General and Comparative Endocrinology 204 (2014) 71–79

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Molecular characterization and analysis of a putative 5-HT receptor involved in reproduction process of the pearl oyster *Pinctada fucata*

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ARTICLE INFO

Article history:
Received 12 December 2013
Revised 18 April 2014
Accepted 6 May 2014
Available online 20 May 2014

Keywords:
5-HT receptor
Oocyte maturation
Spawning
Pinctada fucata

ABSTRACT

5-HT (5-hydroxytryptamine; serotonin) has been linked to a variety of biological roles including gonad maturation and sequential spawning in bivalve molluscs. To gain a better understanding of the effects of 5-HT on developmental regulation in the pearl oyster *Pinctada fucata*, the isolation, cloning, and expression of the 5-HT receptor was investigated in this study. A full-length cDNA (2541 bp) encoding a putative 5-HT receptor (5-HT_{pf}) of 471 amino acids was isolated from the ovary of the pearl oyster. It shared 71% and 51% homology, respectively, with the *Crassostrea gigas* 5-HT receptor and the *Aplysia californica* 5-HT_{1ap}. The 5-HT_{pf} sequence possessed the typical characteristics of seven transmembrane domains and a long third inner loop. Phylogenetic analysis also indicated that 5-HT_{pf} was classified into the 5-HT_1 subtype together with other invertebrate 5-HT_1 receptors. Quantitative RT-PCR showed that 5-HT_{pf} is widely expressed in all tissues tested, is involved in the gametogenesis cycle, embryonic and larval development stages, and expression is induced by E_2 in ovarian tissues. These results suggest that 5-HT_{pf} is involved in the reproductive process, specifically in the induction of oocyte maturation and spawning of *P. fucata*.

1. Introduction

The biogenic amine serotonin, or 5-hydroxytryptamine (5-HT), is widely distributed in animals. It acts through multiple receptors to modulate many complex behaviors in vertebrates and invertebrates (Gerhardt and Heerikhuizen, 1997; Tierney, 2001). In mammals, 5-HT is involved in learning, anxiety, emotion, sleep, locomotion, reproduction and pain perception (Bordukalo-Niksic et al., 2010; Green and Backus, 1990; Wang et al., 2010; Weiger, 1997). In invertebrates several studies have been performed on the function and location of 5-HT, especially in annelids, arthropods and molluscs (Siniscalchi et al., 2004; Walker, 1984).

In molluscs, 5-HT and its receptors are engaged in neuronal functions including feeding (Kawai et al., 2011), circadian rhythm (Levenson et al., 1999), memory (Kandel, 2001), locomotion (Filla et al., 2004), parturition (Fong and Warner, 1995) and development (Panasophonkul et al., 2009). It also plays an important role in reproduction through biosynthesis and release of active egg-laying peptide precursor proteins, control and initiation of gamete release, and gamete maturation in several molluscan species (Vaca and Alfaro, 2000; Zatylny et al., 2000). Administration of 5-HT has been shown to induce oocyte maturation and spawning (Hamida et al., 2004; Tanabe et al., 2010), and to mediate reinitiation of meiosis in prophase-arrested oocytes, as evidenced by germinal vesicle breakdown (GVBD) (Garnerot et al., 2006; Krantic et al., 1992; Krantic and Rivailler, 1996). However, the major function of 5-HT is in the induction of oocyte maturation (Tanabe et al., 2006). The endogenous 5-HT signal is transmitted to the oocyte through 5-HT receptors (Bandivdekar et al., 1991). A number of hormones play key roles in controlling gonad development and secondary sexual characteristics. Testosterone and estradiol both show transient increases during the spawning stage in both sexes of clams (Garnerot et al., 2006). Studies on bivalves have indicated that estrogens potentiate 5-HT-induced spawning which may be mediated through the induction of 5-HT receptor synthesis (Osada et al., 1998, 2004; Wang and Croll, 2006) resulting in oocyte maturation.

In mammals, receptors interacting with 5-HT can be classified into seven different subfamilies (5-HT_1—5-HT_7). All except the

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http://dx.doi.org/10.1016/j.ygcen.2014.05.010
0016-6480/

5-HT_3 receptor, which is a ligand-gated ion channel, belong to the superfamily of G-protein-coupled receptors (GPCRs) (Hoyer et al., 2002). In molluscs, several physiological and pharmacological characterizations have been carried out on 5-HT receptors (Barbas et al., 2002; Hamdan et al., 1999). However, the attempts to classify molluscan 5-HT receptors using vertebrate nomenclature have only been partially successful since these receptors appear to have mixed pharmacological and transductional characteristics in some cases. Accordingly, data from molecular studies are very important, since gene structures and deduced amino acid sequences offer a more definitive way to identify and classify invertebrate 5-HT receptors.

To date, several 5-HT receptors have been identified by molecular cloning in molluscs, to our knowledge: 5-HT_{Lym} and 5-HT_{2Lym} from the pond snail *Lymnaea stagnalis*, which are presently regarded as 5-HT_1-like and 5-HT_2 receptors (Gerhardt et al., 1996; Sugamori et al., 1993). Four were characterized in *Aplysia californica*. Li et al. (1995) cloned two closely-related 5-HT receptors, which were named Ap5-HT_{B1} and Ap5-HT_{B2} and could not be classified according to any of the mammalian subtypes. The third receptor, 5-HT_{1ap}, is categorized in the mammalian 5-HT_1 subgroup (Angers et al., 1998). 5-HT_{ap2} is the fourth receptor identified in *A. californica*. It was demonstrated to be similar to 5-HT_{1ap} and to have an identity 68% similar to 5-HT_{Lym} (Barbas et al., 2002). A cDNA encoding a putative 5-HT receptor was isolated from the tropical abalone *Haliotis rubra*, termed 5-HT_{1ha} (Panasophonkul et al., 2009). A putative 5-HT receptor named 5-HT_{py} was cloned from the Japanese scallop *Patinopecten yessoensis* (Tanabe et al., 2010). 5-HT receptors were also obtained from the Pacific oyster *Crassostrea gigas* (Zhang et al., 2012) and *Mytilus edulis* (Cubero-Leon et al., 2010). Almost all the 5-HT receptors cloned in molluscs are expressed widely in tissues, with 5-HT_{Lym}, 5-HT_{1ap} and 5-HT_{ap2} highly expressed in the CNS, while Ap5-HT_{B1}, 5-HT_{1ha} and 5-HT_{py} showed high expression in the reproductive system. All these findings clearly show that 5-HT receptors mediate a variety of functions in molluscs, but are especially important in reproduction.

The pearl oyster *Pinctada fucata*, a marine bivalve mollusc, cultivated world-wide, has a very high economic value in pearl production in China. However, there are no reports describing the molecular cloning of the 5-HT receptor in this species. To gain insight into the molecular mechanisms of the 5-HT receptors during the reproductive process, we investigated the molecular structure, distribution and induction of expression of a putative 5-HT receptor named 5-HT_{pf}, which was classified into the 5-HT_1 subtype.

2. Materials and methods

2.1. Experimental animals and tissue sampling

Two-years-old pearl oysters *P. fucata* were used as the experimental animal. Samples collected at the growing stage of gonad development were obtained from the Marine Biology Station of the Chinese Academy of Sciences at Daya Bay (Shenzhen, Guangdong, PR China) between October 2011 and November 2012. To clone the 5-HT receptor cDNAs, the ovaries were excised, frozen immediately in liquid nitrogen, and then kept at −80 °C until RNA extraction. The ovary, testis, mantle, visceral ganglion, digestive gland, adductor muscle and gill were also dissected and stored in the same manner for analysis of tissue distribution. The gonadal tissues from different stages were dissected and fixed in Bouin's solution at 4 °C overnight then used to determine gonadal stages.

Gametes were obtained by dissecting the gonads then passing through a 100 μm screen to remove large particles of tissue debris. The eggs were fertilized with sperm in filtered seawater containing 0.005–0.006% (v/v) ammonia at temperatures of 25–26 °C, and different stages of embryos or larvae were collected and stored in liquid nitrogen for RNA extraction.

2.2. Reverse transcription, cloning and sequencing

The total RNA used for reverse transcription was extracted from frozen tissues using an E.Z.N.A. mollusc RNA kit according to the manufacturer's protocol (Omega Biotek Inc., Norcross, GA). One microgram of isolated RNA was used to synthesize first-strand cDNA using the ReverTra Ace-first-strand cDNA synthesis kit (Toyobo Co. Ltd., Osaka, Japan). A BD smart race cDNA amplification kit (Clontech, Mountain View, CA) was used to synthesize SMART cDNA. All the primers used for PCR are listed in Table 1. PCR primers were designed using Primer Premier 5.00 (Premier Biosoft International, Palo Alto, CA).

First, the fragment obtained from the transcriptome of *P. fucata* was amplified using a pair of specific primers, 5HTf1 and 5HTr1. PCR was performed under the following conditions: 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C with a final extension step of 10 min at 72 °C. The resulting 467 bp fragment was used to generate full-length cDNA by 5′-RACE and 3′-RACE using adaptor primers UPM, NUP and gene-specific primers 5HT5′-1, 5HT5′-2, 5HT3′-1 and 5HT3′-2. Primers 5HTf2 and 5HTr2 were used to validate the open reading frame (ORF). All PCR amplifications were performed as follows: denaturation at 94 °C for 3 min, followed by 35–40 cycles at

Table 1
Primers used for 5-HT_{pf} cloning and expression analysis.

No.	Primer name	Usage	Sense/antisense	Primer sequence (5′–3′)
1	5HTf1	RT-PCR	Sense	5′-GGGCGAAAGCATCCAGAATATAAGT-3′
2	5HTr1	RT-PCR	Antisense	5′-TTTGTTTAGCACTTCTTCGTCGGATGT-3′
3	5HT5′-1	5′RACE	Antisense	5′-CACAAGTCCGAACCTAAATACCAG-3′
4	5HT5′-2	Nested 5′RACE	Antisense	5′-TCCGTGACAGCCAACGATAGA-3′
5	5HT3′-1	3′RACE	Sense	5′-CTGGTATTTAGGTTCGGACTTGTG-3′
6	5HT3′-2	Nested 3′RACE	Sense	5′-TCCGACGAAGAAGTGCTAAACA-3′
7	UPM mixture	5′ and 3′RACE	Sense	5′-CTAATACGACTCACTATAGGGCAAGCAGT GGTATCAACGCAGAGT-3′ 5′-CTAATACGACTCACTATAGGGC-3′
8	NUP	5′ and 3′RACE	Sense	5′-AAGCAGTGGTATCAACGCAGAGT-3′
9	5HTf2	RT-PCR(ORF)	Sense	5′-TCGGGTCACCCTTGGTCG-3′
10	5HTr2	RT-PCR(ORF)	Antisense	5′-TGAAAGCAAACATACCAGCAATA-3′
11	5HTf3	Real-time PCR	Sense	5′-CTGGTATTTAGGTTCGGACTTGT-3′
12	5HTr3	Real-time PCR	Antisense	5′-ATTTGTTTAGCACTTCTTCGTCG-3′
13	18S-f	Real-time PCR	Sense	5′-CGTTTCAACAAGACGCCAGTAG-3′
14	18S-r	Real-time PCR	Antisense	5′-ACGAAAAAAAGGTTTGAGAGACG-3′