General and Comparative Endocrinology 205 (2014) 251–259

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (*Dicentrarchus labrax*)

Maria Josep Agulleiro, Raúl Cortés, Esther Leal, Diana Ríos, Elisa Sánchez, José Miguel Cerdá-Reverter *

Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), Ribera de Cabanes, Castellón, Spain

ARTICLE INFO

Article history:
Available online 20 February 2014

Keywords:
Agouti-signaling protein
Agouti-related protein
Melanocortin
Food intake
Pigmentation
Fish

ABSTRACT

The melanocortin system is one of the most complex hormonal systems in vertebrates. Atypically, the signaling of melanocortin receptors is regulated by the binding of endogenous antagonists, named agouti-signaling protein (ASIP) and agouti-related protein (AGRP). Teleost specific genome duplication (TSGD) rendered new gene copies in teleost fish and up to four different genes of the agouti family of peptides have been characterized. In this paper, molecular cloning was used to characterize mRNA of the agouti family of peptides in sea bass. Four different genes were identified: AGRP1, ASIP1, AGRP2 and ASIP2. The AGRP1 gene is mainly expressed in the brain whereas ASIP1 is mainly expressed in the ventral skin. Both ASIP2 and AGRP2 are expressed in the brain and the pineal gland but also in some peripheral tissues. Immunocytochemical studies demonstrated that AGRP1 is exclusively expressed within the lateral tuberal nucleus, the homologue of the mammalian arcuate nucleus in fish. Long-term fasting (8–29 days) increased the hypothalamic expression of AGRP1 but depressed AGRP2 expression (15–29 days). In contrast, the hypothalamic expression of ASIP2 was upregulated during short-term fasting suggesting that this peptide could be involved in the short term regulation of food intake in the sea bass.

1. Introduction

Agouti-signaling (ASIP) and agouti-related (AGRP) protein are endogenous antagonists of melanocortin receptors (MCRs), which compete with agonists derived from a complex precursor, called proopiomelanocortin (POMC), i.e., melanocyte-stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH). The melanocortin receptors were identified as a family of G-protein coupled receptors. In mammals, five paralogues have been cloned (MC1R–MC5R) (reviewed by Schiöth et al., 2005). MC1R is expressed by melanocytes and its activation increases intracellular cAMP levels. Such increases are responsible for the main melanogenic actions of α-MSH, including the transcriptional activation of tyrosinase (TYR), the rate limiting enzyme in melanin synthesis, which results in a switch from the production of phaeomelanin (yellow/red pigment) to eumelanin (black/brown pigment, reviewed by García-Borrón et al., 2005). The expression of the MC2R mRNA is mainly restricted to the adrenal cortex and adipose tissue (Mountjoy et al., 1992; Boston and Cone, 1996). MC2R is pivotal in the synthesis and secretion of adrenal glucocorticoids which mediate the stress response and it is a key point in the peripheral response to stress (Dallman et al., 2004). MC3 and MC4R are expressed mainly in the central nervous system (CNS) to regulate the energy homeostasis. Central activation of MC3R and MC4R is thought to mediate melanocortin effects on energy balance (Cone, 2005, 2006) since both MC3R knockout rat (Chen et al., 2000) and MC4R knockout mice (Huszar et al., 1997) display severe alterations in energy homeostasis. Interruption of α-MSH central signaling by ubiquitous constitutive expression of agouti gene in obese yellow mice (Ay) results in hyperphagia, hyperinsulinemia, increased linear growth, maturity-onset obesity and yellow fur (Lu et al., 1994). A similar metabolic syndrome is also observed in transgenic mice ubiquitously overexpressing agouti or AGRP genes (Klebig et al., 1995; Ollmann et al., 1997), and in the MC4R knockout mice (Huszar et al., 1997). MC5R has a wide distribution in the brain and peripheral tissues, but always shows low expression levels (Schiöth et al., 2005; Cone, 2006). Deletion of the MC5R gene in mice results in nearly total loss of MSH binding sites in skeletal muscle and the Haderian, lachrymal and preputial glands indicating that MC5R represents the major MCR in these tissues. MC5R knockout mice exhibit a severe dysfunction of exocrine secretion, affecting hair follicle-associated sebaceous, Harderian, lachrymal and preputial glands. The absence

* Corresponding author. Address: Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), 12595 Torre de la Sal, Ribera de Cabanes, Castellón, Spain. Fax: +34 964319509.

E-mail address: jm.cerda.reverter@csic.es (J.M. Cerdá-Reverter).

http://dx.doi.org/10.1016/j.ygcen.2014.02.009
0016-6480/

of MC5R expression results in reduced hair lipid content, which provokes defects in water repulsion, and reduced coat insulation against cold environments, resulting in an impaired thermoregulatory function (Chen et al., 1997).

In mice, ASIP is expressed by dermal papillae cells and acts within the hair follicle microenvironment to control the switch between the production of eumelanin (black–brown pigment) and phaeomelanin (yellow–red pigment) by antagonizing α-MSH effects on MC1R in the follicle melanocytes (reviewed by Cone, 2006). In contrast, AGRP is expressed mainly in the central nervous system (CNS) where it antagonizes α-MSH at central MC3R and MC4R to regulate food intake and energy expenditure (Ollmann et al., 1997). AGRP works also as an inverse agonist in constitutively activated MC4R (Nijenhuis et al., 2001; Sánchez et al., 2009a,b; Ersoy et al., 2012). ASIP and AGRP molecules share a cysteine-rich C-terminal domain (cystine knot), which are essential for the conformational stability and biological functions of the molecule. The N-terminal region of ASIP is filled by a basic domain that leads a proline-rich area that immediately precedes the cystine knot. AGRP sequences lacks both regions but, in contrast, exhibits a processing site prior to the cysteine domain where the propetide is cleaved (Creemers et al., 2006).

Mammals have a single copy of ASIP and AGRP genes but the genome of teleost antecessor doubled once more [3R or teleost specific genome duplication (TSGD)], resulting in an expansion of the receptor/peptide systems. This event, together with particular tetraploidization events (e.g., salmonids), resulted in the additional copies of agouti peptides observed in the genome of teleost fish. Sequencing projects have led to the conclusion that teleost fish exhibit paralogue genes of ASIP (ASIP1 and ASIP2) and AGRP (AGRP1 and AGRP2) (Kurokawa et al., 2006). The evolutionary relationship among peptides has still not been unraveled (Braasch and Postlethwait, 2011; Schiöth et al., 2011; Guillot et al., 2012; Vastermark et al., 2012) and alternative evolutionary hypotheses suggest that a different nomenclature should be used for these peptides. Here, we use the nomenclature proposed by Kurokawa and collaborators, i.e., ASIP1, AGRP1, ASIP2 and AGRP2. Both AGRP1 and ASIP1 share a cystine knot with the structure C-x(6)-C-x(6)-C, whereas AGRP2 and ASIP2 exhibit a shortened version of the cystine knot C-x(6)-Cx(5)-C and were originally named as A2 peptides (Klovins and Schioth, 2005). AGRP1 is expressed in the tuberal hypothalamus of all studied species, in a homologous area of the mammalian arcuate nucleus, but also in some peripheral tissues, including the skin (Cerdá-Reverter and Peter, 2003; Song et al., 2003; Forlano and Cone, 2007). In contrast, AGRP2 is only expressed in the pineal gland of the zebrafish (Zhang et al., 2012), although quantitative PCR (qPCR) studies in salmon and carp have demonstrated wider AGRP2 expression at the peripheral tissues, including gill, eye and pituitary (Murashita et al., 2009; Wan et al., 2012). Hypothalamic expression of AGRP1 regulates the energy balance (Cerdá-Reverter and Peter, 2003; Song and Cone, 2007; Zhang et al., 2012), whereas pineal expression of AGRP2 is involved in pigment background adaptation (Zhang et al., 2010). ASIP1 is expressed mainly in the ventral skin, where it inhibits melanogenesis to regulate the dorso-ventral pigment pattern (Cerdá-Reverter et al., 2005; Guillot et al., 2012). RT-PCR studies have demonstrated the expression of ASIP2 in the eye, testis, heart and skin of the pufferfish (Kurokawa et al., 2006) but its function remains unknown.

Previous work in our lab focused the characterization of the MCRs in sea bass (*Dicentrarchus labrax*) (Agulleiro et al., 2010; Sánchez et al., 2009a,b, 2010). Pharmacological studies using zebrafish synthetic peptide demonstrated that AGRP1 works both as an endogenous antagonist and inverse agonist at MC1R (Sánchez et al., 2010), and MC4R (Sánchez et al., 2009b). In the present study, we focused on the endogenous melanocortin antagonist in the sea bass, identifying and characterizing four different agouti-related genes whose deduced peptides contain the typical agouti structural features, i.e., the cystine knot C-x(6)-C-x(6)-C for ASIP1 and AGRP1 and the shortened version, C-x(6)-C-x(5)-C, for A2 peptides (ASIP2 and AGRP2). We also studied the tissue expression and fasting response of paralogues expressed in the brain, AGRP1 and AGRP2, which were up-regulated and down-regulated, respectively, by long-term fasting. In contrast, ASIP2 is highly up-regulated during short-term fasting but no effects were recorded during long-term fasting.

2. Materials and methods

2.1. Cloning procedure and gene structure of sea bass agouti-family genes

Total brain RNA was extracted with Tri-reagent (Sigma) and treated with RQ1-DNaseI (Promega). Subsequently, 5 μg of total RNA were used as template for cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) primed with random hexamers (Invitrogen). A fragment of 115 bp was amplified by RT-PCR using degenerated primers (Supplementary Table 1). The fragment homology to AGRP sequences was confirmed by sequencing and phylogenetic studies. A search was carried out for the sea bass ASIP2 sequence in NCBI databases (http://www.ncbi.nlm.nih.gov/pubmed), using the tblast program with a small fragment of fugu ASIP2 (gene bank accession number: AB261602). A chromosome sequence corresponding to linkage group 18 (gene bank accession number: FQ310508) of 180 pb was obtained in sea bass showing a high degree of homology with ASIP2 in different species. The 5′ and 3′ regions of both genes were amplified by rapid amplification of cDNA ends (RACE) from brain mRNA isolated with PolyATtract® mRNA isolation systems (Promega) using the SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacture's recommendation. Primer sequences are shown in Supplementary Table 1. Sea bass AGRP2 sequence was obtained from Aquagenomics database (http://www.aquagenomics.es). The sea bass ASIP1 sequence was obtained from NCBI (genebank accession number: FM021895). Specific primers were designed to clone and verify sea bass sequences. Sequence alignments were performed using public domain ClustalX 2.1 and edited with GeneDoc software. A phylogenetic tree was derived using CulstalX and SeaView, which use the Neighbor-Joining method on a matrix of distances and maximum likelihood, respectively. The cleavage site for removal of the hydrophobic signal peptide was predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).

2.2. Tissue expression experiments

Animals were anesthetized in 2-phenoxy-ethanol (0.1%) for 2 min before any manipulation and were killed by rapid decapitation. All experiments were approved by the bioethics committee of the Scientific Investigation Superior Council and carried out in the Institute of Aquaculture of Torre de la Sal (registration code 36271-42-A), in accordance with the principles published in the European animal directive (86/609/EEC) concerning the protection of experimental animals. Total RNA was purified from fresh tissues (brain, pituitary, pineal, liver, head kidney, posterior kidney, spleen, gill, fat, intestine, testis, ovary, ventral and posterior skin, muscle and heart). It was extracted as above and 5 μg (2 μg and 1 μg in the case of pineal and pituitary, respectively) were used as template for cDNA synthesis as before but primed with a mix of random hexamers and oligo(dT)12-18 (Invitrogen). The cDNA of five sample of each tissue from different animals ($n = 5$/tissue) except for ovary ($n = 3$/tissue for ovary) was subsequently used as template for quantitative real-time PCR (qPCR).