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Behind melanocortin antagonist overexpression in the zebrafish brain: A behavioral and transcriptomic approach

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ABSTRACT

Melanocortin signaling is regulated by the binding of naturally occurring antagonists, agouti-signaling protein (ASIP) and agouti-related protein (AGRP) that compete with melanocortin peptides by binding to melanocortin receptors to regulate energy balance and growth. Using a transgenic model overexpressing ASIP, we studied the involvement of melanocortin system in the feeding behaviour, growth and stress response of zebrafish. Our data demonstrate that ASIP overexpression results in enhanced growth but not obesity. The differential growth is explained by increased food intake and feeding efficiency mediated by a differential sensitivity of the satiety system that seems to involve the cocaine- and amphetamine- related transcript (CART). Stress response was similar in both genotypes. Brain transcriptome of transgenic (ASIP) vs wild type (WT) fish was compared using microarrays. WT females and males exhibited 255 genes differentially expressed (DEG) but this difference was reduced to 31 after ASIP overexpression. Statistical analysis revealed 1122 DEG when considering only fish genotype but 1066 and 981 DEG when comparing ASIP males or females with their WT counterparts, respectively. Interaction between genotype and sex significantly affected the expression of 97 genes. Several neuronal systems involved in the control of food intake were identified which displayed a differential expression according to the genotype of the fish that unravelling the flow of melanocortinergic information through the central pathways that controls the energy balance. The information provided herein will help to elucidate new central systems involved in control of obesity and should be of invaluable use for sustaining fish production systems.

Introduction

The melanocyte-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH) are the main melanocortin peptides. All of them are encoded in a common precursor called proopiomelanocortin (POMC), which is expressed mainly in the pituitary (Castro and Morrison, 1997). In the rat brain, POMC is also expressed in two discrete neuronal groups, the arcuate nucleus (Ac) of the hypothalamus, and the caudal region of the nucleus of the tractus solitarius (NTS) of the medulla (Bangol et al., 1999). Melanocortin signaling is mediated by binding to a family of specific G protein-coupled receptors that positively couple to adenylyl cyclase. Five melanocortin receptors (MC1R-MC5R) have been characterized in tetrapods but only *mc3r* and *mc4r* are abundantly expressed within the mammalian central nervous system (CNS). Subtype 2 receptor binds ACTH, whereas the other four MCRs distinctively recognize MSHs (Schiöth et al., 2005; Cortés et al., 2014). Atypically, melanocortin signaling is not exclusively regulated by the binding of endogenous agonists, as naturally occurring antagonists, agouti, designed as agouti-signaling protein (ASIP) in species different form mouse, and agouti-related protein (AGRP) compete with melanocortin peptides by binding to MCRs. ASIP is a potent melanocortin antagonist at MC1R and MC4R (Cone, 2005, 2006). In mice, ASIP is exclusively produced within the hair follicle, where it locally regulates the production of pigment in follicular melanocytes by antagonizing the effects of α-

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MSH on MC1R (Lu et al., 1994). In contrast, AGRP is mainly produced within the hypothalamic arcuate nucleus and the adrenal gland, and it is potent in inhibiting melanocortin signaling at MC3R and MC4R, but inactive at MC1R (Ollmann et al., 1997). Strong evidence has demonstrated that the central melanocortin system is a nodal point in controlling the energy balance in mammals (Girardet and Butler, 2014). Central activation of MC3R and MC4R is thought to mediate melanocortin effects on the energy balance (Cone, 2005, 2006) since both *mc3r* knockout rats (Chen et al., 2000) and *mc4r* knockout mice (Huszar et al., 1997) display severe alterations in energy homeostasis. The 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 *mc4r* knockout mice (Huszar et al., 1997). However, mice with multiple copies of *agouti* gene expressed under the control of a skin-specific promoter do not exhibit the obesity-related phenotype but manifest yellow fur (Klebig et al., 1995). This suggests that the central antagonism α-MSH signaling by agouti protein in obese yellow mice is responsible for this metabolic syndrome. Accordingly, the central administration of the C-terminal fragment of AGRP (Rossi et al., 1998) or chemical antagonists for MC3R and MC4R increase food intake in rodents (Fan et al., 1997; Kask et al., 1998), and intracerebroventricular (icv) injections of the melanocortin receptor agonist MTII (melanotan-II) produce a dose-dependent reduction in food intake in mice (Fan et al., 1997). However, *mc4r* deficient mice do not respond to the anorectic effects of MTII, suggesting that endogenous melanocortins inhibit feeding primarily by activating MC4R (Marsh et al., 1999). In several species including sea bass (Sánchez et al., 2009), mice (Nijenhuis et al., 2001) and humans (Tolle and Low, 2008), in vivo and in vitro experiments have demonstrated that MC4R signaling does not require binding agonist but it is constitutively activated. AGRP binding reduces the constitutive activity of the receptor as an inverse agonist does. It suggests that hunger is constitutively inhibited by MC4R signaling and AGRP binding to the receptor overcomes the MC4R-induced inhibition to promote feeding (Tolle and Low, 2008).The structure of the melanocortin system in fish diverges from that reported in tetrapods as the genome of the teleost antecessor doubled once more (3R), resulting in an expansion of the receptor/peptide systems (Cerdá-Reverter et al., 2011; Cortés et al., 2014). Therefore, the zebrafish genome has two *pomc* (Gonzalez-Nunez et al., 2003), *agrp* paralogue genes, a single copy of *asip* gene (Klovins and Schiöth, 2005) and six different *mcrs* since *mc5r* duplicated (Logan et al., 2003). In contrast, neuronal pathways expressing *pomc* (Cerdá-Reverter et al., 2003a; Forlano and Cone, 2007), AGRP (Cerdá-Reverter and Peter, 2003; Forlano and Cone, 2007) and mc4r (Cerdá-Reverter et al., 2003b) are well conserved, as is the involvement of the melanocortin system in the energy balance regulation (Cerdá-Reverter et al., 2011). The central administration of melanocortin agonist severely inhibits food intake in fasted goldfish (Cerdá-Reverter et al., 2003 a,b), whereas MC4R antagonists stimulate food intake in fed animals (Cerdá-Reverter et al., 2003b). Fasting increases *agrp* expression in the lateral tuberal nucleus, the homologue of the mammalian arcuate nucleus of goldfish (Cerdá-Reverter and Peter, 2003) and zebrafish (Song et al., 2003). Overexpression of *agrp* in transgenic models resulted in increased linear growth and adipocyte hypertrophy, suggesting that zebrafish could serve as a model for obesity research (Song and Cone, 2007). The AGRP-mediated suppression of MC4R activity seems critical for early larval growth (Zhang et al., 2012). In addition, non-functional Y-linked *mc4r* copies are associated with the larger males of *Xhiphophorus* and act as dominant-negative mutations, delaying the onset of puberty (Lampert et al., 2010).

In recent experiments, we generated a transgenic zebrafish strain over-expressing goldfish *asip* (Ceinos et al., 2015). Because ASIP is an antagonist (Cerdá-Reverter et al., 2005) of the constitutively activated MC4R (Sánchez et al., 2009), *asip* overexpression could reduce the activation of the central MC4R resulting in enhanced growth. This model offers an excellent scenario to analyze the phenotype induced by decreased activity of the central melanocortin system, and to study the neuronal pathways downstream of the melanocortin system. Here, we demonstrate that *asip*-overexpressing zebrafish exhibit an enhanced linear growth that could be mediated by increased food intake because of a decreased satiety but also as a result of increased food efficiency. Transcriptomic analysis reveals several candidate neuronal pathways that could mediate the melanocortin effect on food intake. Total lipid levels and profiles remained unaltered, suggesting that enhanced food intake does not result in an obese phenotype.

Material and methods

Animals and reagents

Wild type (WT) TU and ASIP [Tg(Xla.Eef1a1:Cau.Asip1)iim04, overexpressing *asip1* (Ceinos et al., 2015)] zebrafish strains were raised at 24–28 °C, with 14 h light/10 h dark cycle. *mc4r*$^{-/-}$ mutant strain *sa122* generated on Tupfel long fin background were obtained from the Sanger Institute Zebrafish Mutation Project and genotyped as previously described (Zhang et al., 2012). Prior any manipulation, animals were netted and anaesthetized for 1 min in 2-phenoxy-ethanol (0.05%) in the sampling tank. When required, animals were sacrificed by rapid decapitation after anaesthesia or cold exposition. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee (project number AGL2013-46448-C3-3-R). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis, MO, USA). Primers used in the experiments are summarized in supplementary Table 1.

Cell culture and transfection

HEK cells were maintained in DMEM media (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin mixture (Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C. Transient transfections were carried out using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions with 25 ng of each construct, and total amounts of DNA were kept constant in 2 μg with pBSSK plasmid.

Pharmacological experiments

A HEK-293 cell clone (clone Q), stably expressing β-galactosidase under the control of a vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP responsive elements (CRE) was used to evaluate receptor activation (CRE-GAL) (Sánchez et al., 2009). MCR constructs alone or in combination were transiently transfected in the clon Q. A construct carrying luciferase gene under the control of a constitutive promoter was also transfected to standardize transfection levels. The following day, cells were split up into 96-well plates and stimulated with human α-MSH (Bachem) ranging from 10^{-6} to 10^{-12} M or forskolin 10^{-6} in assay medium at 48 h post-transfection. After 6 h, the medium was removed, cells were lysed and galactosidase activity measured as previously described (Sánchez et al., 2009). The effect of human ASIP (Phonenix Pharmaceuticals Inc, USA) 10^{-7} M on MTII-stimulated MCRs (Bachem, Switzerland) activity was studied also. Measurements were normalized for the protein content, the luciferase activity and forskolin-induced galactosidase activity. Protein content was determined using the BCA protein assay kit (Pierce). Luciferase activity was determined using the luciferase assay kit (Promega) following provider instructions. Receptor activation assays were performed in quadruplicate wells and repeated at least