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Characterization of a $GABA_A$ receptor β subunit in the abalone *Haliotis asinina* that is upregulated during larval development

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ABSTRACT

In the tropical abalone *Haliotis asinina*, the neurotransmitter γ-aminobutyric acid (GABA) is a potent inducer of larval settlement, a process beginning with the onset of a behavioral search for a suitable substratum and ending with metamorphosis. In the natural environment, larvae can encounter GABA or GABA-like molecules through association with conspecific foot mucus and crustose coralline algae. To understand the role of GABA in the molecular process leading to settlement required identification and analysis of GABA's cognate receptor. We now have isolated the first abalone full-length $GABA_A$ receptor (*Has*-$GABA_A$R) β subunit gene, which encodes a protein of 485 amino acids, from juvenile *H. asinina* neural tissue. Similar to other metazoan $GABA_A$Rs, the abalone $GABA_A$R contains four transmembrane domains, a conserved cysteine loop in the N-terminal extra-cellular domain, and highly conserved sequence motifs. The *Has-GABA$_A$R* gene is expressed at extremely low levels in unfertilized eggs, but increases significantly just prior to settlement, peaking at 120 h post fertilization (hpf). We further demonstrate that during the period of larval competence (96–144 hpf), gene transcripts and the encoded *Has*-$GABA_A$R were localized in a cluster of cells along the dorsal and lateral edges of the foot, as well as the posterior epithelium. In functional settlement assays using GABA and 5-AVA, we found that there was significantly lower settlement of veligers pre-treated with antibodies to an external domain of the *Has*-$GABA_A$R than those treated with preimmune serum, or untreated veligers. We postulate that this receptor may act as a highly sensitive chemical sensor, whose activation is necessary to trigger chloride-mediated sensory neuron activation or inhibition, leading to the initiation of settlement and metamorphosis events.

1. Introduction

For many animals, chemosensory detection of marine waterborne molecules is crucial for sensing the external environment and controlling subsequent behavior and development. In the gastropod *Haliotis* (abalone), development has been identified as being a continuous process which following spawning and fertilization involves gradual morphogenic changes and growth that varies among species (Hahn, 1989). One of the key stages in the life-cycle of *Haliotis* spp. is the preveliger stage, which generally lasts for 1 to 3 days, during which time the larvae alternately creep and suspend searching for a favorable settlement place. After settlement, the larvae begin to secrete mucus from the foot sole (Laimek et al., 2008), firmly adhering to the substratum. They also begin diatom feeding and proceed to develop an early shell as they metamorphose into juveniles (Hahn, 1989; Singhagraiwan and Doi, 1993). The neurotransmitter γ-aminobutyric acid (GABA), as well as GABA-like molecules, have been recognized as key chemical mediators for the induction of settlement and subsequent metamorphosis (Gapasin and Polohan, 2004; Laimek et al., 2008; Morse and Morse, 1984; Morse, 1992; Roberts and Nicholson, 1997; Stewart et al., 2008). Induction of larval settlement and metamorphosis by GABA also occurs in other molluscan species, including a variety of gastropods (Hatakeyama and Ito, 2000; Hernádi, 1994; Ierusalimsky and Balaban, 2001; Jing et al., 2003).

In mammalian systems, there are three classes of GABA receptors (GABAR), $GABA_A$ and $GABA_C$ (fast response) receptors, which are ligand-gated ion-channels, and $GABA_B$ (slow response) receptors, which are G-protein-coupled receptors. GABA exhibits conformational

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flexibility, because the internal bonds can rotate freely (Johnston, 1968), thereby permitting interactions with different types of GABA receptors (Andrews and Johnston, 1979; Chebib and Johnston, 1999). In invertebrate systems, $GABA_A$ and $GABA_B$ receptor subunits have been identified in a number of species, including *Drosophila melanogaster* (Henderson et al., 1993), *Caenorhabditis remanei* (GenBank accession no. AAZ42341), *Aedes aegypti* (GenBank accession no. XP_001654541), and *Culex quinquefasciatus* (GenBank accession no. EDS38916). In molluscs, only $GABA_A$Rs have been described in a few species by molecular cloning, including *Lymnaea stagnalis* (Hutton et al., 1993), *Sepia officinalis* (Kirby et al., 1997), and *Aplysia californica* (Moroz et al., 2006).

The first $GABA_A$R gene isolated in a mollusc was from the freshwater gastropod *L. stagnalis* (Hutton et al., 1993); its four transmembrane (TM) domains and six intron–exon splicing sites are similar to those found in vertebrate $GABA_A$R genes, indicating a high degree of conservation (Hutton et al., 1993). In later studies, the *L. stagnalis* $GABA_A$R β subunits were found to share high nucleotide sequence identity with those described in the cephalopod, *S. officinalis* (Kirby et al., 1997). Kirby (2000) also identified another two types of $GABA_A$R subunits, exhibiting about 50% identity to both vertebrate $GABA_A$R β subunits and the invertebrate $GABA_A$R β subunits previously described (Hutton et al., 1993). Studies on molluscan $GABA_A$Rs show that the GABA-receptor interaction is physiologically and pharmacologically similar to that observed in mammals. For example, the $GABA_A$Rs of *L. stagnalis* are sensitive to the blocking effect of bicuculline, a vertebrate $GABA_A$R antagonist (Hutton et al., 1993). Also, the action of GABA on the feeding rhythm generator of *L. stagnalis* can be mimicked by baclofen (which activates the $GABA_B$ receptors in mammalian neurons), but is not sensitive to bicuculline (which is a $GABA_A$R antagonist in mammals). Conversely, bicuculline competitively inhibits the GABA-induced excitation of the tentacle motor neurons in *L. stagnalis* (Arshavsky et al., 1993).

In our previous work on Haliotids, we found that GABA was secreted naturally into the mucus of juvenile and adult abalone, as well as other gastropods (Laimek et al., 2008). This observation led us to postulate that there might be a GABA receptor expressed in larvae, and that this receptor might be necessary for the induction of settlement and subsequent metamorphosis. No GABA receptor has previously been identified in abalone, thus there has been no opportunity to elucidate the interaction of a receptor with GABA. The identification of a GABA receptor in abalone would provide the means to clarify the molecular regulatory mechanisms associated with GABA-induced settlement behavior, and could significantly expand our understanding of chemically-mediated behavioral changes in abalone larvae.

Here we report the isolation of a *Has-GABA$_A$R-β* gene, encoding a $GABA_A$R β subunit of 485 amino acids, from the nerve ganglia of juvenile *Haliotis asinina*. This *Has-GABA$_A$R* β gene shares the entire ligand-gated ion channel receptor motifs characteristic of a typical $GABA_A$R β subunit. We use quantitative RT-PCR, whole mount *in situ* hybridization and immunohistochemistry to document the temporal and spatial expression of this receptor in developing larvae, with a focus on the critical stages of competence, settlement and metamorphosis.

2. Materials and methods

2.1. Cloning of a H. asinina GABA receptor (Has-GABA$_A$R β subunit)

Juvenile animals (12 months old) were collected from the Coastal Aquaculture Development Center, Department of Fisheries, Prachaub Khiri Khan Province, Thailand. Once in the laboratory, they were anesthetized on ice. Cerebral and pleuropedal ganglia were dissected out and rinsed with 0.1 mol l^{-1} PBS pH 7.8, then immediately frozen in liquid nitrogen and stored at −80 °C until used for RNA extractions. Total RNA was isolated using TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions. The RNA precipitate was dissolved in RNase-free distilled water and the quality and quantity of total RNA calculated by UV spectrophotometry, as well as RNA gel electrophoresis.

Full-length double-stranded RACE cDNA was synthesized using a two-step process, based on a protocol adapted from the SMART™ (Switching Mechanism) protocol. The sequences of oligonucleotide primers (OP) and RACE primers used to clone a $GABA_A$R β subunit were based on degenerate primers designed from sequence alignment of *L. stagnalis* (GenBank access no. X58638) and *S. officinalis* (GenBank accession no. AY005810), *OP1*: ATT GTC GAC TIT CNT GGG TNW SNT TYT GG, *OP2*: GCY GAG AAR ATC TGG GTY CCA GAC ACR TTC YTY GCC AAC, *OP3*: CCN AGA GTG CAG GAC GTG AAN, *OP4*: GCG TAY TTG TCS ATC GTR TTC ACG TCC TGC AC, *OP5*: GTG ATG TCR TGS ARG AAA G (these oligonucleotide primers were all synthesized by Proligo Australia Pty. Ltd.; where I denotes Inosine, K = G or T, M = A or C, R = A or G, S = C or G, W = A or T, Y = C or T, N = A or C or G or T, V = A or C or G). Unless specified elsewhere, PCRs were performed as a 2-step process to amplify low copy number transcripts. The first PCR reaction was performed using 1× PCR buffer, 3 mmol l^{-1} $MgCl_2$, 100 μmol l^{-1} dNTPs, 0.5 μmol l^{-1} of forward and reverse primers, 1.25 U of Taq DNA polymerase (Scientifix), 1 μl of cDNA and sterile H_2O to a final volume of 25 μl. The PCR reaction was conducted in a 96-well cooled-gradient palm-cycler with an initial hot start PCR denaturation step at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 50–55 °C for 30–60 s, and an extension at 72 °C for 1–1.30 min. This was followed by a final extension step at 72 °C for 10 min. The second PCR was performed by taking 5 μl of previous amplified products and added to a 0.2 ml PCR tube containing the mixture of 1× PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 100 μmol l^{-1} dNTPs, 0.5 μmol l^{-1} of forward and reverse primers, 1.25 units of Taq DNA polymerase (Scientifix), and sdH_2O to a final volume of 25 μl. The PCR reaction was performed as above, except a 5 °C increase in annealing temperature.

Cloning of PCR products was achieved after separation using 2% agarose gel electrophoresis with 0.5× TBE, visualization under UV after ethidium bromide staining, and purification using an Ultra™ GelSpin™ DNA Purification Kit (Mol Bio laboratories, Inc.), as per the manufacturer's instructions. Purified fragments were ligated into pCR®2.1-TOPO® 3.9 kb vector (Invitrogen) using a TOPO TA Cloning kit (Invitrogen), according to the manufacturer's instructions. After transformation into competent *E. coli* Top10F′ cells using a One Shot® chemical transformation kit (Invitrogen), the plasmids were isolated from white colonies using an Aurum™ Plasmid Mini Kit (Bio-Rad), and insert sizes were analyzed by PCR. Inserts of appropriate sizes were sequenced by the Australian Genome Research Facility in Brisbane, Australia.

2.2. Phylogenetic analysis of the Has-GABA$_A$R β subunit sequence

Cloned PCR sequences were analyzed and homologies compared with known sequences in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST). Alignments were performed using Clustal X, 8.1 (Thompson et al., 1997). Nucleotide sequences were translated into amino acid sequence using the Gene Runner 3.05 program. Hydrophobic transmembrane (TM) domains were predicted using the TMHMM 2.0 program and the signal sequence of the mature protein was identified by the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). The unrooted tree was generated with 1000 bootstrap trials using the neighbor-joining method (Saitou and Nei, 1987), and presented with a cut-off bootstrapping value of 50.

2.3. Spawning and larval culture of H. asinina for temporal and spatial expression of Has-GABA$_A$R-β

Spawning of adult *H. asinina* and larval culture were carried out at the University of Queensland Heron Island Research Station on the