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The expression of dopa decarboxylase and dopamine beta hydroxylase and their responding to bacterial challenge during the ontogenesis of scallop *Chlamys farreri*

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ABSTRACT

Dopa decarboxylase (DDC) and dopamine beta hydroxylase (DBH) is responsible for the synthesis of dopamine and norepinephrine, respectively. In the present study, dopa decarboxylase (CfDDC) and dopamine beta hydroxylase (CfDBH) were selected as indicator to investigate the development of catecholaminergic nervous system in the larvae of scallop *Chlamys farreri*. The CfDDC and CfDBH transcripts were all detectable during the whole ontogenesis expect for the CfDDC transcripts in 2-cell embryos stage. The expression level of CfDDC and CfDBH mRNA increased significantly in the veliger stage, and reached the peak in late (35.64-fold, $P < 0.05$) and mid-veliger (400.21-fold, $P < 0.05$) larvae, respectively. By immunofluorescence, two CfDDC immunoreactive areas were observed in the trochophore and D-hinged larvae, and then three CfDDC immunoreactive areas and two immunopositive fibres formed in early and late veliger larvae, respectively. Two CfDBH immunopositive fibers appeared initially in the early D-hinged stage, and another two similar fibers developed in the late D-hinged stage. The bacteria *Vibrio anguillarum* challenge could induce the mRNA expression of CfDDC and CfDBH in different developmental stage. The significantly increase of CfDDC mRNA was observed in the trochophore larvae at 12 h (8.61-fold, $P < 0.05$) and in late D-hinged larvae at 24 h (1.56-fold, $P < 0.05$) post challenge. The expression level of CfDBH mRNA decreased significantly in late D-hinged larvae at 6 h (0.45-fold, $P < 0.05$), whereas it increased significantly in late veliger larvae at 12 h after bacterial challenge (14.52-fold, $P < 0.05$). These results concluded that the scallop catecholaminergic nervous system appeared firstly as the form of dopaminergic neurons in the trochophore larvae, and the developing catecholaminergic nervous system in the trochophore, D-hinged and veliger larvae of scallop could respond to the immune stimulation in different patterns.

1. Introduction

The catecholaminergic nervous system consists mainly of the dopaminergic, norepinephrinergic and epinephrinergic neurons, which is distributed in the catecholaminergic region of brain and the sympathetic nervous system in mammal, and this system can be activated at a basal level to maintain host homeostasis [1]. For example, it can be activated by immune mediators to repress excessive immune response, repair immunologic injury and restore host homeostasis [2].

In recent years, catecholaminergic nervous system has been identified in adult invertebrate animals, including arthropod, mollusc, and nematode [3–5], and some crucial catecholamine metabolism enzymes have been characterized, such as dopa decarboxylase (DDC) and dopamine beta hydroxylase (DBH) [6–8]. Meanwhile, the development of catecholaminergic nervous system has also been investigated in some invertebrate animals [9–13]. For example, the development of catecholaminergic nervous systems have been outlined in larvae of the pond snail *Lymnaea stagnalis* and mussel *Mytilus trossulus* [14–17], and the catecholaminergic neurons are first detected in the veliger stage, and distributed in oesophagus, velum, foot and posterior regions [16]. Since the formaldehyde glutaraldehyde fluorescent technique used in these reports cannot discriminate the types of different catecholaminergic neurons, the development of catecholaminergic nervous system in larvae is still not well understood.

The catecholaminergic nervous system can interact with the immune system through immune mediators and catecholamines

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during the whole life history of vertebrate [18]. In the adult stage, catecholaminergic nervous system helps host to optimize the immune response and maintain the homeostasis [19,20]. When the host suffers from pathogen invasion, the catecholaminergic nervous system will be activated by immune mediators such as proinflammatory cytokines to release catecholamines into blood plasma [2], and these released catecholamines can modulate innate and adaptive immune response to eliminate the invasive pathogens and repair immunologic injury [21–23]. During the ontogenesis of vertebrates, the catecholaminergic nervous system is able to modulate the processes development and function of immune system [18,24,25]. Similar interaction between the catecholaminergic nervous system and immune system has also been reported in the adult invertebrates [26,27]. For example, catecholamines concentration increased significantly in the haemolymph of mollusc after bacterial challenge, and these catecholamines could regulate most of immune parameters, such as the total frequency and phagocytosis of haemocytes, and the activities of immune-related enzymes in haemolymph [27,28]. However, the knowledge of the interaction between the catecholaminergic nervous system and immune system is limited in the larvae of invertebrates.

The scallop *Chlamys farreri* is a dioecious bivalve native to the coast of China, Korea and Japan, and contributes weightily to the aquaculture industry of northern China. In recent years, the failure of scallop hatching has resulted in the significant decrease of larvae supply for aquaculture. The information about the development of catecholaminergic nervous system and its response to immune response would be helpful to find out the internal cause of scallop hatching failure. The purposes of this study were to (1) examine the temporal expression of CfDBH and CfDDC during in the early development stages of scallop *C. farreri*, (2) observe the CfDDC and CfDBH immunoreactivity in the scallop larvae, and (3) survey the alteration of CfDDC and CfDBH mRNA expression response against bacterial challenge in the different larval stage.

2. Materials and methods

2.1. Embryo, larvae and bacterial challenge

All embryos and larvae were sampled from the Yixiang farm in Rongcheng, Shandong Province, China in April and May of 2011. The samples from fourteen stages were identified microscopically, including oosperm, 2-cell embryos (1 h and 25 min post-fertilization), 4-cell embryos (2 h and 40 min post-fertilization), 8-cell embryos (3 h and 45 min post-fertilization), 16-cell embryos (4 h and 20 min post-fertilization), morula (6 h and 45 min post-fertilization), blastula (11 h and 25 min post-fertilization), gastruta (18 h and 25 min post-fertilization), trochophore larvae (22 h post-fertilization), early D-hinge larvae (50 h post-fertilization), late D-hinge larvae (74 h post-fertilization), early veliger larvae (113 h post-fertilization), mid-veliger larvae (168 h post-fertilization) and late veliger larvae (552 h post-fertilization). The samples from thirteen stages excepted for late D-hinge larvae were collected with six repeat sampling after addition of 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction. For immunofluorescence assay, the larvae from five stages including trochophore, early D-hinged, late D-hinged, early veliger and late veliger larvae were collected, and relaxed by gradually adding a 7.5% $MgCl_2$. After relaxation, the larvae were fixed in fresh 4% paraformaldehyde in phosphate buffered saline (PBS, 377 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 8.09 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, pH 7.4) for 1 h, washed in PBS three times (each time for15 min), and transferred into 70% ethanol and stored at −20 °C for subsequent immunofluorescence.

Two hundred thousand larvae in trochophore, late D-hinged and late veliger stages were transferred into 2.0 L aquarium filled with aerated seawater containing 10^8 CFU mL^{-1} of hot-killed bacteria *Vibrio anguillarum*, and each treatment were employed six times as the challenge group. The larvae from the same stages were transferred into six aquarium filled with aerated seawater, which were employed as the control group. At 6, 12 and 24 h after bacterial challenge, about sixty thousand larvae were collected from bacterial challenge group and control group for RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the embryos and larvae using Trizol reagent (Invitrogen) according to its protocol. The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo(dT)-adaptor as primer (Table 1). The reaction was performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:10 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR.

2.3. Quantitative real-time PCR

The quantitative real-time PCR was carried out in a total volume of 25 μL, containing 12.5 μL of 2 × SYBR Green Master Mix (Applied Biosystems), 2 μL of the 10 times diluted cDNA, 0.5 μL of each primers (10 mmol L^{-1}), 9.5 μL of DEPC-treated water. For the measurement of CfDDC mRNA, a 110 bp product was amplified with the sense primer P1 and the antisense primer P2 (Table 1) from cDNA template [7]. The sense primer P3 and the antisense primer P4 (Table 1) were used to amplify a 139 bp product to quantify CfDBH mRNA [8]. Two CfEF-1α (elongation factor 1 alpha from scallop *C. farreri*) primers, sense primer P5 and antisense primer P6 (Table 1), were used to amplify an 86 bp fragment as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The quantitative real-time PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) as described by Zhang et al. [29].

2.4. Immunofluorescence assay

The Whole-mount immunofluorescence assay was conducted as described by Voronezhskaya et al. [16]. In brief, all stored larvae were rinsed in PBS for 3 × 15 min. The shells of older larvae (day 2 and up) were decalcified in 5% EDTA in PBS for 30 min, rinsed in PBS 3 × 15 min, and blocked overnight in the blocking solution (10% normal goat serum, 0.25% bovine serum albumin, 1% TritonX-100 and 0.03% sodium azide in PBS). Then larvae were labeled by incubating them in anti-CfDDC or anti-CfDBH antibody (which were acquired via immunization 6-week old rats with CfDDC and CfDBH recombinant protein and confirmed to be specific by the western blotting, diluted 1:500 in the blocking solution) for 1–3 days at 4 °C [7,8]. These larvae were then washed (3 × 1 h) in PBS-TX and incubated for 1 day in goat anti-rat IgG conjugated to Alexa 488 (Invitrogen) diluted 1:100 in PBS. After incubation in secondary antibodies, all specimens were washed in PBS for 3 × 1 h,

Table 1
Sequence of the primers used in the experiment.

Primer	Sequence (5′–3′)	Sequence information
P1 (forward)	TGTTAGCCAGACCGTCAG	CfDDC specific primer
P2 (forward)	ATTATCTCCTTCTTCGTCCTCC	CfDDC specific primer
P3 (forward)	AGACCTTAACCAGAAACACCA	CfDBH specific primer
P4 (reverse)	TCAACAGAAACACTATCACCCT	CfDBH specific primer
P5 (reverse)	ATCCTTCCTCCATCTCGTCCT	CfEF-1α specific primer
P6 (reverse)	GGCACAGTTCCAATACCTCCA	CfEF-1α specific primer