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Characterization of red pigment concentrating hormone (RPCH) in the female mud crab (*Scylla olivacea*) and the effect of 5-HT on its expression

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ABSTRACT

Red pigment concentrating hormone (RPCH) is a member of the chromatophorotropic hormones and, in crustaceans, it is synthesized in the eyestalk. We have isolated a full-length cDNA for a RPCH preprohormone gene (*Scyol-RPCH*) from the eyestalks of female mud crabs, *Scylla olivacea*. The open reading frame consists of 642 nucleotides, and encodes a deduced 108 amino acid precursor protein, which includes a signal peptide, the RPCH (pQLNFSPGWamide), and an associated peptide. We show that the mud crab RPCH peptide exhibits 100% identity with 15 other decapods. Expression of *Scyol-RPCH* within adult mud crab takes place in the eyestalk, brain, and ventral nerve cord, comprising subesophageal ganglion, thoracic ganglion, and abdominal ganglion. *In situ* hybridization demonstrates specific expression within neuronal clusters 1, 2, 3, and 4 of the eyestalk X-organ, clusters 6, 8, 9, 10, and 17 of the brain, and in neuronal clusters of the ventral nerve cord. We found that administration of 5-HT up-regulates RPCH gene expression in the eyestalk, suggesting that RPCH may play a role as a downstream hormone of 5-HT.

1. Introduction

Red pigment concentrating hormone (RPCH) is a peptide hormone that was first discovered in crustaceans, and involved in the distribution of the pigments and light–dark adaption (reviewed by [28]). In decapod crustaceans, RPCH is synthesized by the X-organ and sinus gland, both of which are located in the eyestalks [20,31]. RPCH was first identified within eyestalks of the pink shrimp, *Pandalus borealis* [6]. Subsequently, RPCH sequences were identified in several other crustacean species, including crabs and crayfish [10,11,22,43]. Based on amino acid composition and its chromatographic characteristics, RPCHs of several other decapod crustaceans are known to be identical [31], exhibiting the same octapeptide sequence (pQLNFSPGWamide), suggesting structural conservation among decapod crustaceans.

RPCH-immunopositive cells have been detected in the eyestalk (ES), brain (BR), and ventral nerve cord (VNC), comprising the subesophageal ganglion (SEG), thoracic ganglion (TG), and abdominal ganglion (AG). They were present in the ES, BR and TG of the common shore crab, *Carcinus maenas* [20], where it is thought to act as a neurotransmitter. In the red swamp crayfish, *Procambarus clarkii*, one mode of its action is to promote the synthesis of methyl farnesoate in the mandibular organs [13], and functions as a hormone in regulating crustacean reproduction [14,30]. This is further supported by experiments showing that RPCH injected into the animal increases ovarian index and oocyte diameter [35]. Moreover, exposure of ovarian explants to RPCH leads to ovarian maturation [35].

Comparative sequence studies across a variety of species shows that RPCH belongs to the same family as adipokinetic hormone (AKH), gonadotropin-releasing hormone (GnRH), and corazonin (Crz), and it was recently proposed through several lines of evidence that these hormones should be grouped into one GnRH superfamily [29]. Importantly, the AKH/RPCH family shares a wide variety of functions, and all act as neurohormones, neurotransmitters, and/or neuromodulators. AKH can concentrate pigments in some crustaceans, whereas RPCH can stimulate lipid metabolism in some insects [9,24,41]. Recently, it was reported that the AKH and GnRH signaling system probably arose very early in metazoan evolution [17,29]. Moreover, AKH stimulates egg-laying through a GnRH receptor in *Caenorhabditis elegans* [17]. Crz was grouped within the GnRH superfamily, based on its structural relationship with the others in the GnRH superfamily [29]. There is little understanding for the role of Crz in arthropods, although it is regarded as a candidate stress hormone [3].

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The mud crab, *Scylla olivacea*, is an economically important species and has become increasingly popular for its meat quality and large size [12,25]. This species is found in estuaries and mangrove regions throughout the Indo-Pacific region, from East and South Africa to Southeast Asia. In Thailand, they are widely distributed along the coasts of the Andaman Sea and the Gulf of Thailand, but the numbers have been steadily decreasing over the past 10 years due mainly to high demand for both domestic and export markets [8]. Very little is known about how RPCH regulates reproduction of the mud crab, or whether it is involved in other important functions.

Serotonin (5-HT) is well known as a neurotransmitter that plays a role in invertebrate reproduction, and is present in the crustacean CNS, and gonad, where it induces gonad maturation [23,32,38–40,42]. In crustaceans, 5-HT has been postulated to stimulate reproduction through induction of a putative gonad-stimulating hormone (GSH) [7]. However, the mechanism by which 5-HT stimulates gonad maturation remains unclear.

Therefore, in this study we firstly identified the mud crab RPCH gene and then defined its tissue-specific, spatial expression throughout the central nervous system (CNS). We then showed in a preliminary study that the expression of RPCH increases following injections of 5-HT.

2. Materials and methods

2.1. Experimental animals

Mature mud crabs, *S. olivacea* (400 ± 50 g body weight and 100–120 mm carapace width), were obtained from a commercial farm, Chanthaburi province, Thailand. They were kept in concrete tanks with seawater at 26–28 °C, and approximately 50% of the sea water was changed every day. The crabs were kept under a photoperiod of 12:12 h light–dark and fed with food pellets twice daily for 10 days before experimental analyses.

2.2. RNA preparation and gene identification

Eyestalks were collected and immediately frozen in liquid nitrogen for storage at −80 °C. Total RNA was prepared from each tissue using Trizol reagent (Invitrogen, CA, USA), following the manufacturer's protocol, and kept at −80 °C, until used. The purity and quantity of RNA was measured by a spectrophotometer at 260 and 280 nm. DNase I (Invitrogen, CA, USA) was used to digest single- and double-stranded DNA. Total RNA (1 μg) and 100 ng of random hexamer primers (Fermentas, Maryland, USA) were used for first-strand cDNA synthesis, using SuperScript III Reverse Transcriptase (Invitrogen, CA, USA), following the manufacturer's protocols. Primers for PCR amplification of RPCH were designed from an alignment of RPCH sequences of the crustacean species, *C. maenas* and *Callinectes sapidus* (Fig. 2), and first-strand cDNA was used as a template in PCR, using the forward and reverse primers shown in Table 1. Thermocycling conditions used for PCR amplification was: one cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 48 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

To identify a full-length RPCH gene sequence, 3′ RACE and 5′ RACE were performed using the SMART™ cDNA library construction kit (Clontech, CA, USA), and a 5′-Full RACE Core Set (Takara, USA), respectively, following the manufacturer's protocols. Universal primers (SMIIIB) provided in the kit and gene-specific primers (5RPCHR 1–2 and 3RPCHF 1–2) were used to obtain the complete gene sequence (Table 1). Thermocycling conditions were: one cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

All amplification products were analyzed using 1.5% agarose gel electrophoresis. The predicted full-length size amplicon was purified using a QIAquick gel extraction kit (Qiagen, Germany), and cloned into a pDrive cloning vector (Qiagen, Germany). Plasmids with insert sequences were purified using GeneJET™ Plasmid Miniprep Kit (Fermentas, USA), and sequenced by Macrogen (Korea).

2.3. Bioinformatic analyses

The full-length RPCH nucleotide sequence was used to search the NCBI GenBank database for similar sequences, and was translated into a deduced amino acid sequence by the Expasy bioinformatics resource portal-translate tool (http://web.expasy.org/translate/). Similar protein sequences of other species were retrieved from Entrez (NCBI). Sequence comparison to other species was performed using the ClustalW2 program. The signal peptide was predicted using the signal IP program 4.0 [27], and predicted cleavage sites using the Neuropred program (http://neuroproteomics.scs.illinois.edu/neuropred.html). A phylogenetic analysis of peptide hormone sequences for the GnRH/AKH/RPCH and Crz superfamily was performed using the neighbor-joining method, and evaluated using the interior branch test method with MEGA version 5 software [33,37]. Percentages of the associated taxa clustered together in the bootstrap test are shown next to branches.

2.4. Tissue distribution by RT-PCR

RNA was extracted from various tissues of five female crabs and five male crabs, using Trizol reagent. The RNAs of eyestalk (ES), brain (BR), ventral nerve cord (VNC), ovary stage 1 (OV1), ovary stage 5 (OV5), heart (HT), hemocyte (HC), gut (G), gill (GI), hepatopancrease (HP), muscle (MU), penis (PE), vas deferens (VA), proximal spermatic duct (PSD), middle spermatic duct (MSD), and distal spermatic duct (DSD), were used as templates in RT-PCR. Each PCR was performed using specific primers of the *S. olivacea* RPCH gene, Sp-RPCH-F and Sp-RPCH-R (Table 1). No addition of templates (i.e., reactions without RNA), were used as negative controls (N), and positive controls utilized amplification of the β-actin gene.

2.5. Tissue distribution by in situ hybridization

Using the plasmids, the mud crab RPCH gene was PCR-amplified using M13 forward (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primers (5′-AACAGCTATGACCATG-3′). PCR products were extracted using QIAquick Gel Extraction Kit (QIAGEN), and eluted by DEPC-treated H_2O. The RNA was labeled with a DIG-oligonucleotide labeling kit (Roche, Germany). Mud crab eyestalk, brain and ventral nerve cords were dissected out and fixed with Davidson's fixative and then embedded into paraffin blocks. Consecutive paraffin sections of 5–6 μm thick were cut and mounted onto silane-coated slides. The tissues were dewaxed in fresh xylene (twice for 10 min each) and rehydrated with a decreasing concentration of ethanol solutions. The sections were treated with a TE buffer (100 mM Tris–HCl, 50 mM EDTA, pH 8.0) containing RNAse-free proteinase K (5 μg/ml) (Roche, Germany), at 37 °C for 30 min. Post-fixation was performed with DEPC-treated PBS containing 4% paraformaldehyde, at 4 °C for 5 min. The sections were then incubated in prehybridization buffer (4× SSC containing 50% deionized formamide), at 37 °C for 10 min, to prevent non-specific binding of the probe to sections. After prehybridization, the buffer was removed, and each section overlaid with 200 μl of hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA) containing 10 ng of sense or anti-sense DIG-labeled RNA probe. Sections were overlaid with hybridization buffer and incubated at 42 °C