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Involvement of melanin-concentrating hormone 2 in background color adaptation of barfin flounder *Verasper moseri*

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ABSTRACT

In teleosts, melanin-concentrating hormone (MCH) plays a key role in skin color changes. MCH is released into general circulation from the neurohypophysis, which causes pigment aggregation in the skin chromatophores. Recently, a novel MCH (MCH2) precursor gene, which is orthologous to the mammalian MCH precursor gene, has been identified in some teleosts using genomic data mining. The physiological function of MCH2 remains unclear. In the present study, we cloned the cDNA for MCH2 from barfin flounder, *Verasper moseri*. The putative prepro-MCH2 contains 25 amino acids of MCH2 peptide region. Liquid chromatography-electrospray ionization mass spectrometry with a high resolution mass analyzer were used for confirming the amino acid sequences of MCH1 and MCH2 peptides from the pituitary extract. *In vitro* synthesized MCH1 and MCH2 induced pigment aggregation in a dose-dependent manner. A mammalian cell-based assay indicated that both MCH1 and MCH2 functionally interacted with both the MCH receptor types 1 and 2. *Mch1* and *mch2* are exclusively expressed in the brain and pituitary. The levels of brain *mch2* transcript were three times higher in the fish that were chronically acclimated to a white background than those acclimated to a black background. These results suggest that in *V. moseri*, MCH1 and MCH2 are involved in the response to changes in background colors, during the process of chromatophore control.

1. Introduction

Melanin-concentrating hormone (MCH) was originally identified from the chum salmon, *Oncorhynchus keta,* as a pituitary peptide that concentrates melanin granules in the melanophores of the skin (Kawauchi et al., 1983). Later studies indicated that teleost MCH is synthesized in the hypothalamus, transported to the nerve terminal in the pituitary neural lobe, and released into the blood (Amano and Takahashi, 2009). MCH has subsequently been identified in the mammalian brain, and shown to act as a neuromodulator regulating feeding behavior, energy homeostasis, stress, reproduction, sensory perception, and neuroendocrine responses (Griffond and Baker, 2002; Nahon, 2006; Saito and Nagasaki, 2008; Sherwood et al., 2012; Wu et al., 2009). The functions of the original teleost MCH (designated as MCH1 in this text) have been well investigated, especially pigment aggregation and cooperation with various pituitary hormones (Kawauchi, 2006). MCH1 has also been implicated in feeding behavior in teleosts, but this function is inconsistent amongst different species. Intracerebroventricular injection studies have suggested that MCH1 has an anorexic function in the goldfish, *Carassius auratus* (Matsuda et al., 2006, 2007); whereas, white background color enhances *mch1* expression and feeding behavior in the barfin flounder, *Verasper moseri*, suggesting a possible orexigenic function in this fish (Sunuma et al., 2009).

Recently, a gene encoding another MCH (termed MCH2 in this text) has been identified as an ortholog of mammalian *mch*, in teleosts (zebrafish, *Danio rerio*; medaka, *Oryzias latipes*; three-spined stickleback, *Gastierosteus aculeatus*; torafugu, *Takifugu rubripes*; winter flounder, *Pseudopleuronectes americanus*; and starry flounder, *Platichthys stellatus*) (Berman et al., 2009; Tuziak and Volkoff, 2012; Kang and Kim, 2013). Very few studies have been conducted to elucidate the physiological functions of MCH2 as compared to

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MCH1. In *D. rerio*, *mch2* is expressed in the lateral tuberal nucleus (NLT) within the hypothalamus where *mch1* is also expressed (Berman et al., 2009). In *D. rerio* and *P. americanus*, the up-regulated expression of *mch2* in the hypothalamus is induced by fasting (Berman et al., 2009; Tuziak and Volkoff, 2012), suggesting the possible involvement of *mch2* in the regulation of feeding in teleosts. The elevated expression of *mch2* upon exposure to white background in *D. rerio* and *P. stellatus* suggests that MCH2 acts on background color-adaptation (Berman et al., 2009; Zhang et al., 2010; Kang and Kim, 2013). However, to date the MCH2 peptide has not been identified, and the physiological functions of MCH2 involved in body color changes remain unknown.

V. moseri is an interesting model organism for investigating the molecular mechanisms of MCH systems because the roles of MCH1 in the regulation of skin color changes (Mizusawa et al., 2011) and feeding/growth behavior (Sunuma et al., 2009; Takahashi et al., 2004; Yamanome et al., 2005) have been thoroughly investigated. In addition, two MCH receptors MCH-R1 and MCH-R2 have also been characterized. MCH-R1 is exclusively expressed in the brain, whereas MCH-R2 is expressed in the brain as well as several peripheral tissues including the skin (Takahashi et al., 2007). MCH-R2 is expressed in skin melanophores and xanthophores (but not in other dermal cells) where MCH1 was shown to induce pigment aggregation (Mizusawa et al., 2011). The present study was undertaken to elucidate the properties of MCH2 using *V. moseri* as to the following five items; (i) molecular cloning of the prepro-MCH2 cDNA, (ii) identification of the MCH2 peptide derived from prepro-MCH2, (iii) characterization of the pigment-aggregation activities of MCH2, (iv) characterization of the pharmacological properties of MCH2, and (v) characterization of the expression levels of MCH2 in response to background color.

2. Materials and methods

2.1. Fish

Immature *V. moseri* were purchased from the Iwate Cultivating Fishery Association (Iwate, Japan), or kindly provided by the Hokkaido National Fisheries Research Institute (Hokkaido, Japan). All experiments were conducted in accordance with the Kitasato University guidelines for the care and use of animals. The photoperiods and water temperatures were maintained at natural conditions. Tissue sampling was performed on fish that were anesthetized by immersion, in 0.05% 2-phenoxyethanol for nucleotide analysis or in ice-cold seawater for the *in vitro* assay of chromatophores.

2.2. Amplification and sequence determination of cDNA

Total RNA was extracted from a single brain using the Isogen II kit (Nippon Gene, Tokyo, Japan). First-strand cDNAs were synthesized from total brain RNA with the SMARTer RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA). Custom oligonucleotides and TaqMan probes were synthesized at Nihon Gene Research Labs (Sendai, Japan; Table 1). Polymerase chain reactions (PCRs) for amplifying the DNA fragments were performed using a HotStarTaq Master Mix (Qiagen, Hilden, Germany), in a thermal cycler (MJ Mini, BIO-RAD, Hercules, CA, USA) under conventional conditions. For the 5′-RACE, two degenerate antisense primers, MCH2-5DR1 for the first PCR and MCH2-5DR2 for the nested PCR were designed based on the prepro-MCH2 amino acid sequences in teleosts (*D. rerio*, *G. aculeatus*, *O. latipes*, and *T. rubripes*). A DNA fragment was amplified from the 5′-RACE-ready cDNA by nested PCR, using the antisense primers and the sense adaptor primers UPM and NUPM provided in the SMARTer RACE Kit. Amplified DNA fragments were subcloned and sequenced as follows. Briefly, the PCR-amplified DNAs were separated by electrophoresis on NuSieve GTG agarose gel (Cambrex Bio Science, Rockland, ME, USA). The DNA was then extracted from the agarose gel by using a QIAEX II Gel Extraction Kit (Qiagen), ligated into a plasmid pT7Blue T-vector (Novagen, Madison, WI, USA), and transferred into JM109 competent cells. Recombinant plasmid DNA was isolated from the cells by the alkaline-SDS method and sequenced using a capillary DNA sequencer (3100-*Avant* Genetic Analyzer; Life Technologies, Carlsbad, CA, USA) and a BigDye Terminator v3.0 Cycle Sequencing Ready Kit (Life Technologies). Two gene-specific sense primers, MCH2-3F1 for the first PCR and MCH2-3F2 for the nested PCR, were designed from the sequenced 5′-untranslated region (UTR) of the cDNA fragment. The full-length reading frame was amplified from the 3′-RACE-ready cDNA by nested PCR using these sense primers and adaptor primers, and then subcloned and sequenced as described above. To confirm the sequence of the full reading frame, a fragment of the prepro-MCH2 cDNA was amplified using MCH2-OF and MCH2-OR primers, which were designed from the 5′- and 3′-UTR sequences, and then subcloned and sequenced as described above.

Table 1
Primers and a probe used in cDNA cloning and real-time RT-PCR. Nucleotide sequences are represented by IUPAC code.

cDNA cloning and RT-PCR		
	MCH2-5DR1	5′-CCA RCA IGG ICK RTA IAC ICK ICC-3′
	MCH2-5DR2	5′-TAI ACI CKI CCI AYC ATR CAI CKI AG-3′
	MCH2-3F1	5′-CTG GAC AAC AGC ATC AGG GAT GAA GAC-3′
	MCH2-3F2	5′-CAG GGA TGA AGA CGG GAA CCC TAA GA-3′
	MCH2-OF	5′-AGA TCA GTC ACG GCC TTC AG-3′
	MCH2-OR	5′-ACA CGA GGA AAA GCT CTC CA-3′
	Actin-F	5′-TGA AGT ACC CCA TCG AGC AC-3′
	Actin-R	5′-TAC AGG TCC TTA CGG ATG TC-3′
RT-PCR and Real-time quantitative RT-PCR		
	MCH1-QF	5′-AAC GGC CTT TCC CTG TAC AAG-3′
	MCH1-QR	5′-CCA CCA TGC ACC TCA TGT TG-3′
	MCH1-QP	5′-(FAM) CGG ACC ACA GCG AGC AGG TGA C (Tamra)-3′
	MCH2-QF	5′-CAA GAC GGC CTC AGT TCC TTT-3′
	MCH2-QR	5′-TCT GCC TCA TGT CCG AGA TG-3′
	MCH2-QP	5′-(FAM) TGA CGA ACC CAT GAT CGA GCA GGC (Tamra)-3′

2.3. Sequence data analysis

BioEdit Sequence Alignment Editor v.7 was used for processing the nucleotide and amino acid sequences, as well as for calculating sequence identities (http://www.mbio.ncsu.edu/bioedit/bioedit.html) (Hall, 1999). All the gaps in the sequences were designated as identical dummy amino acids before calculating the sequence identities (e.g., "GRR–DFD" was changed to "GRRXXDFD", where "-" represents a gap). ClustalX 2.1 (http://www.clustal.org/clustal2/) was used for amino acid sequence alignments and for constructing a phylogenetic tree using the Neighbor-Joining method (Larkin et al., 2007). The signal peptide domains for prepro-MCH2 were predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

2.4. Peptide extraction for mass spectrometry (MS)

A single pituitary was homogenized in 700 μL of 80% acetonitrile containing 12 mM HCl by using an ultrasonic processor, followed by centrifugation at 19,000 *g* for 15 min at 4 °C. The lyophilizate from one quarter volume of supernatant that contained peptides was redissolved in 20 μL of 1.5× Invitrosol containing 200 mM triethylammonium bicarbonate buffer. Cystine disulfide bonds were reduced by the addition of 2 μL of 200 mM tris(2-carboxyethyl)phosphine for 30 min at 55 °C. After cooling