Fish and Shellfish Immunology 93 (2019) 911–916

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Neuroendocrine immune-regulatory of a neuropeptide *Ch*GnRH from the Hongkong oyster, *Crassostrea Hongkongensis*

Qingsong Huang, Qiuhong Li, Hongmei Chen, Baohua Lin, Dongbo Chen*

School of Life Sciences, Bioparmaceutics of Guangdong Pharmaceutical University, Guangzhou, 510006, China

ARTICLE INFO

Keywords:
GnRH
Neuroendocrine-immune interactions
Oyster
cAMP signaling pathway

ABSTRACT

It is increasingly appreciated that neuroendocrine-immune interactions hold the key to understand the complex immune system. In this study, we explored the role of a reproductive regulation-related hormone, GnRH, in the regulation of immunity in Hong Kong oysters. We found that *vibrio* bacterial strains injection increased the expression of *Ch*GnRH. Moreover, *Ch*GnRH neuropeptide promotes the phagocytic ability and bacterial clearance effect of hemocytes which regarded to be the central immune organ. The content of cAMP after incubation with *Ch*GnRH peptide was increased, which could be blocked by adenylyl cyclase inhibitor SQ 22,536. Furthermore, the stimulated effect of *Ch*GnRH peptide on the phagocytosis and bacterial clearance was also blocked by SQ 22,536, H89 and enzastaurin, strongly demonstrating that cAMP dependent PKA and PKC signaling pathway was involved in *Ch*GnRH mediated immune regulation. In conclusion, this study confirms the presence of neuroendocrine-immune regulatory system in marine invertebrates, which contributes to understand the complexity of oyster immune defense system.

1. Introduction

The mammalian endocrine and immune systems interact and form a network to maintain the homeostasis of the body, which is bidirectionally regulated by signal molecules such as hormones and cytokines [1,2]. The cross-talk involving the endocrine and immune systems is now largely established, which named neuroendocrine-immune regulatory system (NEI system). It was reported classical hormones such as prolactin (PRL), growth hormone (GH) and even glucocorticoids (GC) could be produced by immune cells [3,4]. In fish, growth hormone (GH) enhances many aspects of immune functions including non-specific defences; cytotoxic, phagocytic, haemolytic and lysozyme activities [5,6]. GH has been found to increase particle ingestion by fish leucocytes *in vitro*, indicating an activation of phagocytic activity [7,8]. *In vivo* and *in vitro* administration of GH enhanced superoxide anion production as a killing mechanism following phagocytosis [8–10]. GH also increased mRNA levels of superoxide dismutase, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide [11]. All of these suggests an immune role of endocrine involving in the cell immunity.

The gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LHRH), is a decapeptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) that plays an essential role in the regulation of reproduction [12]. GnRH is responsible for the synthesis and secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) [13], which effects the gonadal steroid hormone production and gametogenesis [14]. GnRH is also considered as a neurohormone, mainly produced in the preoptic area of the hypothalamus, where contains most of the GnRH-secreting neurons. The length of GnRH subtype is almost decapeptides that contains the highly conserved amino acid sequences in the N-terminal (Glp-His-Trp-Ser) and C-terminal (Pro-Gly-Gly-NH2) regions, indicating that this molecular feature is crucial for receptor binding and activation [15]. In mammals, GnRH is found in organs outside of the hypothalamus and pituitary, such as the lymphocytes of the spleen and thymus [16]. With the deepening research on the interaction between the nervous system and the immune system, the role of GnRH in immune system has attracted more attention. Treatment with various concentrations of GnRH increased interleukin-2 receptor γ-chain mRNA in a dose-dependent manner, indicating that GnRH may be involved in lymphocyte activation [17]. Splenic and thymus lymphocytes have the capacity to produce immunoactive GnRH, further strengthening an association between the endocrine and immune systems [18–20].

More recently, new GnRH homologs have been identified in a variety of invertebrates [21–23]. In mollusks, GnRH mRNA is widely expressed in the central nervous system and accessory sex organs [24]

* Corresponding author.
E-mail address: chendb1966@163.com (D. Chen).

https://doi.org/10.1016/j.fsi.2019.05.055
Received 28 February 2019; Received in revised form 15 May 2019; Accepted 23 May 2019
Available online 24 May 2019

or oocytes [22]. The GnRH in octopus has a high expression level in brain and acts as a multifunctional modulatory factor in memory processing, sensory, movement, autonomic functions and feeding [25,26]. In the pacific oyster, two GnRH-related peptides (*Cg*GnRH-a and *Cg*GnRH-G) are characterized by mass spectrometry, which are high conserved with other mollusks or vertebrates [27]. However, it is reviewed that the functions of the invertebrate GnRH-like peptides are not necessarily related to reproduction [28]. Considering the relationship between the endocrine and immune system [5,29,30], and the fact that vertebrates GnRH have been proved to involve in the process of immune regulation, we hypothesize that the neuroendocrine immune-regulatory of GnRH may be traced to lower invertebrate, such as mollusks. The Hongkong oyster *Crassostrea Hongkongensis*, as an important global economic shellfish, mainly depends on innate immune system to defense invading microorganisms. Phagocytosis is one conserved cellular immune response for host to against microbes, or even apoptotic and necrotic cells [31,32]. It is well documented in oyster that defense against foreign bacterial is mediated by phagocytic cells which circulates in hemolymph. In this study, we discuss the role of neuropeptide *Ch*GnRH in oyster immune system and its influence on the ability of oyster hemocytes to engulf the fluorescent bacterial, in order to discuss the exist of cross-talk between endocrine and immune system in invertebrate.

2. Materials and methods

2.1. Oyster collection and reagent

The Hongkong oysters, *Crassostrea Hongkongensis* (two years old with an average 100 mm shell length) were obtained from Zhanjiang, Guangdong Province, China, and maintained at 22–25 °C in tanks with re-circulating seawater before experiments. Naive and pathogen-free oysters were chosen for the experiments, independently of their genetic background. The oysters were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*. They were held for two weeks prior to experimentation. *Ch*GnRH peptide were synthesized by GL biochem Ltd. Forskolin, SQ 22,536, H89 and enzastaurin were purchased from Sigma-Aldrich.

2.2. Tissue collection and bacterial challenge

To analyze the tissue distribution of *Ch*GnRH, total RNA was extracted from gill, mantle, adductor muscle, digestive gland, gonads and hemocytes of three healthy *Crassostrea Hongkongensis* individuals. To study the immune response of the *Ch*GnRH, one hundred oysters were randomly divided into 2 groups and placed in 2 tanks: the bacteria challenge and control group. Oysters were challenged by injecting 100 μl bacteria *Vibrio alginolyticus* or *Vibrio parahaemolyticus* (1×10^8) bacteria suspended with Phosphate buffer saline (PBS) into adductor muscles. The control groups were injected with equal volume of PBS. Hemolymph was collected at scheduled intervals (3, 6, 12, 24, 48, 72 h after pathogenic challenge) from the pericardial cavity through the adductor muscle and immediately centrifuged ($700 \times g$ for 10 min at 4 °C) to separate the hemocytes from plasma. Four individuals were randomly sampled in each group at every time point after injection.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's directions, and quantified by measuring absorbance at 260 nm. The integrity of RNA was checked by agarose gel electrophoresis. Purified RNA sample were diluted to 1 μg/μl and pooled to perform cDNA synthesis using PrimerScript™ First Strand cDNA Synthesis kit (TAKARA Bio Inc. Japan) following manufacturer's protocol.

2.4. Bioinformatics analysis of ChGnRH

Amino acid sequence of *Ch*GnRH was aligned with GnRH protein sequences of different species obtained from BLAST analysis using BioEdit Sequence Alignment Editor. The phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) version 3.0 with the neighbor-joining method with 1000 bootstrap replicates.

2.5. Transcription analysis of ChGnRH by quantitative real-time PCR (qRT-PCR)

qRT-PCR analysis was used to determine the expression of *Ch*GnRH mRNA in various tissues and during bacterial challenge using the gene-specific primers (Supp Table 1). Each assay was performed in triplicate with β-actin mRNA as internal control, as it is a gene which is least affected by bacterial infection and is independent of tissue type [33]. The qRT-PCR was conducted using a LightCycler 480 (Roche) in a reaction volume of 10 μl containing 1 μl of template cDNA, 5 μl of 2 × SYBR Green Mix, 1 μl of each primer (10 pmol/μl), and 3 μl of PCR-grade water. The qRT-PCR cycle program consist of one cycle of 95 °C for 1 min, following by 40 cycles of amplification 95 °C for 15 s, 55 °C for 15 s, 72 °C for 20 s, 85 °C for 20 s for signal collection in each cycles. The dissociation curve analysis of amplification products was performed to confirm the specificity at the end of each PCR reaction. The relative expression of *Ch*GnRH gene was calculated using the $2^{-\Delta\Delta CT}$ method [34]. All data are given in terms of relative mRNA, expressed as means ± standard error of mean (SE).

2.6. Phagocytosis assay

Vibrio alginolyticus and *Vibrio parahaemolyticus* labeled with FITC (fluorescein isothiocyanate) could emit green fluorescence. Then the hemocytes were incubated with GnRH peptide for one hour. The bacteria *V. alginolyticus* and *V. parahaemolyticus* were incubated with the hemocytes cultured in the 24 well plates for 15 min in a 50:1 ratio. Next, hemocytes were washed three times with Tris buffer (pH8.0, 50 mM) and suspended in PBS + 15% EDTA solution. Finally, flow cytometry assay was performed to detect the phagocytosis of oyster hemocytes. Hemocytes phagocytosis was monitored using at least a total of 10,000 events.

Moreover, SQ 22,536 (500 μM), H89 (50 μM), enzastaurin (25 μM) were added to hemocytes with *Ch*GnRH peptide (10^{-5} g), respectively and incubated for two hours, following by detecting the hemocytes phagocytosis as described above.

2.7. Bacteria clearance assay

The bacterial clearance assay was performed as described previously with some modification [35,36]. Two strains, *Vibrio parahaemolyticus*, *Vibrio alginolyticus,* (the concentration is probably OD = 0.2) were first prepared. Oyster hemocytes were collected and cultured in 24-well plates (~2.5×10^5 cells) for an hour with GnRH peptides, and then exposed to bacteria in a 50:1 ratio. After half hour incubation, extracellular bacteria were removed by washing with 0.02% trypsin-EDTA for four times. Immediately, the hemocytes were lysed in 1 mL PBS buffer containing 0.05% Triton X-100. Finally, 50 μl of lysate was used for the purpose of culturing on LB plates to achieve bacterial counts. For each group, three wells were used to perform bacteria clearance assay and each experiment was repeated three times. The survival rates of *Vibrio* was calculated by the formula: the survival rates = the number of survival bacterial/the total number of adding bacterial, which was reported previously [37].

Moreover, SQ 22,536 (500 μM), H89 (50 μM), enzastaurin (25 μM) were added to hemocytes with ChGnRH peptide (10^{-5} g), respectively and incubated for two hours, following by detecting the bacterial

clearance rate as described above.

2.8. ChGnRH stimulated the production of cAMP in hemocytes

Cyclic AMP (cyclic adenosine 3′, 5′-monopthosp, MW 351.2) is one of the most important intracellular mediators. In order to examine the effect of *Ch*GnRH on intracellular cAMP production, a cAMP - Gs dynamic assay kit (Cisbio, 62AM4PEB) was used. Oyster hemocytes were collected and cultured for half an hour, followed by incubation with different concentrations (10^{-7} g, 10^{-6} g, 10^{-5} g) of *Ch*GnRH peptide for 2 h. *Ch*GnRH peptide were synthesized by GenScript Biotech Corp.

Next, hemocytes were lysed for detection of the cAMP concentration according to manufacturer's instructions. Different concentrations of SQ 22,536 and *Ch*GnRH peptide (10^{-5} g) were added to hemocytes and incubated for two hours, following by detecting the cAMP concentration as described above.

3. Result

3.1. Sequence analysis and phylogenetic analysis of ChGnRH

Amino acid sequence alignments (Supp Fig.1A) of the GnRH precursor protein shows that 11 amino acids form the mature peptides and have a high degree of identity with the GnRH in mammals, amphibians, fish and other shellfish. Phylogenetic tree was generated based on the GnRH precursor protein from eleven species, including *Homo sapiens, Gallus gallus, Mus musculus, Sus scrofa, Sparus aurata, Xenopus laevis, Oryzias latipes, Danio rerio, Mizuhopecten yessoensis, Crassostrea virginica* and *Crassostrea Hongkongensis* (Supp Fig.1B). The evolutionary tree revealed GnRH had one ancient origin prior to divergences between protostomia-deuterostomia.

3.2. Tissue distribution and temporal expression of ChGnRH following bacterial challenge

qRT-PCR was used to detect the distribution of *Ch*GnRH mRNA in different tissues. The results revealed that *Ch*GnRH was constitutively expressed in all tissues including mantle, gill, digestive gland, hemocytes, heart, adductor muscle and gonads, supporting that *Ch*GnRH had a broad distribution range in cells and tissues [38,39]. Moreover, the expression level of *Ch*GnRH was higher in hemocytes and gill than gonads as show in Supp Fig.2.

Next, we investigated the temporal mRNA expression of *Ch*GnRH in the hemocytes post bacterial challenge (Fig. 1). After injection with *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, the expression level of *Ch*GnRH was up regulated within 24 h, and it was significantly changed at 6, 12 and 24 h. The highest expression level of *Ch*GnRH could be observed at 12 h and 24 h respectively, post *V. alginolyticus* or *V. parahaemolyticus* infection. Then the expression level recovered to the rest state. The control group injected with PBS maintained the normal expression.

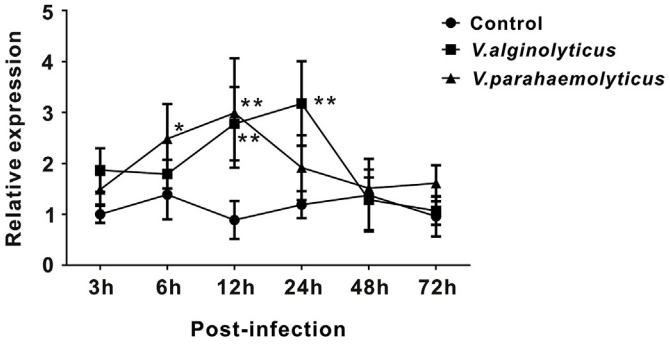

Fig. 1. *Ch*GnRH mRNA expression after bacterial challenge in hemocytes. *Ch*GnRH mRNA expression of each tissue was calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as the internal control. Error bar represents the means ± SE (N = 4).

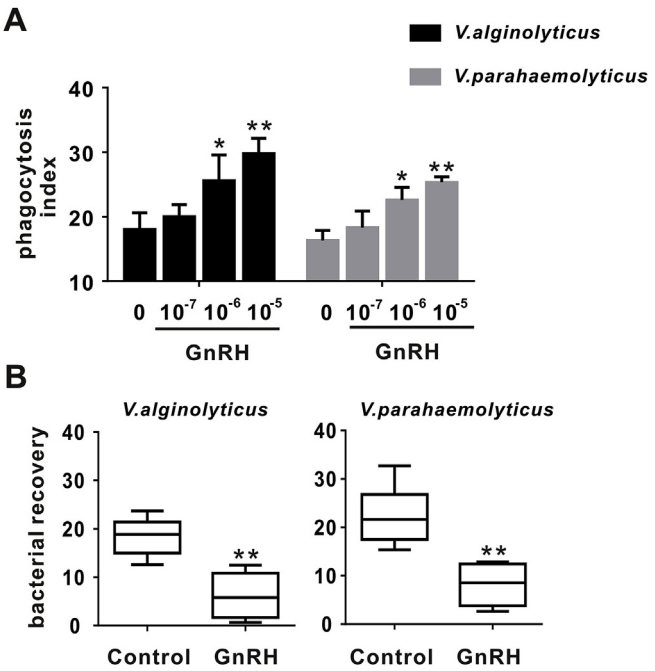

Fig. 2. *Ch*GnRH peptide regulates innate immunity in hemocytes. (A) Data analysis was performed using GraphPad Prism 5 software and vertical bars represent the mean ± SE (N = 3). The phagocytosis percentage of *v. alginolyticus* (*left panel*) and *v. parahaemolyticus* (*right panel*) of the *Ch*GnRH (10^{-7}~10^{-5}μM) incubation group are higher than those of the control group. (B) The clearance capacity of hemocyte to bacteria (*Vibrio parahaemolyticus*, and *Vibrio alginolyticus*) at treatment of 10^{-6}μM *Ch*GnRH. Data represent the mean ± SE (N = 3). Significant differences were indicated with * at $P < 0.05$ and ** at $P < 0.01$.

3.3. ChGnRH promotes bacteria phagocytesis and clearance

Phagocytosis is a basic strategy of immune defense for oyster hemocytes. To investigate the possible effect of GnRH on the phagocytic ability of oyster hemocytes, the synthesized *Ch*GnRH peptide was incubated into primary hemocyte culture. As shown in Fig. 2A, the results showed that the phagocytic ability was significantly increased to either *V. alginolyticus* or *V. parahaemolyticus* after incubation with *Ch*GnRH dose of 10^{-6} and 10^{-5}μM, indicating the key role of *Ch*GnRH in the process of hemocytes immunity. Meanwhile, the dose-dependent effect of *Ch*GnRH on the enhancement of hemocytes phagocytosis were also observed and the highest phagocytic proportion to *v. alginolyticus* and *v. parahaemolyticus* the reached 29.8% and 26.2% at dose of 10^{-5}μM GnRH, which increased 1.7- and 1.6-fold when compared to control, respectively.

Bacterial clearance is an effective way of hemocytes to eliminate invaders pathogens. The results found that *Ch*GnRH significantly enhanced the clearance of *v. alginolyticus* and *v. parahaemolyticus* (Fig. 2B). After 30min of hemocytes phagocytosis, the viable *v. alginolyticus* and *v. parahaemolyticus* occupies 18.4% and 21.8% of the bacteria amount initially added, which means that majority of bacteria were cleared by hemocytes. In the experimental group incubated with *Ch*GnRH, only 6.2% and 8.5% of *v. alginolyticus* and *v. parahaemolyticus* survived.

3.4. ChGnRH enhanced the production of cAMP in the hemocytes

To investigate the signaling pathway of *Ch*GnRH in oyster hemocytes, we examined its activation effect on cAMP production. Forskolin

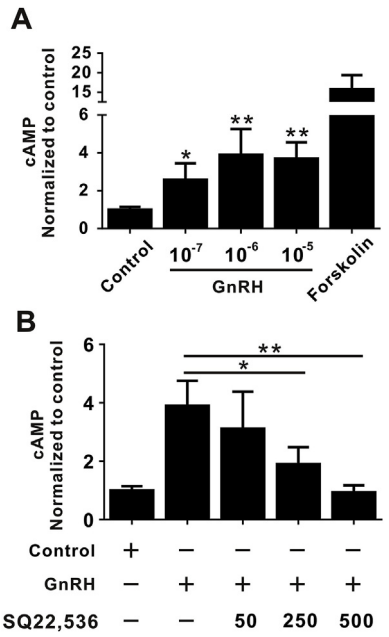

Fig. 3. *Ch*GnRH peptide stimulates cAMP production in hemocytes. (A) The effect of ChGnRH peptide on the cAMP production at different concentration (10^{-7}~10^{-5} M). Treatment of forskolin (5 μM) was as a positive control to stimulate the cAMP production. The adenylyl cyclase inhibitor SQ 22,536 (50–500 μM) was used to block cAMP production. Data represent the mean ± SE (N = 3). Significant differences were indicated with * at P < 0.05 and ** at P < 0.01.

is one adenylyl cyclase agonist to stimulate the cAMP production. After half of hour stimulation by *Ch*GnRH, the hemocytes lysis was collected to detect the concentration of cAMP. As shown in Fig. 3A, it is clearly observed that the content of the cAMP was dose-dependent and significantly enhanced as the concentrations of *Ch*GnRH increases, which reached 2.8-fold–3.9-fold higher than that of the control group. Meanwhile, the cell-permeable adenylyl cyclase inhibitor SQ 22,536 was co-incubated and significantly blocked the stimulation effects of *Ch*GnRH in the dose dependent effects (Fig. 3B), confirmed cAMP production in GnRH stimulated hemocytes.

3.5. *ChGnRH enhanced the hemocytes immunity via cAMP signaling pathway*

In order to further investigate which cAMP signaling pathway involves *Ch*GnRH mediated immune defense oysters, the selective inhibitor of cAMP-dependent protein kinase (PKA)-H89 and the protein kinase C beta inhibitor-enzastaurin, were used to co-incubation with *Ch*GnRH, respectively. As shown in Fig. 4A, *Ch*GnRH significantly enhanced the phagocytosis percentage of *v. alginolyticus* and *v. parahaemolyticus* and reached at 26.7% and 23.8%, and co-incubation with SQ 22,536 dramatically decreased the phagocytosis capability. Meanwhile, both H89 and enzastaurin could effectively block the stimulated effect of *Ch*GnRH on phagocytosis, suggesting involvement of both PKA and PKC signaling pathway in immune defense. Next, we also test whether cAMP pathway regulates the ability of bacterial clearance in oyster. Similarly, *Ch*GnRH could enhance the bacterial clearance to either *v. alginolyticus* or *v. parahaemolyticus*. However, this enhancement was blocked when co-treatment with SQ 22,536, H89 (50 μM) and enzastaurin (25 μM) (Fig. 4B). Taken together, these experiments demonstrated that cAMP dependent PKA and PKC signaling pathway was involved in *Ch*GnRH mediated immune regulation.

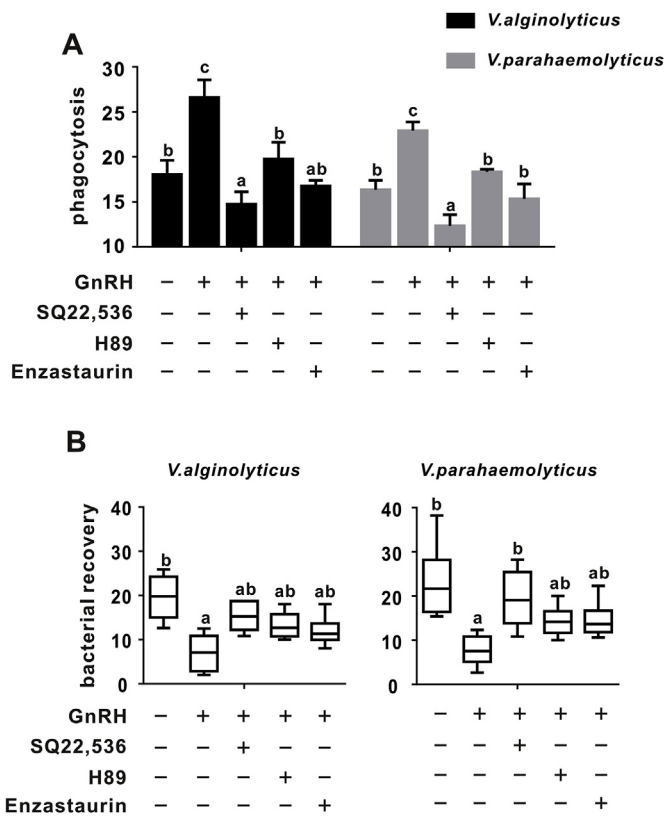

Fig. 4. cAMP dependent signaling pathway involves in *Ch*GnRH mediated immune defense. Effects of SQ22,536, H89 and enzastaurin on *Ch*GnRH stimulated phagocytosis (A) and bacterial clearance (B). Data represent the mean ± SE (N = 3). Different letters refers to significant differences.

4. Discussion

Bidirectional communication between the immune and neuroendocrine systems is responsible for the infection with complex adaptations. Gonadotropin-releasing hormone (GnRH) is a highly conserved decapeptide hormone that plays a key role in the neuroendocrine system of vertebrates and also participates in the host's immune response process. It is known to possess direct immunomodulatory effects *in vivo* and *in vitro* in castrated rats and have been shown to exist in spleen and thymus, resembling a cytokine [18,40]. The presence of GnRH in human peripheral T-cells ($CD4^+$, $CD8^+$) has also been extensively reported, which suggest a potential role of GnRH in immune regulation [41]. Similar studies about cross-talk between endocrine and immune system have also been conducted in many marine invertebrate [42,43]. Our study reveals that *Ch*GnRH has a high expression level in oyster immune organs, indicating a cross-talk between endocrine and immunity in mollusk.

Advances in molecular approaches in immunology prove that cross-talk involving the endocrine and immune systems is now largely established in vertebrate and invertebrate including oyster. However, the signaling pathway involved in GnRH dependent immune activation are still unclear. Generally, GnRH could stimulate cAMP second messenger pathway in varied cell types [44,45]. In agreement with this, our study was undertaken and demonstrated that low dose of Hongkong oyster GnRH peptide could elicit cAMP synthesis in the hemocytes cells, while adding SQ 22,536 (inhibitor of adenylyl cyclase) can effectively weaken the activation effect of *Ch*GnRH on cAMP production. GnRH was also reported to increase cAMP levels and stimulated a cAMP-responsive promoter in rats that affected hormone release and synthesis [46]. The potential relationship of GnRH and cAMP has been strengthened recently observed, suggesting the conserved signaling pathway of GnRH

from human to oyster.

Cyclic AMP regulates a number of different cellular processes such as cell growth and differentiation, ion channel conductivity, synaptic release of neurotransmitters, and gene transcription [47]. Recent studies has demonstrated that activation of cAMP-dependent signaling pathways is necessary for control phagocytosis through actin rearrangements in human neutrophils [48], which plays a crucial role in engulfment of invaders and apoptotic cells, and maintaining the cell homeostasis [49,50]. GnRH was examined in phagocytic leucocytes of rainbow trout (*Oncorhynchus mykiss*), which indicated GnRH stimulates phagocytosis and superoxide production in fish leucocytes through a GnRH-receptor-dependent pathway [51]. Similar result is observed in Hongkong oyster that *Ch*GnRH also harbors the capacity of improving phagocytosis and bacterial clearance in hemocytes to regulate the immune responses, which could be blocked through co-incubation of cAMP synthesis inhibitor SQ 22,536. So, these evidences strongly suggested that *Ch*GnRH dependent phagocytosis activation is mediated through cAMP signaling pathway in oyster hemocytes. Downstream cAMP signaling is transduced by its interactions with effector molecules, such as protein kinase A (PKA), which increased expression of pro-inflammatory mediators [47,52]. cAMP analogues mediate the translocation of PKC to the nucleus and function in the regulation of gene expression [53]. Inhibition of PKA in Hongkong oyster with H89 has effect on the ability of bacterial clearance and phagocytosis. The same phenomenon could be observed when PKC was blocked by enzastaurin. These findings lead us to hypothesize that GnRH could constitute a mechanism for hemocytes phagocytosis and bacterial clearance via cAMP-dependent PKA/PKC signaling pathway.

In conclusion, this study for the first time confirms the presence of GnRH dependent immune regulatory system in the Hongkong oyster. GnRH could strongly promotes the hemocytes phagocytosis and bacterial clearance through cAMP and its downstream signaling pathways, PKA and PKC. Therefore, the cross-talk between endocrine and immune system in invertebrate not only benefits to revealing the complexity of neuroendocrine immunology in marine invertebrates, and contributes to new strategies in disease control in oyster aquaculture.

Acknowledgments

This work was supported by the National Science Foundation of China (NO. 31701183) and Science and Technology Planning Project of Guangdong (2012A020602059).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.055.

References

[1] H.O. Besedovsky, A. del Rey, Immune–Neuroendocrine Network, (1986), pp. 578–587.
[2] E.M. Sternberg, Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens, Nat. Rev. Immunol. 6 (2006) 318–328.
[3] L.Y. Yu-Lee, Prolactin modulation of immune and inflammatory responses, Recent Prog. Horm. Res. 57 (2002) 435–455.
[4] T. Wilckens, R. De Rijk, Glucocorticoids and immune function: unknown dimensions and new frontiers, Immunol. Today 18 (1997) 418–424.
[5] T. Yada, T. Nakanishi, Interaction between endocrine and immune systems in fish, Int. Rev. Cytology - a Survey of Cell Biology 220 (2002) 35–92 220.
[6] T. Yada, Growth hormone and fish immune system, Gen. Comp. Endocrinol. 152 (2007) 353–358.
[7] M. Sakai, M. Kobayashi, H. Kawauchi, In vitro activation of fish phagocytic cells by GH, prolactin and somatolactin, J. Endocrinol. 151 (1996) 113–118.
[8] J.A. CalduchGiner, A. SitjaBobadilla, P. AlvarezPellitero, J. PerezSanchez, Growth hormone as an in vitro phagocyte-activating factor in the gilthead sea bream (Sparus aurata), Cell Tissue Res. 287 (1997) 535–540.
[9] T. Yada, T. Azuma, Y. Takagi, Stimulation of non-specific immune functions in seawater-acclimated rainbow trout, Oncorhynchus mykiss, with reference to the role of growth hormone, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129 (2001) 695–701.
[10] T. Yada, K. Uchida, S. Kajimura, T. Azuma, T. Hirano, E.G. Grau, Immunomodulatory effects of prolactin and growth hormone in the tilapia, Oreochromis mossambicus, J. Endocrinol. 173 (2002) 483–492.
[11] T. Yada, H. Kaiya, K. Mutoh, T. Azuma, S. Hyodo, K. Kangawa, Ghrelin stimulates phagocytosis and superoxide production in fish leukocytes, J. Endocrinol. 189 (2006) 57–65.
[12] L.W. Cheung, A.S. Wong, Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues, FEBS J. 275 (2008) 5479–5495.
[13] C.K. Cheng, P.C. Leung, Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans, Endocr. Rev. 26 (2005) 283–306.
[14] S. Su, X. Sun, X. Zhou, F. Fang, Y. Li, Effects of GnRH immunization on the reproductive axis and thymulin, J. Endocrinol. 226 (2015) 93–102.
[15] P. Limonta, M. Manea, Gonadotropin-releasing hormone receptors as molecular therapeutic targets in prostate cancer: current options and emerging strategies, Cancer Treat Rev. 39 (2013) 647–663.
[16] D.A. Chambers, K. Schauenstein, Mindful immunology: neuroimmunomodulation, Immunol. Today 21 (2000) 168–170.
[17] H.F. Chen, E.B. Jeung, M. Stephenson, P.C.K. Leung, Human peripheral blood mononuclear cells express gonadotropin-releasing hormone (GnRH), GnRH receptor, and interleukin-2 receptor gamma-chain messenger ribonucleic acids that are regulated by GnRH in vitro, J. Clin. Endocrinol. Metab. 84 (1999) 743–750.
[18] N.V. Emanuele, M.A. Emanuele, J. Tentler, L. Kirsteins, N. Azad, A.M. Lawrence, Rat spleen lymphocytes contain an immunoactive and bioactive luteinizing hormone-releasing hormone, Endocrinology 126 (1990) 2482–2486.
[19] N. Azad, N.V. Emanuele, M.M. Halloran, J. Tentler, M.R. Kelley, Presence of luteinizing hormone-releasing hormone (LHRH) mRNA in rat spleen lymphocytes, Endocrinology 128 (1991) 1679–1681.
[20] B. Marchetti, V. Guarcello, M.C. Morale, G. Bartoloni, G. Palumbo Jr., F. Raiti, et al., A physiological role for the neuropeptide luteinizing hormone-releasing hormone (LHRH) during the maturation of thymus gland function, Int. J. Neurosci. 51 (1990) 287–289.
[21] J.A. Tello, N.M. Sherwood, Amphioxus: beginning of vertebrate and end of invertebrate type GnRH receptor lineage, Endocrinology 150 (2009) 2847–2856.
[22] C. Di Cristo, E. De Lisa, A. Di Cosmo, GnRH in the brain and ovary of Sepia officinalis, Peptides 30 (2009) 531–537.
[23] R.K. Rastogi, M.M. Di Fiore, A. D'Aniello, L. Iela, M. Fiorentino, GnRH in the invertebrates: an overview, Prog. Brain Res. 141 (2002) 19–29.
[24] J.A. Veenstra, Neurohormones and neuropeptides encoded by the genome of Lottia gigantea, with reference to other mollusks and insects, Gen. Comp. Endocrinol. 167 (2010) 86–103.
[25] E. Iwakoshi-Ukena, K. Ukena, K. Takuwa-Kuroda, A. Kanda, K. Tsutsui, H. Minakata, Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (Octopus vulgaris) by in situ hybridization and immunohistochemistry, J. Comp. Neurol. 477 (2004) 310–323.
[26] H. Minakata, S. Shigeno, N. Kano, S. Haraguchi, T. Osugi, K. Tsutsui, Octopus gonadotrophin-releasing hormone: a multifunctional peptide in the endocrine and nervous systems of the cephalopod, J. Neuroendocrinol. 21 (2009) 322–326.
[27] L. Bigot, C. Zatylny-Gaudin, F. Rodet, B. Bernay, P. Boudry, P. Favrel, Characterization of GnRH-related peptides from the pacific oyster Crassostrea gigas, Peptides 34 (2012) 303–310.
[28] G.J. Roch, E.R. Busby, N.M. Sherwood, Evolution of GnRH: diving deeper, Gen. Comp. Endocrinol. 171 (2011) 1–16.
[29] G. Goldsworthy, L. Mullen, K. Opoku-Ware, S. Chandrakant, Interactions between the endocrine and immune systems in locusts, Physiol. Entomol. 28 (2003) 54–61.
[30] T.P. Su, E.D. London, J.H. Jaffe, Steroid binding at sigma-receptors suggests a link between endocrine, nervous, and immune-systems, Science 240 (1988) 219–221.
[31] A.E. Wright, S.R. Douglas, Further observations on the role of the blood fluids in connection with phagocytosis, Proc. Roy. Soc. Lond. 73 (1904) 128–142.
[32] T.P. Stossel, Phagocytosis (first of three parts), N. Engl. J. Med. 290 (1974) 717–723.
[33] P.P. Xiao, Y.H. Hu, L. Sun, Scophthalmus maximus cystatin B enhances head kidney macrophage-mediated bacterial killing, Dev. Comp. Immunol. 34 (2010) 1237–1241.
[34] K.J.L.T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (2001) 402–408.
[35] X.W. Wang, X.F. Zhao, J.X. Wang, C-type lectin binds to beta-integrin to promote hemocytic phagocytosis in an invertebrate, J. Biol. Chem. 289 (2014) 2405–2414.
[36] M. Duperthuy, P. Schmitt, E. Garzon, A. Caro, R.D. Rosa, F. Le Roux, et al., Use of OmpU porins for attachment and invasion of Crassostrea gigas immune cells by the oyster pathogen Vibrio splendidus, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 2993–2998.
[37] J. Li, Y. Zhang, Y. Liu, Y. Zhang, Z. Xiang, F. Qu, et al., A thymosin beta-4 is involved in production of hemocytes and immune defense of Hong Kong oyster, Crassostrea hongkongensis, Dev. Comp. Immunol. 57 (2016) 1–9.
[38] N. Cimerman, P.M. Brguljan, M. Krasovec, S. Suskovic, J. Kos, Serum concentration and circadian profiles of cathepsins B, H and L, and their inhibitors, stefins A and B, in asthma, Clin. Chim. Acta 310 (2001) 113–122.
[39] J. Kos, T.T. Lah, Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (Review), Oncol. Rep. 5 (1998) 1349–1361.
[40] C.C. Maier, B. Marchetti, R.D. LeBoeuf, J.E. Blalock, Thymocytes express a mRNA that is identical to hypothalamic luteinizing hormone-releasing hormone mRNA, Cell. Mol. Neurobiol. 12 (1992) 447–454.

[41] N. Azad, N. La Paglia, K.A. Jurgens, L. Kirsteins, N.V. Emanuele, M.R. Kelley, et al., Immunoactivation enhances the concentration of luteinizing hormone-releasing hormone peptide and its gene expression in human peripheral T-lymphocytes, Endocrinology 133 (1993) 215–223.
[42] S. Aladaileh, M.G. Mohammad, B. Ferrari, S.V. Nair, D.A. Raftos, In vitro effects of noradrenaline on Sydney rock oyster (Saccostrea glomerata) hemocytes, Comp. Biochem. Physiol. Mol. Integr. Physiol. 151 (2008) 691–697.
[43] S. Aladaileh, S.V. Nair, D.A. Raftos, Effects of noradrenaline on immunological activity in Sydney rock oysters, Dev. Comp. Immunol. 32 (2008) 627–636.
[44] P. Cassina, J. Sellers, J.D. Neill, Effect of cAMP on GnRH stimulated LH secretion from individual pituitary gonadotropes, Mol. Cell. Endocrinol. 114 (1995) 127–135.
[45] G.A. Bourne, D.M. Baldwin, Evidence for camp as a mediator of gonadotropin-secretion from male pituitaries, Am. J. Physiol. 253 (1987) E296–E299.
[46] T.C. Liu, P.S. Wang, G.L. Jackson, Effects of gnrh and drugs that affect camp levels on lh synthesis and release, Am. J. Physiol. 241 (1981) E14–E21.
[47] B.S. Skalhegg, K. Tasken, Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA, Front. Biosci. 5 (2000) D678–D693.
[48] L. Ydrenius, M. Majeed, B.J. Rasmusson, O. Stendahl, E. Sarndahl, Activation of cAMP-dependent protein kinase is necessary for actin rearrangements in human neutrophils during phagocytosis, J. Leukoc. Biol. 67 (2000) 520–528.
[49] L. Canesi, G. Gallo, M. Gavioli, C. Pruzzo, Bacteria-hemocyte interactions and phagocytosis in marine bivalves, Microsc. Res. Tech. 57 (2002) 469–476.
[50] S. Greenberg, S. Grinstein, Phagocytosis and innate immunity, Curr. Opin. Immunol. 14 (2002) 136–145.
[51] T. Yada, Effect of gonadotropin-releasing hormone on phagocytic leucocytes of rainbow trout, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155 (2012) 375–380.
[52] A. Medeiros, C. Peres-Buzalaf, F. Fortino Verdan, C.H. Serezani, Prostaglandin E2 and the suppression of phagocyte innate immune responses in different organs, Mediat. Inflamm. 2012 (2012) 327568.
[53] J.C. Cambier, M.K. Newell, L.B. Justement, J.C. McGuire, K.L. Leach, Z.Z. Chen, Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes, Nature 327 (1987) 629–632.