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Research paper

Molecular characterization, expression analysis, and functional properties of multiple 5-hydroxytryptamine receptors in Pacific abalone (*Haliotis discus hannai*)

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ABSTRACT

Neurotransmitters such as serotonin (5-hydroxytryptamine; 5-HT) in the central nervous system regulate diverse physiological functions, including reproduction, feeding, learning, and memory, in diverse animal phyla. 5-HT and the 5-HT1 subtype receptor play important roles in sexual maturation and in the initiation of gamete release in mollusks. However, little is known about the involvement of other 5-HT receptor subfamilies in the reproduction process. In the present study, we identified the cDNAs encoding eight subtypes of 5-HT receptors from the ganglia tissues of the Pacific abalone *Haliotis discus hannai* (Mollusca; Gastropoda; Haliotidae), and examined the gonadal expression of the transcripts of 5-HT receptors. A phylogenetic analysis indicated that the molluskan 5-HT receptors are largely classified into four major clades: 5-HT1/5/7, 5-HT2, 5-HT4, and 5-HT6. Among the *H. discus hannai* (Hdh) *5-HT1-7* transcripts, *Hdh5-HT1B*, *4A*, *4B*, and *6* were the major subtypes detected in the mature ovary. Estradiol-17β injection into the pedal sinus induced the downregulation of *5-HT4B* and upregulation of *5-HT6* transcripts in the ovary of mature abalone within 72 h. In HEK293 cells overexpressing Hdh5-HT1B, forskolin-stimulated cAMP response element luciferase (CRE-Luc) reporter activity was inhibited by 5-HT in a dose-dependent manner, whereas serum response element luciferase (SRE-Luc) activity was not affected. In Hdh5-HT4A-expressing HEK293 cells, forskolin-stimulated CRE-Luc and SRE-Luc reporter activities were both marginally increased by treatment with a high dose of 5-HT. Our results provide new insights into the roles of 5-HT through diverse G protein-coupled 5-HT receptors in the reproductive process of mollusks.

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that acts in the central nervous system (CNS) and the peripheral nervous system, as well as in non-neuronal tissues. 5-HT is one of the evolutionarily oldest neurotransmitters and exerts its various physiological functions through seven 5-HT receptor subfamilies (5-HT1-7) that differ with respect to structural features and signaling mechanisms (Hannon and Hoyer, 2008). The 5-HT receptors are further classified into at least 14 distinct receptor subtypes, 13 of which are G protein-coupled receptors (GPCRs) and one ligand-gated cation channel (McCorvy and Roth, 2015). The major classes of 5-HT receptors are estimated to have appeared about 750 million years ago, long before cholinergic, adrenergic, or dopaminergic receptors, although the GPCR family is estimated to have arisen over 1 billion years ago (Hannon and Hoyer, 2008). 5-HT and its receptors play important roles in the regulation of numerous biological events, including behavioral and neuropsychological processes, gastrointestinal and endocrine function, and cardiovascular and pulmonary physiology (Berger et al., 2009). With regard to the reproductive processes of humans and rats, 5-HT has been shown to be involved in the regulation of uterine contraction (Minosyan et al., 2007), urethrogenital reflex activity (Giraldi et al., 2004), and female sexual response through multiple 5-HT receptors (Goldstein et al., 2017). In males, 5-HT increases the ejaculatory latency and delays orgasm through 5-HT2C and 5-HT1B receptors but can also decrease ejaculatory latency through the 5-HT1A receptor (de Jong et al.,

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); AC, adenylate cyclase; CNS, central nervous system; CRE, cAMP response element; DMEM, Dulbecco's modified Eagle medium; E2, estradiol-17β; EL, extracellular loop; FBS, fetal bovine serum; FKN, forskolin; GPCR, G protein-coupled receptor; Hdh, *Haliotis discus hannai* (Pacific abalone); PPG, pleuro-pedal ganglion; SRE, serum response element

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2006). Thus, altered 5-HT signaling involving various 5-HT receptor subtypes is implicated in the regulation of reproductive systems, e.g., the mammary gland, uterus, and penis of humans and rodents (Berger et al., 2009).

Several 5-HT receptors have also been identified and characterized in mollusks, including 5-HTLym and 5-HT2Lym from *Lymnaea stagnalis* (Gerhardt et al., 1996; Sugamori et al., 1993); Ap5-HTB1, Ap5-HTB2, 5-HTap1, 5-HTap2, and 5-HTapAC1 from *Aplysia californica* and *Aplysia kurodai* (Angers et al., 1998; Barbas et al., 2002; Lee et al., 2009; Li et al., 1995); and 5-HT1-like receptors from *Haliotis rubra* (Panasophonkul et al., 2009), *Patinopecten yessoensis* (Tanabe et al., 2010), *Mytilus edulis* (Cubero-Leon et al., 2010), and *Pinctada fucata* (Wang and He, 2014). The molluskan 5-HT1-like receptors are expressed in diverse tissues, including the neural ganglia and the gonads (Angers et al., 1998; Panasophonkul et al., 2009; Tanabe et al., 2010; Wang and He, 2014). In contrast, the expression of 5-HTap2 and Ap5-HTB2 was shown to be restricted to the CNS, whereas Ap5-HTB1 was found to be strongly expressed in the ovotestis and not in the CNS (Barbas et al., 2002; Li et al., 1995). With regard to their pharmacological and transductional properties, it is difficult to directly compare the molluskan 5-HT receptors with mammalian 5-HT receptor subfamilies, since it is likely that characteristics of more than one receptor subtype have been retained in certain cases, e.g., for *A. californica* and *P. yessoensis* 5-HT receptors (Barbas et al., 2002; Osada et al., 1998).

5-HT is also known to play pivotal roles in molluskan reproductive systems, including induction of gamete maturation and spawning, and reinitiation of meiosis, i.e., germinal vesicle breakdown (Garnerot et al., 2006; Hamida et al., 2004; Kim et al., 2018; Krantic et al., 1993; Matsutani and Nomura, 1982; Tanabe et al., 2006; Yuan et al., 2012). In addition, studies using the scallops *P. yessoensis* and *Placopecten magellanicus* indicated that estrogens promote 5-HT-induced spawning, which may be achieved through 5-HT1-like receptors (Osada et al., 1998; Wang and Croll, 2006). In mature *M. edulis* mussels, the expression level of the 5-HT1-like receptor was shown to decrease following estradiol-17β(E2) treatment, whereas the opposite trend was observed at immature stages (Cubero-Leon et al., 2010). Most of these studies suggest that E2 may affect 5-HT-induced gamete maturation and spawning by influencing the synthesis of 5-HT1-like receptors in marine bivalves.

However, little is known about the diversity of molluskan 5-HT receptors and their function in reproduction. Thus, in this study, we aimed to gain insight into the potential role of 5-HT receptors in the reproductive processes of the Pacific abalone *Haliotis discus hannai* (Hdh) through sequence and gene expression analyses. Among the major transcripts of Hdh5-HT receptor subtypes in the mature ovary (Hdh5-HT1B, 4A, 4B, and 6), Hdh5-HT1B and 4A receptor-mediated signaling pathways were explored using a heterologous GPCR reporter assay system. Since rhodopsin-like (class A) GPCRs can be functionally characterized according to their coupling to secondary messengers via G-proteins (Hannon and Hoyer, 2008), we selected two representative and contrasting receptors, Hdh5-HT1B and 4A, which are most likely linked to Gi/o and Gs, respectively.

2. Materials and methods

2.1. Database searching and phylogenetic analysis of 5-HT receptor orthologs in Pacific abalone

Individual genes encoding 5-HT receptor orthologs were identified in the Hdh transcriptome databases (Kim et al., 2017a) using a tBLASTn algorithm against the National Center for Biotechnology Information (NCBI) or UniProtKB/SwissProt databases (Table S1). The amino acid sequence identities of the putative Hdh 5-HT receptors were then analyzed by the BLAST or UniProtKB/SwissProt tools. The selected Hdh5-HT genes were translated using the online ORF finder tool of the NCBI. Amino acid sequences used for phylogenetic analysis were identified by protein-protein BLAST searches of the NCBI database with the deduced amino acid sequence of Hdh5-HTs as the "query" (Table S1). The amino acid alignment was carried out by CLUSTALW with MEGA6 (Tamura et al., 2013), and the dendrogram was constructed using the maximum-likelihood method based on the JTT matrix-based model (Jones et al., 1992). Branch supports were provided using 1000 bootstrap replicates.

2.2. cDNA cloning and plasmid construction

Total RNA was extracted from the pleuro-pedal ganglion (PPG) of mature female abalone using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) as previously described (Kim et al., 2017a,b). To amplify the cDNAs encoding Hdh5-HT receptors, each pair of primers (Table S2) was designed based on the abalone 5-HT receptor sequences (Table S1). The polymerase chain reaction (PCR) products were digested with MunI/EcoRI and XbaI restriction enzymes and inserted into the EcoRI and XbaI sites of the pcDNA3-HA plasmid (Invitrogen, Waltham, MA, USA) using T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA). Standard procedures for PCR, cDNA cloning, and plasmid purification are described previously (Kim et al., 2017b, 2018; Ko et al., 2007). All of the constructs were sequenced to verify the correct sequence and orientation. The sequences generated in this study have been deposited in the NCBI GenBank database (accession nos. MK370919-26).

2.3. Quantitative (q)PCR analysis

Hdh5-HTs mRNA expression was analyzed by qPCR using cDNA from various tissues of mature female abalone (N = 5, shell length: 8.6 ± 0.9 cm, total body weight: 87.0 ± 24.7 g). The mature stage (stage III and IV, ripe and partial spent stage, respectively) of the abalone ovary was classified according to a previous study (Kim et al., 2017b). To examine the tissue-specific expression of Hdh5-HT receptor mRNAs, the PPG and cerebral ganglion (CG) as well as the ovary, gills, intestine, and adductor muscle were dissected and immediately frozen in liquid nitrogen before storage at −80 °C. Total RNA extraction, reverse transcription, the qPCR assay, and analysis of relative mRNA levels were performed as previously described (Kim et al., 2017a,b; López-Landavery et al., 2014; Wan et al., 2011), except for the gene-specific primer sets (Table S2).

2.4. In vivo E2 injection

In July-August 2018, adult female abalones were purchased from a local dealer (Gangneung, Gangwon-do, Korea). Mature females (N = 35, 8.54 ± 0.28 cm, 72.96 ± 3.9 g) were randomly divided into three groups: 100 μL of 0.05 or 1.0 μg/μL E2 (Sigma-Aldrich, St. Louis, MO, USA). E2 was dissolved in dimethyl sulfoxide (DMSO) and then diluted in mollusk physiological saline (Kim et al., 2018) prior to injection into the pedal sinus of the two experimental groups (N = 10 per group), and the same volume of saline was injected into the control group (N = 15). The concentration of DMSO was maintained below 0.1% in saline throughout the study. After injection, individual groups were placed in tanks filled with filtered seawater (14 °C). At 0, 24, and 72 h post-injection, the ovarian tissues were dissected and immediately frozen in liquid nitrogen before storage at −80 °C.

2.5. Cell culture, transient transfection, and reporter assay

Human embryonic kidney 293 (HEK293) cells were grown in monolayer culture in Dulbecco's modified Eagle medium (DMEM; Gibco, Loughborough, UK) supplemented with 10% fetal bovine serum (FBS, HyClone, GE Healthcare, Chicago, IL, USA) and 1% penicillin/streptomycin (Invitrogen) at 37 °C, 5% CO_2. HEK293 cells were seeded in 24-well plates and transfection was performed using a formulated polyethylenimine solution as previously described (Shin and Sohn,

2014). Hdh5-HT receptor expression plasmids, pcDNA3-HA-Hdh5-HT1B or Hdh5-HT4A, luciferase reporter plasmids containing the cAMP response element (CRE-Luc) or serum response element (SRE-Luc), and the pRSV-β galactosidase expression plasmid (internal control) were co-transfected into HEK293 cells (Ko et al., 2007; Oh et al., 2005). The total amount of plasmids used in each transfection was adjusted to 300 ng with pcDNA3-HA. At approximately 36 h post-transfection, the cells were maintained in FBS-free DMEM for starvation for 16 h. The cells were then treated with 5-HT, forskolin (FKN; a PKA pathway stimulator), and 12-O-tetradecanoylphorbol-13-acetate (a PKC pathway stimulator) (all from Sigma-Aldrich) in DMEM and incubated for further 6 h. The negative control cells were treated with the same amount of DMEM. Following treatment, the cells were harvested and luciferase activities were assayed using a microplate luminometer (Berthold, Bad Wildbad, Germany) as previously described (Ko et al., 2007). To normalize luciferase activity, aliquots of cell lysates were incubated with o-nitrophenyl-β-D-galactopyranoside solution at 37 °C for 2 h and β-galactosidase activities were analyzed using a microplate reader at 405 nm (Tecan, Männedorf, Switzerland).

2.6. Statistical analysis

Data were compared with one-way analysis of variance followed by Tukey's post-hoc test for three or more groups and with a two-tailed Student's *t*-test between two groups. Statistical significance was performed using IBM SPSS v.25.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Sequence analysis of Hdh5-HT receptors

A search of the Hdh transcriptome database with the sequences of the 5-HT receptors revealed eight putative orthologs of 5-HT receptor genes. Molecular phylogenetic analysis placed the Hdh5-HT receptors in the 5-HT1, -2, -4, -6, and -7 subfamilies (Fig. 1), designated as Hdh5-HT1 (1A and 1B), Hdh5-HT2, Hdh5-HT4 (4A and 4B), Hdh5-HT6, and Hdh5-HT7. In addition, an Hdh5-HT-like receptor was identified, which cannot be readily classified by any of the 5-HT receptor subtype nomenclatures. The prediction of transmembrane helices showed that all eight Hdh5-HT receptors have seven predicted trans-membrane (TM-1-7) domains, containing well-conserved sequences and motifs essential for the three-dimensional structure, ligand binding, and signal transduction of rhodopsin-like (class A) GPCRs (Bockaert and Pin, 1999; Costanzi, 2012), e.g., N in TM-1, LXXXD (X is any amino acid) in TM-2, W and P in TM-4, FXXXWXP in TM-6, and NPXXY in TM-7 (Fig. S1). Two cysteine residues in the extracellular loop (EL) 1 and 2, a GPCR signature tripeptide (E/DRY/F) at the interface of TM-3 and intracellular loop 2, and consensus sites for post-translational modifications were also found to be well conserved among vertebrate and invertebrate 5-HT receptors (Figs. S2–S6).

3.2. Expression pattern of Hdh5-HT receptor transcripts

Conventional PCR was first performed to confirm Hdh5-HT receptors expression patterns in the ganglia and the ovary in mature abalone. All eight *Hdh5-HT* cDNA amplicons were detected from the reverse-transcribed cDNAs of the PPG, whereas *Hdh5-HT1B*, *4A*, *4B*, and *6* were the major subtypes detected in the ovary (Fig. S7). Next, we examined the tissue distribution of the mRNA expression of *Hdh5-HT1B*, *4A*, *4B*, and *6* subtypes in mature female abalone. The mRNA expression levels of *Hdh5-HT1B* and *Hdh5-HT6* were significantly higher in the ganglia (CG and/or PPG) compared to those of the ovary, gills, intestine, and adductor muscle (Fig. 2A and D). *Hdh5-HT4A* and *4B* also showed a tendency of higher expression levels in the ganglia than in other examined tissues, although the differences were not statistically significant (Fig. 2B and C). Exceptionally, a significantly higher expression level of *Hdh5-HT4A* was detected in the adductor muscle (Fig. 2B).

3.3. Effects of E2 injection on Hdh5-HT receptor transcripts

The expression levels of the *Hdh5-HT* receptor genes were measured in the ovaries of the mature females following E2 injection. As shown in Fig. 3, *Hdh5-HT1B* and *4A* mRNA levels did not change significantly following injection of two doses (5 and 100 μg) of E2 until 72 h post-injection (Fig. 3A and B). *Hdh5-HT4B* mRNA expression showed a tendency of downregulation due to E2 injection compared to that of the saline-injected abalone, and the *Hdh5-HT4B* level showed a significant decrease by the high dose of E2 at 24 h post-injection (Fig. 3C). In contrast, *Hdh5-HT6* transcript levels showed an increasing tendency following E2 injection, and significant upregulation was detected by both low and high doses of E2 after 72 h (Fig. 3D).

3.4. Effects of 5-HT on Hdh5-HT1B and 4A receptor signaling

CRE- and SRE-driven reporter systems were applied to evaluate the potential 5-HT signaling pathways coupled to 5-HT receptors in Pacific abalone. Transiently expressed CRE- and SRE reporters in HEK293 cells were not affected by 5-HT treatment (10 μM) when 5-HT receptors were not overexpressed (Fig. S8). Immunocytochemical analysis revealed that Hdh5-HT1B and 4A were mainly expressed in the membrane of HEK293 cells, although the immunoreactive signals were also detected in the cytoplasm (Fig. S9). For Hdh5-HT1B receptor, FKN-stimulated CRE-Luc activities were significantly inhibited by 5-HT treatment in a dose-dependent manner (Fig. 4A), whereas SRE-Luc reporter activities were not affected by 5-HT (Fig. 4B). In the case of Hdh5-HT4A, the highest tested dose of 5-HT (5 μM) significantly increased the CRE-Luc reporter activity in FKN-stimulated HEK293 cells (Fig. 4C) and the SRE-Luc reporter activity in HEK293 cells (Fig. 4D).

4. Discussion

The 5-HT receptor family may represent an example of the complexity and redundancy of GPCRs with suspected diversity; however, the specific functions of some of the many subtypes in human health and/or disease remain to be elucidated (Hannon and Hoyer, 2008). Furthermore, 5-HT receptor subfamily members in mollusks do not exactly correspond to their vertebrate orthologs because of at least the partially diversified and mixed characteristics of their subtypes (Barbas et al., 2002; Osada et al., 1998). Recently, a transcriptome analysis of the sea slug *Hermissenda crassicornis* demonstrated the presence of putative orthologs of 5-HT1, -2, -4, and -7, and a novel receptor 5-HT6 (Tamvacakis et al., 2015). To clarify the 5-HT receptor classification in mollusks, we identified eight 5-HT receptor subtypes in the neural ganglia of the gastropod mollusk, Pacific abalone. The phylogenetic analysis showed that the molluskan 5-HT receptors are largely classified into four major clades, 5-HT1/5/7, 5-HT2, 5-HT4, and 5-HT6, in line with earlier studies (Tanabe et al., 2010; Wang and He, 2014). In mammals, the 5-HT1 receptor class is composed of five receptors (5-HT1A, 1B, 1D, 1E, and 1F), which, in humans, show 40–63% overall sequence identity (Hannon and Hoyer, 2008). The diverged molluskan 5-HT1 subtypes, including Hdh5-HT1A and B, indicate that the 5-HT1 gene has undergone a duplication event in mollusks, resulting in new subtypes (Nagakura et al., 2010). Detailed examination of 5-HT2 subtypes showed that the Ala residue in the middle of TM-5 is conserved in molluskan 5-HT2, which was suggested to confer agonist (D-lysergic acid diethylamide) selectivity for human 5-HT2A (Almaula et al., 1996). Most of the predicted sequences and motifs in Hdh5-HT receptors are relatively well conserved in terms of their overall architecture with regard to rhodopsin-like (class A) GPCR features (Bockaert and Pin, 1999; Costanzi, 2012; McCorvy and Roth, 2015). However,

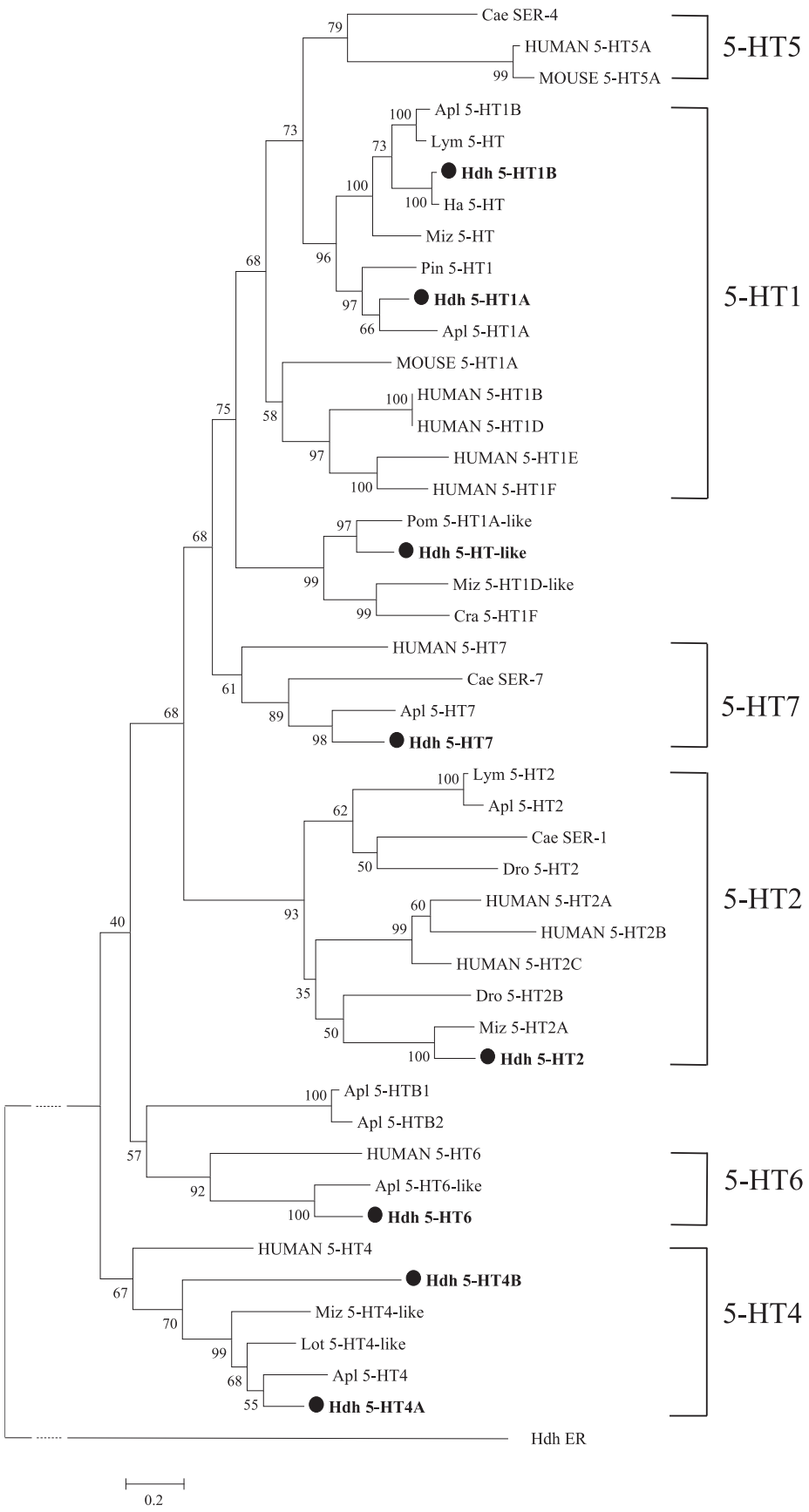

Fig. 1. Maximum-likelihood phylogenetic tree showing relationships between 5-HT receptor subtypes. The bootstrap values from 1000 replicates are given at each branch node. The scale bar indicates 0.2 amino acid replacements per site. Each sequence is denoted by the species from which it was isolated followed by its 5-HT receptor subtype. Groups of 5-HT receptors are distinguished by brackets. 5-HT receptors from *H. discus hannai* are shown in bold and marked by black circles. See Table S1 in the online supporting information for the nomenclature and the corresponding GenBank accession number of the reference sequences. Hdh (*Haliotis discus hannai*), Apl (*Aplysia californica*), Cae (*Caenorhabditis elegans*), Cra (*Crassostrea gigas*), Dro (*Drosophila melanogaster*), Ha (*Haliotis asinina*), HUMAN (*Homo sapiens*), Lym (*Lynmnaea stagnalis*), Lot (*Lottia gigantea*), Miz (*Mizuhopecten yessoensis*), MOUSE (*Mus musculus*), Pin (*Pinctada fucata*), Pom (*Pomacea canaliculata*). An estrogen receptor (Hdh ER) was included as an outgroup.

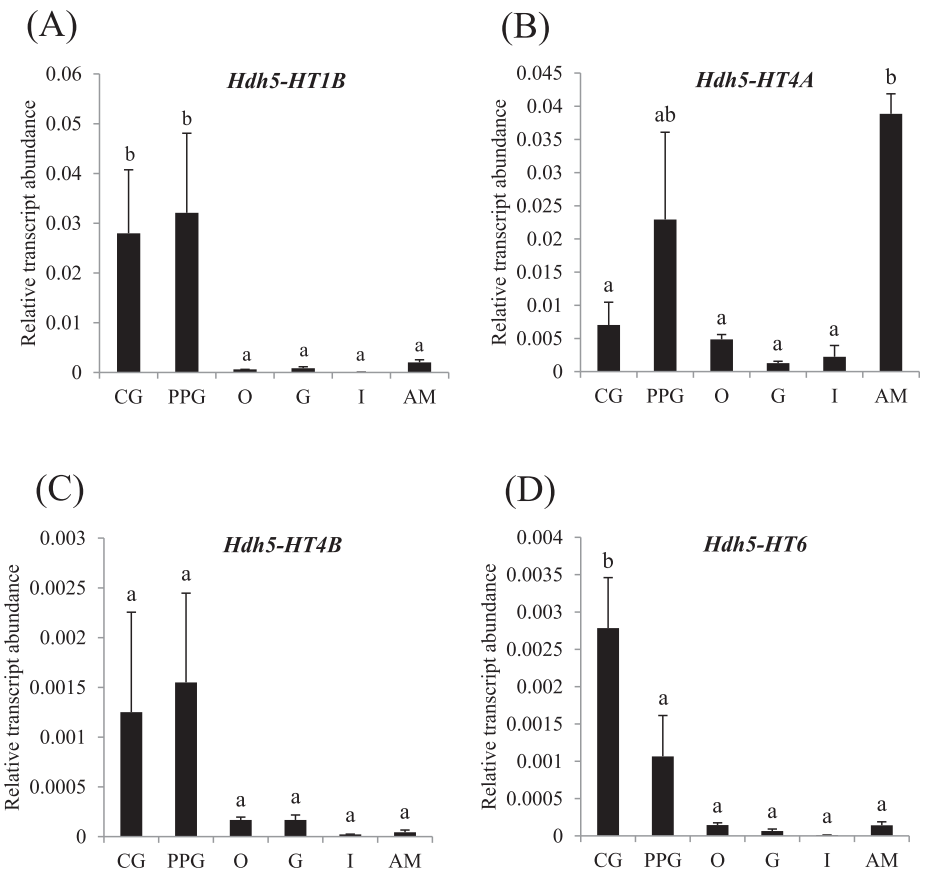

Fig. 2. Relative expression levels of Hdh5-HT receptor transcripts in various tissues of mature female abalone. The receptor mRNA levels of *Hdh5-HT1B* (A), *Hdh5-HT4A* (B), *Hdh5-HT4B* (C), and *Hdh5-HT6* (D) were quantified by qPCR and described as means ± SEM (N = 4–5). Different lowercase letters on the bars indicate significantly different values ($P < 0.05$). *RPL-5* mRNA was used as a reference gene to calibrate the reverse-transcribed cDNA templates for all the samples. CG: cerebral ganglion: PPG: pleuro-pedal ganglion; O: ovary; G: gills; I: intestine; AM: adductor muscle.

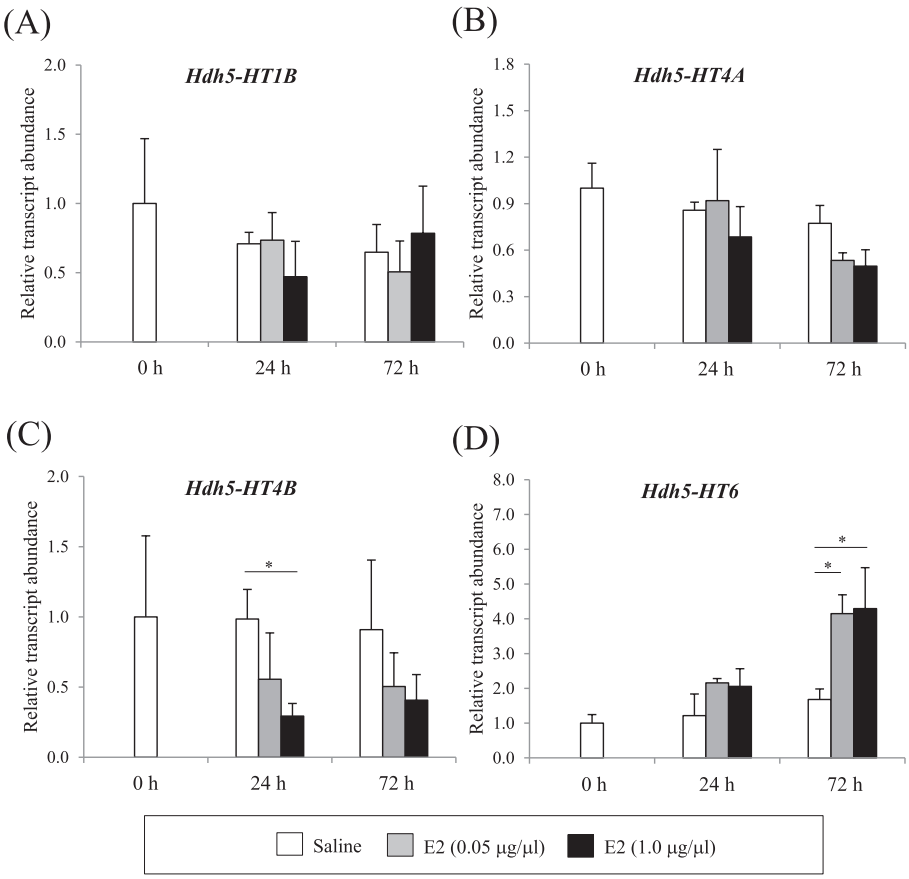

Fig. 3. Effect of E2 injection on expression of Hdh5-HT receptor transcripts in the ovary of mature female abalone. E2-dissolved saline (100 μL) at a concentration of 0.05 or 1 μg/μL was injected into the pedal sinus of each female abalone. The ovarian tissues were collected at 0, 24, and 72 h post-injection. The receptor mRNA levels of *Hdh5-HT1B* (A), *Hdh5-HT4A* (B), *Hdh5-HT4B* (C), and *Hdh5-HT6* (D) were quantified by quantitative real-time PCR as indicated in Fig. 2 and are described as means ± SEM (N = 3–4). Asterisks mean a significantly different value from the saline-injected control ($P < 0.05$).

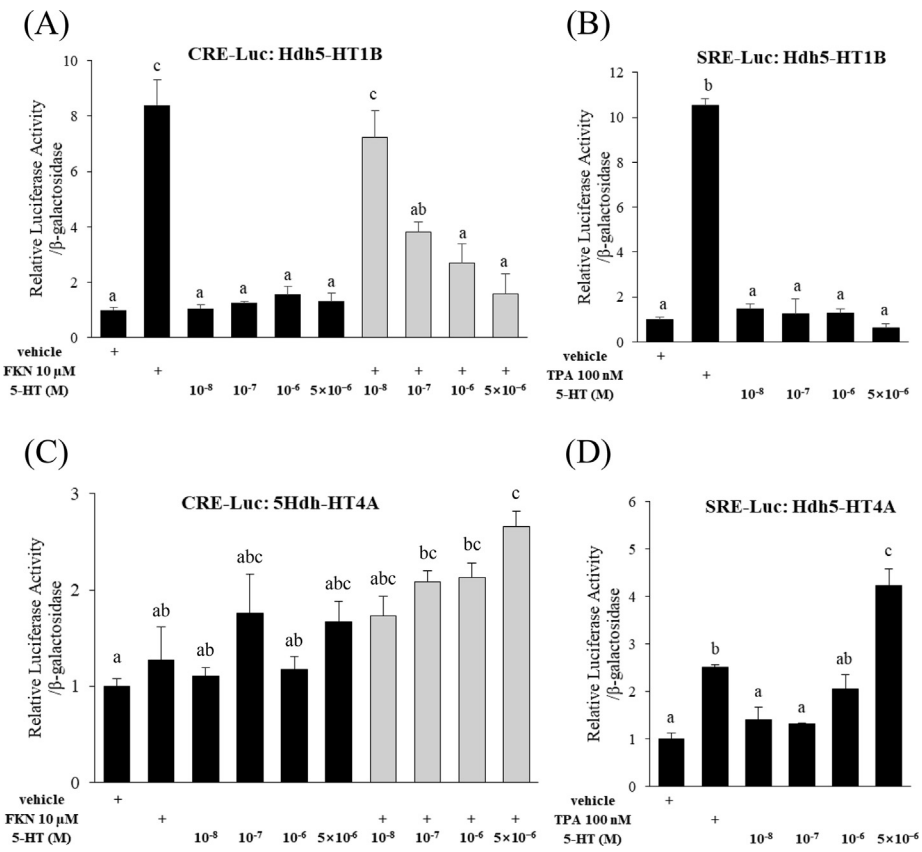

Fig. 4. Effect of 5-HT on CRE- and SRE-luciferase reporter activity in Hdh5-HT-overexpression HEK293 cells. Cells were transiently transfected with Hdh5-HT receptor expression plasmids in combination with CRE- or SRE-Luc reporter plasmids, and pRSV-β galactosidase plasmid (panels A and B for Hdh5-HT1B; panels C and D for Hdh5-HT4A). Approximately 36 h post-transfection, the cells were maintained in serum-free medium for starvation for 16 h and treated with various doses of 5-HT and/or FKN (10 μM) or 12-O-tetradecanoylphorbol-13-acetate (TPA, 100 nM) for 6 h. All data shown are means ± SEM (N = 3). Different lowercase letters on the bars indicate significantly different values ($P < 0.05$).

Hdh5-HT4B is not a typical 5-HT4 subtype in terms of the conserved sequences and motifs, e.g., mutations in E/DRY/F in TM-3, a Cys residue for a disulfide bridge between TM-3 and EL-2, and the so-called P-I-F motif for receptor activation (McCorvy and Roth, 2015). These differences between species may provide insight into the important sequences for 5-HT signal transduction, and will therefore need to be investigated in future studies.

The physiological significance of the 5-HT receptor in the molluskan gonad is still unclear, although earlier studies demonstrated the efficacy of 5-HT for promoting oocyte maturation and spawning of the scallop *P. yessoensis* (Matsutani and Nomura, 1982, 1987; Tanabe et al., 2006). We detected the mRNA expression of *Hdh5-HT1B*, *-4A*, *-4B*, and *-6* receptors in the ovary of Pacific abalone, suggesting that these subtypes are involved in reproductive processes. In mollusks, 5-HT1-like subtypes were previously shown to be highly expressed in the ovary as well as in the nervous systems (Angers et al., 1998; Panasophonkul et al., 2009; Tanabe et al., 2010; Wang and He, 2014). Interestingly, the *Aplysia* Ap5-HTB1 receptor was reported to be dominantly expressed in the ovotestis, and amino acid sequence comparison revealed that Ap5-HTB1 is closely related to mammalian 5-HT6 (Barbas et al., 2002; Li et al., 1995). However, analysis of the pharmacological characteristics showed that Ap5-HTB1 is positively coupled with phospholipase C, indicating that Ap5-HTB1 is functionally grouped into the mammalian 5-HT2 subtype (Hannon and Hoyer, 2008). In the nematode *Caenorhabditis elegans*, *ser-1*, *ser-4*, *ser-5*, and *ser-7* are required for 5-HT-modulated egg laying, as demonstrated by animals containing null mutations in the genes encoding these 5-HT receptors (Carnell et al., 2005; Hapiak et al., 2009). Among these 5-HT receptor orthologs, SER-4 and SER-5 phylogenetically cluster in the 5-HT1 and 5-HT6 subfamilies, respectively, based on analysis of truncated amino acid sequences with the variable N- and C-termini and third intracellular loops deleted (Hapiak et al., 2009). In accordance with this result, the SER-4 sequence was found to be more similar to that of the Hdh5-HT1A and 1B subtypes (30.3% and 34.7%, respectively) than to that of other *Haliotis* 5-HT receptors (23.1–26.1%). Moreover, the high relative expression levels of *Hdh5-HT4A* mRNA detected in the adductor muscle suggest that this receptor subtype is possibly involved in 5-HT-mediated muscle movement in the gonad. This possibility is worthy of further investigation considering that a 5-HT4-specific antagonist (GR 113808) was previously shown to inhibit the 5-HT-induced contraction of human detrusor muscle strips (Tonini et al., 1994), and several bundles of 5-HT-immunoreactive fibers were innervated into the scallop gonad near the adductor muscle (Matsutani and Nomura, 1986).

E2 has been suggested to play a role in the oocyte development of mollusks, although there is conflicting evidence for the presence of sex steroids and biological effects (Scott, 2013). Induction of ovarian vitellogenin and estrogen receptor expression following *in vitro* and *in vivo* treatment of E2 strongly supports the roles of E2 in the control of oocyte development in marine mollusks (Ni et al., 2014; Osada et al., 2003; Tran et al., 2016a,b). Furthermore, E2 could upregulate the expression of 5-HT1 receptors in oocytes during oocyte maturation in the Japanese scallop and oyster species (Osada et al., 1998, Tanabe et al., 2010; Wang and He, 2014), and promoted 5-HT-induced spawning in sea scallop (Wang and Croll, 2006). To the best of our knowledge, the present study provides the first evidence to suggest that E2 selectively influences the transcript-level expression of 5-HT receptor subtypes in the molluskan ovary, with a decrease of *Hdh5-HT4B*, increase of *Hdh5-HT6*, and no alteration of *Hdh5-HT1B* and *4A* levels detected at 72 h post-injection of E2. Since the specific roles of E2 in the control of reproductive activity and 5-HT-induced spawning in mollusks are not yet fully established, this result may broaden the possible involvement of 5-HT receptor subtypes in 5-HT- and E2-mediated oocyte development and maturation in mollusks. As mentioned above, *5-HT1* is the only E2-induced 5-HT receptor subtype detected in mollusks to date, whereas the *5-HT1* expression level in the mature mussel *M. edulis* was found to decrease following E2 treatment (Cubero-Leon et al., 2010). In fact, injections of E2 also decreased 5-HT levels in the gonad of *M. edulis* (Gagné and Blaise, 2003). Thus, further investigation on the different

expression patterns of 5-HT receptors with subtype-specific antagonists is needed to elucidate the molecular mechanism of diverse 5-HT receptors in the abalone ovary *in vivo* and *in vitro*.

Hdh5-HT1B shows high sequence identities with the tropical abalone 5-HT receptor Ha5-HT (97%) (Panasophonkul et al., 2009), Japanese scallop Miz5-HT (60%) (Tanabe et al., 2010), *Lymnaea* Lym5-HT (57%) (Sugamori et al., 1993), *Aplysia* 5-HT1B (5-HTap2) (57%) (Barbas et al., 2002), and human 5-HT1A (41%), suggesting a close association with the mammalian 5-HT1 subfamily. This was confirmed in the present phylogenetic analysis showing that Hdh5-HT1B formed a group with members of the 5-HT1 subfamily. In addition, Hdh5-HT1B-expressing HEK293 cells showed a dose-dependent inhibitory effect of 5-HT on FKN-stimulated CRE-Luc activities, indicating that Hdh5-HT1B is coupled to G_i protein and inhibits adenylate cyclase (AC) and cAMP accumulation, in line with a previous study on *Aplysia* 5-HT1B (Barbas et al., 2002). Nevertheless, the observed Hdh5-HT4A-mediated reporter activation does not exactly correspond with that previously reported for the 5-HT4 subtype, since the mammalian 5-HT4/6/7 subtypes couple preferentially to G_s protein and promote cAMP formation through activation of various ACs (Hannon and Hoyer, 2008). Unexpectedly, Hdh5-HT4A-mediated SRE-Luc activation was observed with treatment of a high dose of 5-HT. Since G_q-coupled receptors can activate SRE-mediated reporter gene transcription via PKC-dependent MAP kinase activation (Hill et al., 2001), Hdh5-HT4A is most likely an atypical G_q-coupled 5-HT receptor. The sequence identity of Hdh5-HT4A is higher with *Aplysia* 5-HT4 (64%) (Nagakura et al., 2010), *Lottia* 5-HT4-like (72%) (Simakov et al., 2013), *Mizuhopecten* 5-HT4-like (60%) (Wang et al., 2017), and human 5-HT4 (43%), indicating that Hdh5-HT4A belongs to a large 5-HT4 subfamily. In accordance with a study using rat 5-HT6-transfected HEK293 cells (Romero et al., 2006), co-incubation with FKN increased the basal CRE-Luc activity in Hdh5-HT4A-transfected HEK293 cells induced by 5-HT with regard to both the amplitude and potency. The SRE-Luc activation by 5-HT in Hdh5-HT4A-transfected cells suggests that Hdh5-HT4A preferentially couples with G_q protein to increase inositol phosphates and cytosolic Ca^{2+} (Hill et al., 2001), similar to mammalian 5-HT2 receptors (Hannon and Hoyer, 2008).

In conclusion, we identified multiple 5-HT receptor subtypes in Pacific abalone that were classified into four major groups: 5-HT1/5/7, 5-HT2, 5-HT4, and 5-HT6. Among the receptors expressed in the ovary, *Hdh5-HT4B* and *Hdh5-HT6* transcript levels were most susceptible to E2 *in vivo*. Based on experiments with receptor-transfected cells along with luciferase reporters, Hdh5-HT1B appears to most likely couple to G_i protein and inhibit cellular cAMP production, whereas Hdh5-HT4A displays a complex combination of the pharmacological characteristics of 5-HT2 and 5-HT4.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.001.

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