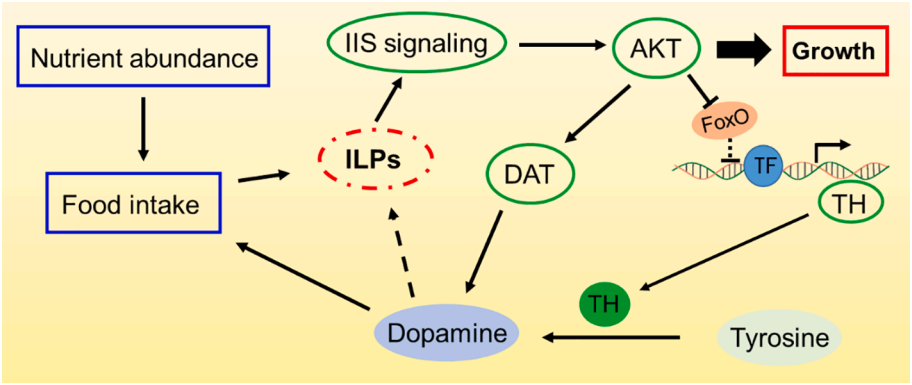

Fig. 7. A proposed model for crosstalk between dopamine and insulin signaling in growth control of the oysters. The nutrient levels affect food intake activity and the levels of insulin-like peptides (ILPs), which activate insulin/insulin-like peptide signaling (IIS) to affect its downstream pathways to regulate growth. The phosphorylation of IIS downstream kinase AKT induces expression of dopamine transporter (DAT) and suppresses FoxO to activate expression of TH through binding to its specific promoter region. The levels of TH are directly associated with synthesis of dopamine which affects food intake activity and possibly the activity of insulin-like peptide producing cells for production of ILPs.

Ghaffari, 2018). Emerging evidence suggests that FoxO also functions to regulate cognitive performance, stem cell maintenance, regeneration, and protection against stress in mammalian nervous system (Maiese, 2015). A recent study reported that FoxO was a key regulator of insulin/leptin-mediated food intake and energy expenditure in central nervous system, and the TH increment by leptin was via STAT3 activation and FoxO inhibition in the midbrain (Son et al., 2019). In addition, the expression pattern of *CgTH* was different from that of the FOXO during the fasting and refeeding process (Li et al., 2021b), indicating a potential transcriptional regulation relationship between FoxO and TH. Further luciferase assay experiment identified a core FoxO binding element from −474 to −295 within the promoter region of *CgTH* gene, suggesting the regulation of insulin signaling on dopamine synthesis through transcriptional regulation of *TH* by FoxO in *C. gigas*.

Taken together, we conclude with a model for the crosstalk between dopamine and insulin signaling in growth control of the oysters (Fig. 7). Specifically, the nutrient levels affect food intake activity and the levels of insulin-like peptides (ILPs), which activate insulin/insulin-like peptide signaling (IIS) to affect its downstream pathways to regulate growth. The phosphorylation of IIS downstream kinase AKT induces expression of DAT and inhibits FoxO to activate expression of TH through binding to its specific promoter region. The levels of TH are directly associated with synthesis of dopamine which affects food intake activity and possibly the activity of insulin-like peptide producing cells for the production of ILPs (Fig. 7). This work provided insights into the crosstalk between dopamine and insulin signaling in growth control of oysters, which will be valuable for further investigation on the molecular mechanism of growth regulation in mollusks and could be also in other invertebrates.

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Author contributions

SL conceived and designed the study. YL, LR, HF, and JT collected the samples and executed the experiments. YL, LR, BY and SL analyzed the data. YL drafted the manuscript, and SL revised the manuscript. ZL and QL provided reagents and materials and supervised the study. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113895.

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