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The neuropeptides of Asian freshwater clam (*Corbicula fluminea*) as new molecular biomarker basing on the responses of organophosphate chemicals exposure

Qi Wang[a,c], Xiangsheng Hong[a,c], Huihui Chen[a], Lilai Yuan[a], Jinmiao Zha[a,b,*]

[a] Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Haidian District, Beijing 100085, China

[b] Beijing Key Laboratory of Industrial Wastewater Treatment and Reuse, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[c] University of Chinese Academy of Sciences, Beijing 100085, China

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ABSTRACT

In the present study, to discover new biomarker of Asian freshwater clam (*Corbicula fluminea*) to assess impact of environmental pollutions, cholecystokinin (CCK), conopressin, and Neuropeptide FF (FFamide) in *C. fluminea* were selected as potent biomarkers. Therefore, their full-length cDNAs were cloned and characterized to investigate the molecular characteristics and expression patterns of neuropeptides in *C. fluminea*. According to the sequence analysis, CCK, conopressin, and FFamide encoded proteins of 173, 152, and 90 amino acids, respectively. Moreover, the multiple sequence alignment revealed that the bioactive regions of these neuropeptides were well conserved among different invertebrates. In addition, under basal conditions, CCK, conopressin and FFamide mRNA were mainly expressed in the visceral mass, whereas the FFamide mRNA was rarely detected in the foot and mantle. Exposure to 20 and 200 μg/L Tris (2-butoxyethyl) phosphate (TBOEP) and tri-butyl-phosphate (TBP) exposure significantly up-regulated the expression of the CCK and FFamide mRNAs in the visceral mass ($p < 0.05$), whereas no significant changes in conopressin mRNA levels were observed in response to any treatment. Therefore, CCK and FFamide of *C. fluminea* neuropeptides are feasible new biomarkers for screening and assessing responses to organophosphate chemicals.

1. Introduction

Neuropeptides are considered the oldest neuronal signaling molecules and are responsible for mediating neurotransmission and behavioral processes within animal nervous systems (Hökfelt et al., 2000). In general, an immature precursor neuropeptide contains an N-terminal signal sequence and single or multiple copies of a bioactive peptide (Zandawala et al., 2017). Neuropeptides are produced from inactive precursor proteins by proteolytic cleavage and further processing and then released into the hemolymph to act as hormones or at synapses to regulate target cells (Oliveira et al., 2015). Neuropeptides have a wide range of functions in the control of neural circuits and physiology, including the modulation of locomotion and rhythmic pattern generators (Vranković and Slavić, 2015), presynaptic facilitation and remodeling of sensory networks (Chen et al., 2015, 2014), and the regulation of reproduction (Tsutsui et al., 2010).

To date, many neuropeptides and neuropeptide families have been identified in vertebrate and invertebrate animals (Hökfelt et al., 2000; Kastin, 2006). In *Aplysia californica*, FMRF-amide, the best known neuropeptide, appears to provide physiological control of gills (Weiss et al., 1984). Bioinformatic analysis based on the conservation of peptides in ganglia is helpful to reveal the variety of neuropeptides in *Crassostrea gigas* (Stewart et al., 2014). In mollusks, only limited information about the role of neuropeptides in the regulation of ciliary beating has been described (Jaworek et al., 2010; Veenstra, 2010). However, research on neuropeptides in freshwater mollusks is not well documented.

The Asian clam (*Corbicula fluminea*), native to Southeast Asia, is a widely distributed freshwater mollusk (Crespo et al., 2015). As a freshwater bivalve, *C. fluminea* displays the biological characteristics of a smaller life radius and high sensitivity to aquatic pollutants, making them an ideal bio-indicator model in field and laboratory toxicity

Abbreviations: CCK, Cholecystokinin; FFamide, Neuropeptide FF; OPFRs, Organophosphate flame retardants; TBOEP, Tris (2-butoxyethyl) phosphate; TBP, Tri-butyl-phosphate

* Corresponding author at: Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, 18 Shuangqing Road, Haidian District, Beijing 100085, China.

E-mail address: jmzha@rcees.ac.cn (J. Zha).

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studies (Chen et al., 2014; Frenzel et al., 2012). Recently, transcriptome sequence assemblies and annotations became available for *C. fluminea*, providing researchers an excellent opportunity to characterize the repertoire of *C. fluminea* neuropeptides (Chen et al., 2013). Therefore, in the present study, we took advantage of a *de novo* transcriptome assembly of gene transcripts expressed in *C. fluminea* and successfully identified three neuropeptide genes: Cholecystokinin (CCK), conopressin, and Neuropeptide FF (FFamide).

CCK was first discovered by Mutt and Jorpes (Mutt and Jorpes, 1968). In vertebrates, CCK is widely distributed in the brain and is reported to effectively reduce food intake (Beinfeld, 2013). The peptide is expressed in satiety centers and regulates feeding (Dufresne et al., 2006; Konturek et al., 2003). In mollusks, CCK may be involved in feeding-related behaviors, sensation, and neurohormonal communication (Gesser and Larsson, 1985; Pisu et al., 2000; Sonetti et al., 1990; Vigna et al., 1984). The immunoreactivity of gastrin/CCK 8 was increased in response to $HgCl_2$ treatments in previous studies, whereas it remained constant following $ZnCl_2$ exposure (Londhe and Kamble, 2014). Conopressin was originally identified in cone snail venom (Cruz et al., 1987) and subsequently described in the oyster and land snail (Stewart et al., 2016, 2014). Conopressin has been reported to be involved in regulating male copulation behavior in *Lymnaea stagnalis* (Cruz et al., 1987). It stimulates the smooth muscles in the vas deferens to spontaneously contract and leads to the ejaculation of semen during copulation (Golen et al., 1995). FFamide is a member of the RFamide peptide family (Fukusumi et al., 2006) and the biological functions suggested for this neuropeptide include pain modulation, food intake, gastrointestinal and hormonal modulation, modulation of opiate tolerance and abstinence, and cardiovascular action (Moulédous et al., 2010; Panula et al., 1996).

The objective of this study was to determine whether *C. fluminea* neuropeptides represent possible new molecular biomarkers of exogenous pollution. Full-length nucleotide sequences for genes encoding the CCK, conopressin and FFamide neuropeptides were cloned and the deduced amino acid sequence homology and evolution with other mollusks were analyzed. Organophosphate flame retardants (OPFRs) are a class of widely used additives in daily production and life. Previous studies have showed that OPFRs can be detected extensively, including air, dust, surface water, and sediments (Hou et al., 2017). Moreover, several toxicological studies have suggested that OPFRs exposure can potentially cause certain adverse effects (Hou et al., 2017). Tris (2-butoxyethyl) phosphate (TBOEP) and tri-butyl-phosphate (TBP) are two representative OPFRs and our laboratory is currently working on them (Hou et al., 2017; Yan et al., 2017). We further clarified their tissue expression patterns and performed a preliminary assessment of the neuropeptide gene expression profiles after exposure to these two OPFRs: TBOEP and TBP. The results provide basic data for the full-length CCK, conopressin and FFamide cDNAs in *C. fluminea* and new evidence for further discussion on the roles of neuropeptides in *C. fluminea*.

2. Materials and methods

2.1. Animals

Healthy *C. fluminea* (20.56 ± 2.05 mm) were collected in May 2017 from Hongze Lake (Jiangsu province, China, 33°18′0″N, 118°42′30″E) and acclimated in an aquarium containing continuous aerated sand-filtered water for one week before being transferred to the laboratory in water tanks with continuous aeration. *C. fluminea* were then maintained at constant temperature (20 ± 1 °C), pH (7.8 ± 0.2), and oxygen saturation (96% ± 2%) in 50 L glass aquaria (8–10 cm water depth, without sediment) on a 12 h:12 h (light: dark) cycle. *C. fluminea* were fed with the single-celled algae *Chlorella vulgaris* and *Scenedesmus obliquus* once per day. All experimental procedures involving *C. fluminea* strictly adhered to the guidelines for the Care and Use of Laboratory Animals of China. The study was approved by the Institutional Animal Care and Use Committee of the Research Center for Eco-Environmental Sciences at the Chinese Academy of Sciences.

2.2. Experimental design

TBOEP (purity 95%) and TBP (purity 99%) were purchased from J& K Chemical Ltd. (Hebei, China) and were dissolved in acetone as stock solutions. The final working concentration of acetone did not exceed 0.01%. Exposure was initiated after two weeks of acclimatization. *C. fluminea* were exposed to various concentrations of TBOEP (20, 200, and 2000 μg/L) or TBP (20, 200, and 2000 μg/L); an unexposed control group (0 μg/L) was used as the positive control, and acetone served as the negative control in the experiments. The total volume of test solution was 20 L. Each group was assembled in triplicate. At each exposure level, 30 healthy *C. fluminea* were randomly separated into three containers. *C. fluminea* were fed once a day and the test solution was replaced daily. None of the groups displayed mortality during the exposure period. After 28 days of exposure, clams were sacrificed and their visceral mass tissues were excised for the gene expression analysis. In addition, gill, visceral mass, mantle, and foot tissues were collected from untreated clams to determine the tissue distribution of CCK, conopressin and FFamide. After collection, all samples were immediately stored at − 80 °C until total RNA extraction.

2.3. TBOEP and TBP concentrations in exposure solutions

The TBOEP and TBP concentrations in the exposure solutions were measured using a previously reported method. Briefly, exposure solutions were filtered through 0.22 μm microfiber filters (Millipore, USA) and analyzed using our previously described procedures (Hou et al., 2017). Before replacing the test solutions, the concentrations of pollutant (mean ± standard deviation; % analyzed/nominal) in the solutions were 18.2 ± 0.6 (91.0%), 180.6 ± 4.8 (90.3%), and 1772.0 ± 9.8 (88.6%) μg/L for TBOEP, and 17.9 ± 0.8 (89.5%), 177.4 ± 5.5 (88.7%), and 1758.0 ± 15.8 (87.9%) μg/L for TBP. The nominal concentrations of TBOEP and TBP are used hereafter.

2.4. Molecular cloning of CCK, conopressin, and FFamide

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. RNA quality was assessed using agarose-gel electrophoresis based on the integrity of the 18 S and 28 S rRNA bands and using Multiskan GO (Thermo Scientific, USA), with an $A_{260\,nm}/A_{280\,nm}$ ratio from 1.8 to 2.1. The cDNAs were synthesized from 2 μg of total RNA by M-MLV reverse transcriptase (Promega, USA) and oligo dT-Adaptor primers at 42 °C for 60 min, according to the manufacturer's protocol. The synthesized cDNAs were stored at − 20 °C until further use. The conservative cDNA fragments of molluscan neuropeptides (CCK, conopressin and FFamide) were obtained from our previously reported *C. fluminea* transcriptome data (accession number of NCBI Sequence Read Archive: SRA062349) (Chen et al., 2013) and amplified using degenerate primers (Table S1). The 5' and 3' RACE-PCRs were performed using the SMART RACE cDNA amplification kit (Clontech, USA). Gene-specific primers (Table S1) were designed using Primer Premier 5.0 software. For 3' CCK, 3' conopressin and 3' FFamide RACE, PCR was performed using 10× Universal Primer A Mix (UPM) and the gene-specific primers CCK-3F1, conopressin-3F1 and FFamide-3F1, respectively, under the following conditions: 1 min of denaturation at 94 °C, 30 cycles of heat denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, polymerization at 68 °C for 1 min, and a 10 min final extension step at 72 °C. Then a nested PCR was performed using UPM primers and the gene-specific primers CCK-3F2 and conopressin-3F2 and FFamide-3F2 under the same annealing conditions. For 5' RACE, CCK-5R1, conopressin-5R1, FFamide-5R1 and UPM (Table S1) were used in the first round PCR and CCK-5R2,