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Research paper

Molecular cloning, tissue distribution, and pharmacological characterization of melanocortin-4 receptor in spotted scat, *Scatophagus argus*

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ABSTRACT

Melanocortin-4 receptor (MC4R) plays an important role in the regulation of food intake and energy expenditure in mammals. The functions of the MC4R in fish have not been investigated extensively. We herein reported on the cloning, tissue distribution, and pharmacological characterization of spotted scat (*Scatophagus argus*) MC4R (*SA*MC4R). It consisted of a 984 bp open reading frame predicted to encode a protein of 327 amino acids. Sequence analysis revealed that *SA*MC4R was highly homologous (>80%) at amino acid levels to several teleost MC4Rs. Phylogenetic analyses showed that *SA*MC4R was closely related to piscine MC4R. Using RT-PCR, we showed that in addition to brain, pituitary, and gonads, *mc4r* mRNA was also widely expressed in peripheral tissues of spotted scat in sexually divergent pattern. With human MC4R (hMC4R) as a control, several agonists including α-melanocyte stimulating hormone (α-MSH), [Nle^4, D-Phe^7]-α-MSH (NDP-MSH), adrenocorticotropic hormone (ACTH) and THIQ (N-[(3R)-1,2,3,4-tetrahydroisoquinolinium3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine), were used to investigate the binding and signaling properties of *SA*MC4R. The results showed that *SA*MC4R bound NDP-MSH with the highest affinity followed by ACTH (1-24) and α-MSH. Similar ranking was also found for hMC4R, although *SA*MC4R had two to five-fold higher affinities for these ligands. THIQ did not displace NDP-MSH from *SA*MC4R, different from hMC4R. α-MSH, NDP-MSH, and ACTH (1-24) were identified as potent agonists to stimulate cAMP generation followed by THIQ in *SA*MC4R. The availability of *SA*MC4R and its pharmacological characteristics will facilitate the investigation of its function in regulating diverse physiological processes in spotted scat.

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1. Introduction

Melanocortin peptides are posttranslational products of the large precursor protein, proopiomelanocortin (POMC), which is expressed in hypothalamic arcuate nucleus and brainstem, pituitary gland, and the skin. They are mainly comprised of α-, β-, γ-, and δ-melanocyte-stimulating hormones (α-, β-, γ-, and δ-MSH), adrenocorticotropic hormone (ACTH), β-endorphin and β-lipotrophic hormone (reviewed in (Dores and Lecaude, 2005; Smith and Funder, 1988)). The melanocortin receptors (MCRs), members of rhodopsin-like Family A G protein-coupled receptors (GPCRs), have been shown to mediate diverse physiological roles such as pigmentation, adrenal steroidogenesis, lipolysis, stress,

Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related protein; ARC, arcuate nucleus; cAMP, cyclic adenosine monophosphate; CCK, cholecystokinin; CNS, central nervous system; GPCR, G protein-coupled receptor; MCR, melanocortin receptor; MC4R, melanocortin-4 receptor; α-MSH, α-melanocyte stimulating hormone; NDP-MSH, [Nle^{4}D-Phe^7]-α-melanocyte stimulating hormone; POMC, proopiomelanocortin; *SA*MC4R, *Scatophagus argus* melanocortin-4 receptor; THIQ, (N-[(3R)-1,2,3,4-tetrahydroisoquinolinium3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine).

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immunomodulation, energy homeostasis, cardiovascular regulation, and sexual function (for reviews, see (Cone, 2006; Gantz and Fong, 2003)). Over the past two decades, since the initial cloning (Gantz et al., 1993; Mountjoy et al., 1994), significant attention has been paid to the melanocortin-4 receptor (MC4R), due to its central expression and roles in regulation of energy homeostasis and body weight. Recent evidence suggested that MC4R is also involved in modulating reproductive function, affecting the secretion of reproductive hormones, and consequent sexual maturation (Khong et al., 2001) (see (Tao, 2010) for a detailed review). Hence, understanding how MC4R mediates the reproductive function in economically important species is important for artificial breeding.

MCRs are primarily coupled to the stimulatory G protein, with receptor activation leading to stimulation of adenylyl cyclase activity and increased production of the intracellular second messenger cyclic adenosine monophosphate (cAMP), triggering downstream signal transduction pathways (Gantz et al., 1993). In tetrapods, five MCRs (MC1R-MC5R) have been identified. Of the five MCRs, only MC3R and MC4R are significantly expressed within the central nervous system, hence these two MCRs are also called neural MCRs (reviewed by (Cone, 2006; Tao, 2005)). They are expressed in the hypothalamic paraventricular nucleus and arcuate nucleus where activation by α-MSH, β-MSH and other POMC-derived peptides regulates feeding behavior, energy homoeostasis, and the partitioning of fuel stores into fat (Butler et al., 2000; Chen et al., 2000; Huszar et al., 1997). MC4R is also expressed in some peripheral tissues (Mountjoy et al., 2003), although the physiological roles in the peripheral tissues are not clear. One study demonstrated that MC4R expressed in enteroendocrine L cells could regulate hormone secretion (Panaro et al., 2014).

In lower vertebrates such as fish, the MC4R is expressed in peripheral tissues, such as gill, spleen, retina, and ovaries in the goldfish, liver, ovary, and testis in the flounder (Cerda-Reverter et al., 2003a; Kobayashi et al., 2008; Ringholm et al., 2002). In addition to endogenous agonists, melanocortin system is also modulated by naturally existing antagonists, agouti and agouti-related protein (AgRP), that compete with melanocortin peptides for binding to MCRs. AgRP, an inverse agonist of MC3R and MC4R, is primarily produced within the hypothalamic arcuate nucleus, participating in the regulation of energy homeostasis by blocking melanocortin signaling (Fong et al., 1997; Graham et al., 1997; Ollmann et al., 1997) (reviewed in (Ilnytska and Argyropoulos, 2008)).

When *MC4R* was first cloned from human brain by degenerate PCR and homology screening in 1993 (Gantz et al., 1993), its physiological functions were unknown. Subsequent studies revealed critical roles of MC4R in regulating energy homeostasis in mammals (reviewed in (Tao, 2010)). *Mc4r* knockout mice exhibit maturity-onset obesity, hyperphagia, increased linear growth, hyperinsulinemia, hyperglycemia, and delayed meal termination and reduced sensitivity to cholecystokinin (Blevins et al., 2009; Fan et al., 2004; Huszar et al., 1997). A similar phenotype is also observed in mice ubiquitously overexpressing *Agouti* or *Agrp* genes (Fan et al., 1997; Graham et al., 1997; Klebig et al., 1995; Ollmann et al., 1997). Central administration of the MC4R agonist α-MSH has been shown to inhibit appetite and increase basal metabolic rate. Conversely, MC4R antagonism by AgRP results in hyperphagia and decreased metabolic activity. Human genetic studies demonstrated that mutations in *MC4R* are the most common form of monogenic obesity, characterized by its early-onset and severity (Farooqi et al., 2003; Hinney et al., 1999; Vaisse et al., 1998; Yeo et al., 1998) (extensively reviewed in (Hinney et al., 2013; Tao, 2005, 2009)). These studies suggest that MC4R plays a key role in regulating energy homeostasis and body weight in mammals including men.

So far several MC4Rs have been characterized in nonmammalian vertebrates including birds and teleosts (Aspiras et al., 2015; Cerda-Reverter et al., 2003a; Jangprai et al., 2011; Klovins et al., 2004; Kobayashi et al., 2008; Lampert et al., 2010; Ringholm et al., 2002, 2003; Sanchez et al., 2009; Sebag et al., 2013; Takeuchi and Takahashi, 1998; Wang et al., 2016a; Wei et al., 2013). Melanocortin system has been shown to be involved in regulating food intake in goldfish (Cerda-Reverter et al., 2003b). Sanchez et al. (2009) reported that similar to its role in mammals, AgRP acts as an inverse agonist at the constitutively active sea bass MC4R (Sanchez et al., 2009). Mutations in fish *mc4r* have also been reported and are related to body size, reproduction, and adaptation to energy availability (Aspiras et al., 2015; Lampert et al., 2010).

Spotted scat, *Scatophagus argus*, a member of *Perciformes*, is an euryhaline subtropical polyphagous fish located on the south and southeast China coast. It is an important fish for aquaculture with high economic value. The complete maturation in female fish cannot be induced artificially, and sexual maturity of male fish is ahead of female fish, which leads to significant difficulty in its artificial propagation (Li et al., 2015). We reported here cloning of *SA*MC4R and tissue distribution of *mc4r* mRNA expression. The results showed that *mc4r* mRNA had a widespread distribution in peripheral and central nervous tissues. Strong signals were observed in the brain, pituitary, ovary, and testis. Previous studies revealed that MC4R is involved in modulating reproductive function in mammals (Klovins et al., 2004; Mountjoy et al., 2003; Van der Ploeg et al., 2002). In fish *mc4r* is also expressed in gonads, and nonfunctional Y-linked *mc4r* copies in larger male swordtails act as dominant negative mutations and delay the onset of puberty (Lampert et al., 2010). Therefore we hypothesize that the MC4R could be associated with reproductive function in spotted scat. In order to guide future experiments, we performed pharmacological characterization of the *SA*MC4R.

2. Materials and methods

2.1. Materials and plasmids

[Nle^4, D-Phe^7]-α-melanocyte stimulation hormone (NDP-MSH) was purchased from Peptides International (Louisville, KY, USA), α-MSH from Pi Proteomics (Huntsville, AL, USA), ACTH (1-24) from Phoenix Pharmaceuticals (Burlingame, CA, USA), and THIQ (N-[(3R)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl) piperidin-1-yl]-2-oxoethylamine) from Tocris Bioscience (Ellisville, MO, USA). ^{125}I-NDP-MSH was iodinated as previously described (Mo et al., 2012) and radiolabeled cAMP was iodinated with chloramine T method (Steiner et al., 1969). The N-terminal c-myc-tagged human MC4R (hMC4R) subcloned into pcDNA3.1 was generated as previously described (Tao and Segaloff, 2003).

2.2. Molecular cloning of SAMC4R

Sequence alignment analysis using DNAman program (Lynnon Corp, Quebec, Canada) suggested that the putative coding region of the *SAmc4r* gene consisted of a single exon of 984 bp. Therefore, spotted scat genomic DNA isolated from brain was used as template for PCR reaction using degenerate primers CPF1 and CPR1 listed in Table 1, designed based on conserved regions of known *mc4r* sequences. The PCR was performed in a total volume of 25 μl containing 100 ng spotted scat genomic DNA, 0.2 mM dNTPs, 0.3 mM of each of the primers, 1 × PCR Buffer for KOD-Plus, 1.0 mM $MgCl_2$, and 1 U KOD-Plus (Toyobo, Shanghai, China) with the following parameters: 3 min at 95 °C for one cycle and 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C for 40 cycles followed by a final cycle at 72 °C for 10 min. The PCR fragment with

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