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Mass spectrometric map of neuropeptide expression and analysis of the γ-prepro-tachykinin gene expression in the medaka (*Oryzias latipes*) brain

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ABSTRACT

Neuropeptides have important roles in modulating behavioral patterns such as social interaction. With the aim to determine the presence of neuropeptides known to be involved in social interaction as well as novel peptides, we used MALDI-TOF/MS to analyze neuropeptide profiles in some medaka brain regions. In the telencephalon, hypothalamus, and pituitary gland, 3, 6, and 10 peaks, respectively, were identified as neuropeptides (Arg-vasotocin [AVT], growth hormone-releasing hormone [GHRH], neuropeptide FF, substance P [SP], somatostatin-1 and -2, melanin-concentrating hormone [MCH], MCH gene-related peptide [Mgrp], melanocyte-stimulating hormone [MSH], corticotropin-like intermediate lobe peptide [CLIP], and β-endorphin). The neuropeptide profile of telencephalon similar to that of the hypothalamus, but completely different from that of pituitary gland. For the future genetic analysis, we identified cDNAs encoding precursor proteins for the identified peptides. We also detect its expression of γ-prepro-tachykinin gene encoding a SP precursor protein in both the telencephalon and hypothalamus. Our results indicated that the medaka brain contains some neuropeptides (AVT, SP, and somatostatins) that may be involved in modulating medaka behaviors such as social interaction.

1. Introduction

The medaka (*Oryzias latipes*) is a freshwater teleost fish native to East Asia that has long been a favorite pet in Japan. Medaka exhibit various social interactions, such as grouping (Nakamura, 1952), intraspecific aggressive behaviors (Magnuson, 1962), and a female mating preference for large males (Howard et al., 1998). The medaka is a model organism for a wide range of biologic studies, such as developmental genetics (Furutani-Seiki and Wittbrodt, 2004). Efficient methods of generating transgenic medaka are now available (Nakamura et al., 2008) and a reverse genetic approach to generate medaka knockout strains was recently established (Taniguchi et al., 2006). The functional analysis of genes involved in modulating social interactions using these advanced genetic methods in medaka will contribute to a better understanding of the neural circuits and intracellular signaling pathways involved in vertebrate social interactions. To date, however, no gene involved in the medaka social interactions has been identified.

Neuropeptides have important roles in modulating social interactions in many animals (Goodson and Bass, 2001; Insel and Young, 2000; Dierick and Greenspan, 2007). In vertebrates, the vasopressin homolog (arginine vasotocin [AVT]), somatostatin, and substance P (SP), one of the most well-characterized tachykinins, modulate reproductive, and/or aggressive behavior patterns in fish (Goodson and Bass, 2001; Thompson et al., 2008; Trainor and Hofmann, 2006). Neuropeptide Y (NPY), oxytocin, and vasopressin are involved in the regulation of social behavior and/or sex-specific behavior in mammals (Baumgartner et al., 2008; Goodson and Bass, 2001; Winslow and Insel, 2004).

In the present study, to identify candidate genes involved in medaka social interactions, we searched for neuropeptides in the medaka brain and identified genes encoding neuropeptides for the future genetic research. We focused on neuropeptides in the telencephalon and hypothalamus in the medaka brain as the telencephalon and hypothalamus are proposed to be involved in vertebrate social interactions from teleosts to mammals (Goodson, 2005; Goodson and Bass, 2001; Insel and Young, 2000; Winslow and Insel, 2004). The telencephalon receives sensory information from different modalities in the fish brain (Finger, 1980). Analysis of the brain structures in Lake Tanganyikan cichlid fish indicates that the development of the telencephalon tends to correlate with the social parameters of these fish (Pollen et al., 2007). The hypothalamus, which produces several neuropeptides (NPY, AVT, SP, and somatostatin) that are important for social interactions, is proposed to be a critical brain structure involved in vertebrate social

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interactions (Goodson, 2005; Goodson and Bass, 2001; Insel and Young, 2000; Winslow and Insel, 2004).

Here, we used direct matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF/MS) (Takeuchi et al., 2003; Yasuda-Kamatani and Yasuda, 2000) to detect the presence of neuropeptides in both the telencephalon and hypothalamus. We identified 6 genes for neuropeptides detected by MALDI-TOF/MS. We also performed *in situ* hybridization to confirm the gene expression of one of the identified genes (γ-prepro-tachykinin [γ-PPT] gene) in both the telencephalon and hypothalamus.

2. Materials and methods

2.1. Fish

Both of female and male adult medaka fish (over 3 months of age) were used throughout the present study. Medaka (*O. latipes*, drR strain) were bred in our laboratory for the direct MALDI-TOF/MS, cDNA cloning, and *in situ* hybridization studies. For the capillary LC–MS/MS analysis, the medaka fish (*O. latipes*) were purchased from a local petshop.

2.2. Direct MALDI-TOF/MS

The brain and pituitary were dissected from medaka that had been anesthetized on ice, and frozen on dry ice in 0.4% NaCl solution. Frozen sections (40-μm thick) were prepared using a cryostat (Microm HM 500O). Sections containing the telencephalon, hypothalamus, or pituitary were placed onto MALDI sample plates. An α-cyano-4-hydroxycinnamic acid matrix was saturated in a solution of acetonitrile/water 50:50 (v/v) containing 0.1% trifluoroacetic acid (TFA). The matrix was rinsed twice to remove excess salts. Fresh matrix solution was added to the sample and the sample was dried. Matrix-coated brain sections were mounted on a MALDI plate, and moved into the MALDI source to expose the area of interest to the fixed position of a laser beam (Takeuchi et al., 2003; Yasuda-Kamatani and Yasuda, 2000). MALDI-TOF/mass spectra were acquired using an Ultraflex III TOF/TOF (Bruker Daltonics).

2.3. Capillary LC–MS/MS analysis

Medaka brains (20 animals) were homogenized with acid–acetone solution (HCl: 90% acetone = 1:30). The extract was lyophilized and subjected to gel filtration on a Superdex 30 column (3 × 500 mm). The fraction containing peptides (500–3000 Da) was further subjected to capillary high pressure liquid chromatography using a TSKgel ODS-100V column (0.5 × 100 mm, particle size 3 mm). Each fraction was analyzed by MALDI-TOF/MS/MS using an Ultraflex III TOF/TOF.

2.4. Molecular cloning of medaka cDNAs for NPFF, MCH, and somatostatin-2

RT-PCR was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan). Total RNA was extracted from the medaka brain using TRIZOL Reagent (Invitrogen, Carlsbad, CA). The primers were as follows: 5′-GCTTGAGATCATGGACACAG-3′ and 5′-ACTACGA CAGACTGTCGAGC-3′ for NPFF; 5′-AGCTCCTGAAAATCTTCACC-3′ and 5′-GCAAATTGTCCTCAGACCTC-3′ for MCH; and 5′-AGGCACA TCCGCTTCACGC-3′ and 5′-GCAGGAGGTGAAGGTCTTCC-3′ for a fragment of somatostatin-2. The amplified PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced.

2.5. cDNA cloning of medaka γ-PPT

Based on the amino acid sequence of the zebrafish tachykinin 1 (GenBank Accession No.: XP_683295), a partial genomic fragment corresponding to medaka *γ-PPT* was identified by the Ensembl Genome Browser (URL: http://www.ensembl.org/Oryzias_latipes/blastview) using TBLASTN algorithm. Total RNA was extracted from the medaka brain using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Rapid amplification of cDNA ends (RACE) was performed with the FirstChoice RLM-RACE kit (Ambion, Austin, TX) following the manufacturer's instructions. The amplification was performed using 5′-CGTATTGTGCTCCACTCGTATGAC-3′ and 5′-CATCAGCCCCACAAAC GAGTTCAC-3′ as outer and inner primers, respectively, for 5′ RACE; and 5′-CAGAGAAATGCTGCTGAGGATGAC-3′ and 5′-ACGCACAGA TCACCAGGAAAAGAC-3′ as gene-specific outer and inner primers, respectively, for 3′ RACE. The medaka brain cDNA for RACE and reverse transcription-polymerase chain reaction (RT-PCR) was synthesized by SuperScript™III reverse transcriptase (Invitrogen, Carlsbad, CA). A γ-PPT cDNA encoding a full open reading frame was amplified by PCR using 5′-CGCTTGAACCATGAAGCTGC-3′ and 5′-AAGATGACAGGTGCTCAGCG-3′ as primers from the medaka brain cDNA. The amplified PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced.

2.6. In situ hybridization analysis

Adult medaka were anesthetized on ice and their brains were dissected. The brains were then fixed with 4% paraformaldehyde in phosphate-buffered saline at 4 °C overnight, dehydrated in graded ethanol-Clear Plus (Falma Company, Tokyo, Japan) series, and embedded in paraffin. Paraffin-embedded brains were sectioned at 10 μm thickness. All sections were mounted on MAS-coated glass slides. Paraffin sections were deparaffinized in Clear Plus and rehydrated. The sections were partially digested with 10 μg/ml proteinase K in 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA for 15 min, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 20 min, treated with 0.2 N HCl for 10 min, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine–HCl at room temperature for 10 min. The hybridization and detection procedures were performed according to conventional methods (Takeuchi et al., 2004). Digoxigenin-labeled antisense and sense probes corresponding to medaka γ-PPT transcript variant-1 (GenBank Accession No.: AB441191, +1 to +372), were synthesized with T7 and SP6 polymerase (Roche, Indianapolis, IN) using DIG RNA Labeling kit (Roche).

3. Results

3.1. Analysis of the neuropeptide profiles in the telencephalon, hypothalamus, and pituitary gland of the medaka brain using MALDI-TOF/MS

In the present study, focused on three brain areas: the telencephalon, hypothalamus, and pituitary gland. The telencephalon and hypothalamus are believed to be brain regions involved in vertebrate social interactions from teleosts to mammals (Goodson, 2005; Goodson and Bass, 2001; Insel and Young, 2000; Winslow and Insel, 2004). The pituitary gland is an endocrine gland, which is innervated by the hypothalamus (Takahashi et al., 2006) and in this present study we analyzed this gland for detection of medaka neurohormone. To analyze neuropeptide profiles of the telencephalon, hypothalamus, and pituitary gland, MALDI-TOF/MS was directly applied to the frozen sections of the medaka brain. There were 7, 17, and 13 major peptide peaks detected in the telencephalon (Fig. 1A), hypothalamus (Fig. 1B), and pituitary gland (Fig. 1C), respectively. The 7 peaks detected in the telencephalon were also