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ScienceDirect
Progress in Natural Science 18 (2008) 691–695
Progress in Natural Science
www.elsevier.com/locate/pnsc

Immunorecognition of estrogen and androgen receptors in the brain and thoracic ganglion mass of mud crab, *Scylla paramamosain*

Haihui Ye *, Huiyang Huang, Shaojing Li, Guizhong Wang

Department of Oceanography, Xiamen University, Xiamen 361005, China

Received 23 November 2007; received in revised form 22 December 2007; accepted 26 December 2007

Abstract

The brain and the thoracic ganglion of a crustacean can synthesize and secrete gonad-stimulating hormone (GSH) which stimulates the maturation of gonad. In the previous experiments, sex steroid hormones (estradiol, testosterone, progesterone, etc.) have been detected from the crustacean. However, the feedback regulation of sex steroid hormones on the brain and the thoracic ganglion of the crustacean has not been reported so far. In the present experiment, monoclonal antibodies were applied to investigate the immunorecognition of estrogen receptor (ER) and androgen receptor (AR) in the brain and the thoracic ganglion mass of *Scylla paramamosain*. The results showed that the distribution of the immunopositive substances of ER and AR was extremely similar. They distributed in the protocerebrum, deutocerebrum and tritocerebrum of the brain, and mainly in protocerebrum. In the thoracic ganglion mass, immunopositive substances distributed in the subesophageal ganglion, thoracic ganglion and abdominal ganglion, and mostly in subesophageal ganglion. Immunopositive substances of ER and AR mostly existed in the cytoplasm of neurons. The present study will provide morphological evidence for the origin and the evolution of ER and AR. In addition, the immunoreactivities of ER and AR suggested that the estrogen and androgen may be involved in the feedback regulation of crustacean neuroendocrine.

Keywords: *Scylla paramamosain*; Estrogen receptor; Androgen receptor; Brain; Thoracic ganglion mass; Immunocytochemistry

1. Introduction

In vertebrates, sex steroid hormones cannot only regulate the gonadal development and reproduction, but also regulate the neuroendocrine by the feedback mechanism. Only by combination with receptors in the brain, can sex steroid hormones regulate the neuroendocrine of the brain and the pituitary. This mechanism is important in the activity of reproductive endocrine [1,2]. So far, there has been few reports about estrogen receptor (ER) and androgen receptor (AR) in invertebrates [3,4]. Crustacean is one of the important communities of invertebrates. Nowadays, estradiol, testosterone and progesterone have been detected from the ovary, hepatopancreas and hemolymph of the crustacean. It was also demonstrated that the hormone level has correlation with gonadal development [5–12]. However, whether sex steroid hormones in crustacean have the same feedback regulation mechanism as in vertebrates is still unknown. Brain and thoracic ganglion are known to be the synthesis center of gonad-stimulating hormone (GSH) in crustacean [13–15], but whether estrogen and androgen have feedback regulation function on the synthesis and the secretion of GSH has not been reported. In this study, the localization of ER and AR in the brain and thoracic ganglion mass of *Scylla paramamosain* was studied using monoclonal antibodies and immunocytochemical methods. It provided morphological evidence that the ER and AR in *S. paramamosain* may be involved in the feedback regulation of crustacean neuroendocrine, which also is important to further study the comparative reproductive endocrinology.

* Corresponding author. Tel./fax: +86 592 2185539.
E-mail address: haihuiye@xmu.edu.cn (H. Ye).

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doi:10.1016/j.pnsc.2007.12.012

2. Materials and methods

2.1. Tissue preparation

A total of 12 adult crabs (*S. paramamosain*) of both sexes, ranging from 6.4 to 8.8 cm in length were used for this study. Crabs were obtained from local vendors in Xiamen. The brain and the thoracic ganglion mass were dissected free, and fixed in Bouin's solution for 10–12 h at 4 °C. The tissues were then embedded in paraffin after routine dehydration in alcohol and clearing in xylene. Serial sagittal and cross sections of 8 μm were mounted on clear glass slides.

2.2. Main reagents

Monoclonal antibodies generated in rabbits against human ER (1:200 dilution) and AR (1:200 dilution) were produced by Santa Cruz Company. 3′,3′-Diaminobenzidine (DAB) was the product of Sigma Company. Streptavidin–biotin-complex (SABC) kit was purchased from Wuhan Boster Biological technology, Ltd.

2.3. SABC immunocytochemistry method

To detect ER and AR, the sections were immunocytochemically stained by SABC method. The following procedure was performed. Sections were: (1) incubated in 3% H_2O_2/PBS for 10 min to inactivate endogenous peroxidase at room temperature, then rinsed in PBS (pH 7.4) for 5 min; (2) immersed in citra buffer (0.01 M, pH 6.0) for thermal-induced antigen retrieval by microwave energy for 15 min at 95 °C. Then allow the buffer to cool down to room temperature, sections were rinsed in PBS for 5 min; (3) incubated in normal goat serum (1:10 dilution) for 10 min to reduce non-specific binding at room temperature; (4) incubated in primary antisera for 1.5 h at 37 °C, then rinsed three times in PBS for 15 min; (5) incubated in biotinylated goat anti-rabbit IgG for 0.5 h at 37 °C, then rinsed three times in PBS for 15 min; (6) incubated in streptavidin–biotin-complex (SABC) for 0.5 h at 37 °C, then rinsed three times in PBS for 15 min; (7) after 5–10 min in 0.06% DAB-0.03% H_2O_2, sections were rinsed thoroughly in tap water, then dyed in hematoxylin, dehydrated in alcohol, cleared in xylene and coverslipped; (8) preparations were observed and photographed with an Olympus BH-2 microscope.

Two negative controls were included by: (1) replacing the primary antibody with normal rabbit serum; (2) omitting the primary antibody in the reaction.

3. Results

Immunopositive substances of ER and AR were brown. Some brown granules were observed in the cytoplasm of immunoreactive neurons. The nucleus of immunoreactive neurons was colorless and became blue after counterstaining with hematoxylin. Thus, the immunoreactive neurons were distinguishable from immunonegative neurons. Nerve fibers seldom reacted positively. The locations of ER and AR immunoreactive neurons were quite similar.

3.1. Immunorecognition of ER and AR in the brain

The brain of *S. paramamosain*, consisting of protocerebrum, deutocerebrum and tritocerebrum, is divided into 11 neuropils and 12 somal clusters (Cluster 6–Cluster 17) [16].

In protocerebrum, many immunoreactive neurons were found in Cluster 6 and 7. The immunoreactive neurons were round and ellipse with different sizes (Fig. 1: 1). In deutocerebrum, sporadical immunoreactive neurons were detected in Cluster 9, 10 and 11 (Fig. 1: 2). In tritocerebrum, some immunoreactive neurons were found in Cluster 17 (Fig. 1: 3). The number of immunoreactive neurons in deutocerebrum and tritocerebrum was less than those in protocerebrum.

3.2. Immunorecognition of ER and AR in the thoracic ganglion mass

The thoracic ganglion mass of *S. paramamosain* is fused by the subesophageal ganglion, the thoracic ganglion and the abdominal ganglion [17].

The number of immunoreactive neurons and the extent of their immunostaining in thoracic ganglion mass exceeded that in brain. In the subesophageal ganglion, a large number of immunoreactive neurons were found. The immunoreactive neurons distributed in both sides (Fig. 1: 4) and the median of subesophageal ganglion (Fig. 1: 5). A few immunoreactive neurons were detected in the thoracic ganglion (Fig. 1: 6). Immunoreactive neurons were also found in the abdominal ganglion, with small neurons showing strong immunoreactivity. The cytoplasm of large neurons had weak immunoreactivity, while their membrane was strongly immunostained (Fig. 1: 7, 8).

4. Discussion

Studies on vertebrate reproductive endocrine have showed that sex steroid hormones regulate the gonadal development and reproductive behavior. Only by combination with receptors in brain, can estrogen and androgen regulate the endocrine of the brain and pituitary of vertebrate. The mechanism played an important role in the activity of reproductive endocrine [1,2]. At all the different evolution stages of vertebrates, ER or AR existed in the brain of mammalia, aves, reptile and osteichthyes [18–22]. In addition, they are also found in the pituitary of fish [23,24]. Both brain and pituitary are involved in regulating gonadotropin secretion. Undoubtedly, sex steroid hormones can regulate the secretion of brain and pituitary. *Amphioxus*, a cephalochordate, is a species of transitional type between invertebrates and vertebrates. ER and AR

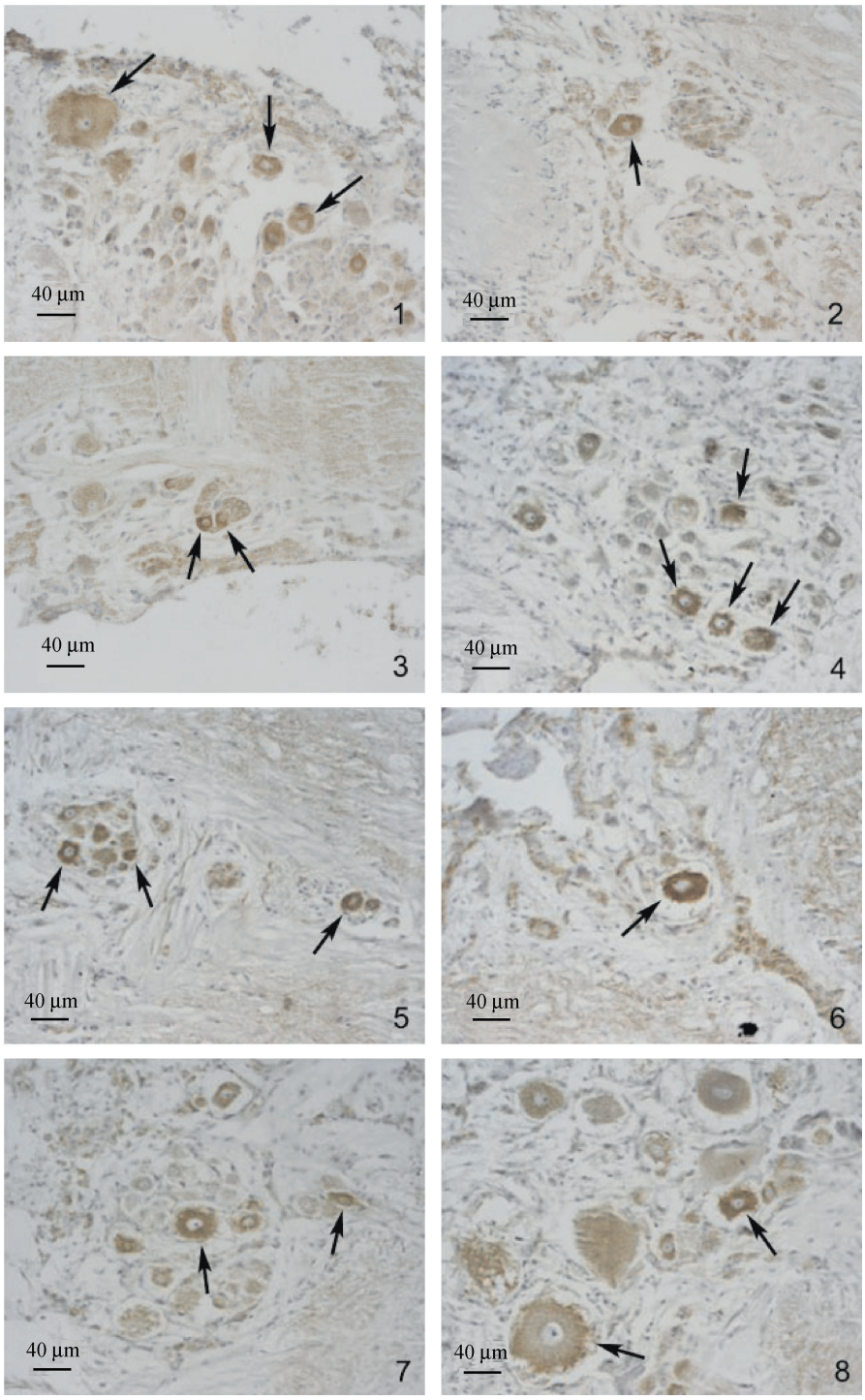

Fig. 1. Immunoreactive neurons in brain and in thoracic ganglion of *Scylla paramamosain*. 1, ER-immunoreactive neurons (arrow) in Cluster 7 in the protocerebrum of *Scylla paramamosain*; 2, ER-immunoreactive neurons (arrow) in Cluster 11 in the deutocerebrum of *Scylla paramamosain*; 3, AR-immunoreactive neurons (arrow) in Cluster 17 in tritocerebrum of *Scylla paramamosain*; 4, ER-immunoreactive neurons (arrow) in the side of subesophageal ganglion of *Scylla paramamosain*; 5, AR-immunoreactive neurons (arrow) in the median of subesophageal ganglion of *Scylla paramamosain*; 6, AR-immunoreactive neuron (arrow) in the thoracic ganglion of *Scylla paramamosain*; 7, ER-immunoreactive neurons (arrow) in the abdominal ganglion of *Scylla paramamosain*; 8, ER-immunoreactive neurons (arrow) in the abdominal ganglion of *Scylla paramamosain*.

were also found in its brain and Hatschek's pit (which is homologous with the pituitary of fish). It is considered that sex steroid hormones can regulate the brain and Hatschek's pit of *Amphioxus* through feedback mechanism [25]. So far, there has been few researches about ER and AR in invertebrates. ER and AR have been detected from the optic ganglion of *S. paramamosain* in our lab [3,4]. Brain and thoracic ganglion, the synthesis center of GSH in crusta-

cean, are of great importance in reproductive endocrinology [13–15]. Recently, the immunopositive substances of follicle stimulating hormone and luteinizing hormone have been detected in the brain of *S. paramamosain*, which further affirmed the reproductive endocrine function of brain in crustaceans [26,27]. Moreover, the correlation between the level of sex steroid hormones (estradiol, 17α-OH progesterone and testosterone) and gonadal development in *S. paramamosain* has been proved [27]. Are there ER and AR located in the brain and thoracic ganglion of *S. paramamosain*? Can they regulate reproductive endocrine function like in vertebrates? In the present study, ER and AR neurons have been detected in the brain and thoracic ganglion mass of *S. paramamosain*. It is suggested that estrogen and androgen may be involved in the feedback regulation of brain and thoracic ganglion. By combining with their receptors, estrogen and androgen may stimulate or inhibit hormone secretion, and keep the balance of hormone level. Whether the feedback mechanism of estrogen and androgen in *S. paramamosain* is similar with that in vertebrates remains to be further investigated. The discovery of ER and AR in *S. paramamosain* is meaningful for understanding the mechanism of reproductive feedback in invertebrate and the evolutionary aspects of reproductive endocrine in animal kingdom.

Sex steroid hormone is the regulator of the hypothalamus–pituitary function, which has been well confirmed in vertebrates [28,29]. ER and AR mostly located in the protocerebrum of *S. paramamosain*. This is consistent with that protocerebrum is the main synthesizing site of GSH [15]. Thoracic ganglion is also an important GSH synthesizing organ as well. However, in this study only a few ER and AR immunopositive neurons are detected there, and this is different from the result in protocerebrum where there are much more receptor neurons. Since ER and AR are also detected in deutocerebrum, tritocerebrum, subesophageal ganglion and abdominal ganglion of *S. paramamosain*, these parts may also be the synthesis site of GSH and they can accept the feedback regulation of estrogen and androgen. Additionally, estrogen and androgen may probably be involved in other nervous activities.

There are two kinds of signaling pathways of sex steroid hormones. One is the genomic action via nuclear receptor which produces comparatively slow effects, the other is the non-genomic action via membrane receptor which can mediate rapid response in cells [30,31]. The ER and AR immunopositive substances located in the membrane, cytoplasm and nerve fibers with few in the nucleus, which is the same with the results of the optic ganglion in *S. paramamosain* [3,4]. Consequently, we presume that estrogen and androgen may feedback through membrane receptor, i.e. after combining with membrane receptor, the hormone-receptor complex quickly triggers a series of downstream signal transduction in cytoplasm, and regulates the function of neurons.

Exogenous steroid hormones can stimulate gonadal development of crustacean. But to date, the results show a great difference about the function of sex steroid hormones [11,12]. It is still a controversial topic whether the mechanism is due to directly triggered gametogenesis, or indirectly, through the steroid hormone receptor in the nervous system. The mechanism of sex steroid hormone deserves further research in crustacean. The detections of ER and AR in *S. paramamosain* might open a new endocrinological field in crustacean.

In this study, ER and AR are only detected in the nervous organs of *S. paramamosain*. The quantitative expression of ER and AR in different reproductive stages remains to be further studied, which will help to understand the mechanism of reproductive endocrine function in *S. paramamosain*.

Acknowledgement

This work was supported by National Natural Science Foundation of China (Grant No. 40776084, 40406030).

References

[1] Yuan H, Deborah AB, Theodore JB, et al. Distribution of occupied and unoccupied estrogen receptors in the rat brain: effects of physiological gonadal steroid exposure. Endocrinology 1995;136:96–105.
[2] Belsham DD. Hormonal regulation of clonal immortalized hypothalamic neurons expressing neuropeptides involved in reproduction and feeding. Mol Neurobiol 2007;35(2):182–94.
[3] Ye HH, Huang HY, Li SJ, et al. Androgen receptors in the optic ganglion of *Scylla paramamosain*. Acta Anat Sin 2004;35(5):547.
[4] Han SZ, Chen XL, Ye HH, et al. Immunorecognition of estrogen receptor in the optic ganglion of *Scylla paramamosain*. J Xiamen Univ (Nat Sci) 2006;45(6):741–2.
[5] Warrier SR, Tirumalai R, Subramoniam T. Occurrence of vertebrate steroid, estradiol 17 beta and progesterone in the reproducing female of the mud crab *Scylla serrata*. Comp Biochem Physiol A Mol Integr Physiol 2001;130(2):283–94.
[6] Couch EF, Hagino N, Lee W, et al. Changes in estradiol and progesterone immunoreactivity in tissues of the lobster, *Homarus americanus*, with developing and immature ovaries. Comp Biochem Physiol 1987;7A(3):65–770.
[7] Quinitio ET, Yamavchi K, Hara A, et al. Profiles of progesterone and estradiol like substance in the haemolymph of female *Pandalus kessleri* during an annual reproductive cycle. Gen Comp Endocrinol 1991;81:343–8.
[8] Quinitio ET, Hara A, Yamauchietal K. Changes in the steroid hormone and vitellogenin levels during the gametogenic cycle of the giant tiger shrimp, *Penaeus monodon*. Comp Biochem Physiol 1994;109(1):21–6.
[9] Jiang RL, Tan YJ, Wu JM, et al. Changes of hemolymph 20-hydroxyecdysone 17β-oestradiol and testosterone levels in *Eriocheir sinensis*. J Fish China 1992;16(2):101–6.
[10] Cai SL, Zhao WX, Li DS, et al. Profile of progesterone and estradiol in hepatopancreas, ovary, and hemolymph of shrimp *Penaeus chinensis* during reproduction cycle. J Fish China 2001;25(4):304–10.
[11] Ghosh D, Ray AK. 17β-Hydroxysteroid dehydrogenase activity of ovary and hepatopancreas of fresh water prawn, *Macrobrachium rosenbergii*, relation to ovarian condition and estrogen treatment. Gen Comp Endocrinol 1993;89:248–54.
[12] Okumura T, Sakiyama K. Hemolymph levels of vertebrate-type steroid hormones in female kuruma prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae) during natural reproductive cycle

and induced ovarian development by eyestalk ablation. Fish Sci 2004;70(3):372–80.
[13] Jin ZX, Ye HH, Li SJ, et al. Role of nervous organs in stimulating ovarian maturation in the mud crab, *Scylla paramamosain*: *in vitro* studies. Mar Sci 2003;27(1):72–4.
[14] Ye HH, Huang HY, Li SJ, et al. *In vitro* study of neuroendocrine regulation over the testicular development in mud crabs, *Scylla paramamosain*. Chin J Oceanol Limnol 2006;24(2):142–6.
[15] Fingerman M. Crustacean endocrinology: a retrospective, prospective and introspective analysis. Physiol Zool 1997;70(3):257–69.
[16] Huang HY, Ye HH, Li SJ, et al. Immunocytochemical localization of serotonin and neuropeptide Y in the brain of *Scylla paramamosain*. J Fish China 2005;29(4):441–6.
[17] Ye HH, Li SJ, Jin ZX, et al. Histological study on the thoracic ganglion mass of the mud crab, *Scylla paramamosain*. J Xiamen Univ (Nat Sci) 2002;41(4):502–5.
[18] Lu SF, McKenna SE, Clifford AC, et al. Androgen in mouse brain: sex differences and similarities in autoregulation. Endocrinology 1998;139:1594–601.
[19] Hamson DK, Jones BA, Watson NV. Distribution of androgen receptor immunoreactivity in the brainstem of male rats. Neuroscience 2004;127(4):797–803.
[20] Zuo MX. Immunohistochemical localization of estrogen receptors in the brain of non-songbird. Acta Zool Sin 1996;42(3):269–74.
[21] Halpern MJ, Morrell I, Pfaff DW. Cellular$^{[3H]}$ estradiol and $^{[3H]}$testosterone localization in the brain of Garter snakes: an autoradiographic study. Gen Comp Endocrinol 1982;46:211–24.
[22] Trudeau VL, Murthy CK, Habibi HR. Effects of sex steroid treatments on gonadotropin-releasing hormone stimulated gonadotropin secretion from the goldfish pituitary. Biol Reprod 1993;48:300–7.
[23] Kim YS, Sarand M, Stumpf WE. Estrogen target cells in the pituitary of platyfish, *Xiphophorus maculates*. Cell Tissue Res 1979;198:435–40.
[24] Schreibman MP, Pertschuk LP, Rainford R, et al. The histochemical localization of steroid binding sites in the pituitary gland of a teleost (the platyfish). Cell Tissue Res 1982;26:523–30.
[25] Weng YZ, Fang YQ, Hu XX. Immunorecognition of estrogen and androgen receptors in the nervous system and Hatschek's pit of amphioxous (*Branchiostoma belcheri*). Acta Zool Sin 2001;47(6):672–6.
[26] Ye HH, Huang HY, Li SJ, et al. Immunorecognition of FSH and LH in the brain of *Scylla paramamosain*. Prog Nat Sci 2006;16(6):768–70.
[27] Han SZ. Studies on the reproductive effect of FSH and LH in *Scylla paramamosain*. Master degree Thesis, Xiamen University; 2007.
[28] Albrecht ED, Billiar RB, Aberdeen GW, et al. Expression of estrogen receptors alpha and beta in the fetal baboon testis and epididymis. Biol Reprod 2004;70(4):1106–13.
[29] Cao AL, Zhang CQ. Sex-specific effects of androgen and estrogen on proliferation of the embryonic chicken hypothalamic neurons. Endocrine 2007;31(2):161–6.
[30] Morrow AL. Recent developments in the significance and therapeutic relevance of neuroactive steroids—introduction to the special issue. Pharmacol Ther 2007;116(1):1–6.
[31] Doweyko AM. Steroid nuclear hormone receptors: the allosteric conversation. Drug Dev Res 2007;68(3):95–106.