



Review

Fish melanocortin system

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ARTICLE INFO

Article history:

Received 14 August 2010

Received in revised form 30 September 2010

Accepted 12 October 2010

Available online 3 January 2011

Keywords:

Melanocortin-stimulating hormone (MSH)

Proopiomelanocortin (POMC)

Agouti-signalling protein (ASIP)

Agouti-related protein (AGRP)

Melanocortin receptor

Melanocortin receptor accessory protein (MRAP)

Food intake

Pigmentation

Stress

ABSTRACT

Melanocortin signalling is mediated by binding to a family of G protein-coupled receptors that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin (MC₁–MC₅) receptors. The number of receptors varies in fish, zebrafish, for example, having six melanocortin receptors, with two copies of the melanocortin MC₅ receptor, while pufferfish have 4 receptors with no melanocortin MC₃ receptor and one copy of melanocortin MC₅ receptor. Fish genomes also exhibit orthologue genes for agouti-signalling protein (ASP) and -related protein (AGRP). AGRP expression is confined to a small area in the hypothalamus but ASP is expressed in the skin. Fish melanocortin MC₂ receptor is specific for ACTH and requires the cooperation of accessory proteins (MRAP) to reach functional expression. The four other melanocortin MC receptors distinctively bind MSHs. The interaction of α -MSH and melanocortin MC₁ receptor plays a key point in the control of the pigmentation and mutations of melanocortin MC₁ receptor are responsible for reduced melanization. Both melanocortin MC₄ and MC₅ receptor are expressed in the hypothalamus, and central melanocortin MC₄ receptor expression is thought to regulate the energy balance through the modulation of feeding behaviour. In addition, the peripheral melanocortin system also regulates lipid metabolism by acting at hepatic melanocortin MC₂ and MC₅ receptors. Both sea bass melanocortin MC₁ and MC₄ receptors are constitutively expressed *in vitro* and both ASP and AGRP work as inverse agonists but only after inhibition of the phosphodiesterase system. Accordingly, the overexpression of AGRP and ASP transgenes promotes obesity and reduces melanization in zebrafish, respectively.

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1. Introduction

Proopiomelanocortin (POMC) gene encodes a complex protein precursor that belongs to the opioid/orphanin gene family, which also includes proenkephalin, prodynorphin and proorphanin. This opioid/

orphanin system was mainly formed by the two genome duplication rounds (2R) and probably a local duplication event during vertebrate evolution (Sundström et al., 2009). Tetrapod POMC precursor comprises three main domains: the N-terminal pro- γ -melanocyte stimulating hormone (MSH), the central adrenocorticotrophic hormone (ACTH) and the C-terminal β -lipotropin. Each domain contains one MSH peptide delineated by a core sequence HFRW: γ -MSH in pro- γ -MSH, α -MSH as N-terminal sequence of ACTH and β -MSH in β -lipotropin domain. The last domain further includes the C-terminal β -endorphin opioid peptide (Nakanishi et al., 1979). The three-domain structure has also been reported in invertebrate species, suggesting

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that this organization represents a plesiomorphic character, although classical melanocortin receptors have been never reported in invertebrates (Stefano et al., 1993). The number of MSH domains varies in fish, depending on the taxonomic class. Ray-finned fish exhibit substitutions in the γ -MSH core (basal groups) or deletion (teleost fish). Lobe finned fish, including lungfish and coelacanth, inherited the three-domain ancestral condition but cartilaginous fish exhibit an additional δ -domain located between α - and β -MSH. An independent duplication event in agnathans coupled to specialization resulted in two genes, POC and POM, showing ACTH and MSHs domains, respectively (Takahashi and Kawauchi, 2006). Avian and mammalian species have a single copy of the POMC gene but the genome of teleost antecessor doubled once more (3R) resulting in an expansion of the receptor/peptide systems. This event, together with particular tetraploidization events (e.g. salmonids), have resulted in additional copies of POMC in the genome of teleost fish. Sequencing projects have led to the conclusion that teleost fish, including zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*) and the pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*) exhibit two POMC (POMCa or α and POMCb or β) paralogue genes (Gonzalez-Nunez et al., 2003; Sundström et al., 2009). In addition, three different forms (a1, a2 and b) have been reported in barfin flounder (*Verasper moseri*; Takahashi et al., 2005). Studies in zebrafish suggest a subfunctionalization of the ancestral POMC sharing functions with the new paralogue gene in teleost fish (de Souza et al., 2005).

Tetrapod POMC is mainly produced in the vertebrate pituitary and its posttranslational processing occurs in a tissue-specific manner. The proteolytic cleavage of POMC by prohormone convertase 1 (PC₁) generates ACTH and β -lipotropin (β -LPH) in the corticotrophs of the anterior pituitary, whereas cleavage by PC₁ and PC₂ produces α -MSH and β -endorphin in the melanotrophs of the *pars intermedia* (Castro and Morrison 1997). As in other vertebrate species, fish POMC is mainly expressed in the pituitary gland. Two pituitary cell types produce melanocortin precursor. Corticotropes or ACTH-producing cells are localized in the rostral *pars distalis*, whereas melanotropes or MSH-producing cells are localized within the *pars intermedia* which is heavily innervated from the neurohypophysis to form the neurointermediate lobe in teleost fish (Cerdá-Reverter and Canosa, 2009). The number of POMC cells is notably smaller within the rostral *pars distalis* than *pars intermedia*, which reflects the preferential isolation of α -MSH, β -MSH and β -endorphin from the whole pituitary extracts (Takahashi and Kawauchi, 2006). Recent studies have demonstrated the differential processing of POMC in the pituitary of teleost fish (Takahashi et al., 2006). Therefore, N-terminal POMC (equivalent to γ -MSH domain of tetrapods), des-acetyl α -MSH, α -MSH, CLIP (corticotropin-like intermediate peptide), N- β -LPH, β -MSH and N-acetyl- β -endorphin has been isolated from the *pars intermedia*. This processing pathway in the *pars intermedia* is similar to that reported in mammalian species (Castro and Morrison, 1997). In the rostral *pars distalis*, N-POMC and ACTH have been isolated, as have des-acetyl α -MSH, CLIP and β -MSH. This suggests that, as in the mammalian system, ACTH is also produced within the *pars distalis* although this peptide may be further cleaved to des-acetyl α -MSH and CLIP, suggesting that corticotropes of the rostral *pars distalis* lack a MSH-acetylating system (Takahashi et al., 2008).

In mammalian species, POMC is also centrally produced and mainly processed to α -MSH and β -endorphin (Castro and Morrison, 1997). In rodents, two discrete groups of neurons in the hypothalamus (arcuate nucleus) and the medulla (nucleus of the *tractus solitarius*) also produce POMC (Bangol et al., 1999). POMC mRNA expression within the mediobasal hypothalamus has been conserved throughout vertebrate evolution, as POMC transcripts have been detected in hypothalamic neuronal systems of amphibians (Tuinhof et al., 1998), birds (Gerets et al., 2000). Our experiments in goldfish have demonstrated that POMC is also expressed within the whole extension

rostro-caudal extension of the lateral tuberal nucleus, which is thought to be the teleostean homologue of the arcuate nucleus (Cerdá-Reverter et al., 2003a). However, no POMC expression within the vagal lobe, the teleostean nucleus of the *tractus solitarius*, has been reported in any teleost species. Unfortunately, no information about POMC processing within the central nervous system is available.

Melanocortin exerts its physiological role by binding to a family of specific G protein-coupled receptors that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin (MC₁–MC₅) receptors. In mammalian systems, melanocortin MC₂ receptor is specific for ACTH. The four other MC receptors bind to MSHs, with melanocortin MC₁ and MC₃ receptors exhibiting the highest affinity for α -MSH and γ -MSH respectively (reviewed by Schiöth et al., 2005). Again, the number of receptors diverges in teleost fish. Zebrafish has six MC receptors, with two copies of the melanocortin MC₅ receptor, while pufferfish have only 4 receptors with no melanocortin MC₃ receptor and only one copy of melanocortin MC₅ receptor (Logan et al., 2003). Interestingly, perciform fish genome, the youngest teleost fish, lacks melanocortin MC₃ receptor and γ -MSH domain in the POMC gene, suggesting co-evolution of the peptide/receptor system.

Atypically, melanocortin signalling is not exclusively regulated by binding of endogenous agonists, as naturally occurring antagonists, agouti-signalling protein (ASP) and agouti-related protein (AGRP), compete with melanocortin peptides by binding to MC receptors. ASP is a potent melanocortin antagonist at melanocortin MC₁ and MC₄ receptors and relatively weak at melanocortin MC₃ receptor. In mice, ASP is exclusively produced within the hair follicle and it locally regulates the production of pigment in follicular melanocytes by antagonizing the effects of α -MSH on melanocortin MC₁ receptor. In contrast, AGRP is mainly produced within the hypothalamic arcuate nucleus and the adrenal gland and it strongly inhibits melanocortin signaling at melanocortin MC₃ and MC₄ receptors, but is not active at melanocortin MC₁ receptor (Cone, 2006).

Several studies have demonstrated that mammalian ASP and AGRP work as inverse agonists at melanocortin MC₄ receptor which is constitutively activated (Nijenhuis et al., 2001; Chai et al., 2003). Therefore, melanocortin MC₄ receptor signals in the absence of agonist binding. AGRP or ASP reduces constitutive melanocortin MC₄ receptor signaling in a dose-dependent manner. The presence of melanocortin endogenous antagonist including AGRP and ASP in fish was first demonstrated in goldfish (Cerdá-Reverter and Peter, 2003; Cerdá-Reverter et al., 2005) and later in several teleost species (Song et al., 2003; Kurokawa et al., 2006; Murashita et al., 2009). Once again, paralogue genes for both ASIPs (ASIP₁ and ASIP₂) and AGRPs (AGRP₁ and AGRP₂) have been demonstrated in teleost (Kurokawa et al., 2006; Murashita et al., 2009). In goldfish, zebrafish and salmon, AGRP₁ is expressed in several peripheral tissues, including skin, but in the central nervous system it is solely expressed within the lateral tuberal nucleus where POMC is also produced (Cerdá-Reverter et al., 2003a, 2003b, 2003c; Forlano and Cone, 2007). In contrast, AGRP₂ is only expressed in the pineal gland of the zebrafish but quantitative PCR (qPCR) studies in salmon have demonstrated wider AGRP₂ expression at the periphery (Murashita et al., 2009). Unlike in the mammalian system, AGRP works as an inverse agonist and competitive antagonist at both sea bass melanocortin MC₁ and MC₄ receptors. ASIP is also widely expressed in the periphery despite a certain degree of central expression. In the periphery, ASIP is mainly expressed in the skin, where it is supposed to participate in the acquisition of the adult pigment pattern (Cerdá-Reverter et al., 2005).

2. Melanocortins and food intake

Studies by Northern blot in goldfish (Cerdá-Reverter et al., 2003a), whole-mount *in situ* hybridization in zebrafish (Song et al., 2003) and qPCR in sea bass have demonstrated that POMC hypothalamic neurons cannot respond to progressive fasting that induces a severe

reduction in weight (Sánchez et al., 2009a). Similar results have also been obtained in mammalian species. Therefore, POMC mRNA remain unchanged in chronically food-restricted sheep suffering about 40% total body-weight loss (Henry et al., 2001) and long-term alterations in adiposity do not affect hypothalamic POMC expression in ovariectomized ewes that have lost about 30% total body weight (Henry et al., 2000). However, intracerebroventricular injections of melanocortin agonist, NDP-MSH [Nle⁴, D-Phe⁷] α -MSH, or MTII (Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH₂), inhibit food intake in goldfish (Cerdá-Reverter et al., 2003a,b) and rainbow trout (Schjolden et al., 2009) in a dose dependent way. Accordingly, intracerebroventricular administration of HS024 (cyclo Ac-Cys-Nle-Arg-His-D-Nal-Arg-Trp-Gly-Cys-NH₂), in fed goldfish increased food intake 4 h after treatment (Cerdá-Reverter et al., 2003b). Similar results were obtained in rainbow trout when using and SHU9119 (Ac-Nle-cyclo (Asp-His-D-2-Nal-Arg-Trp-Lys)-NH₂), although the former had a more pronounced effect on food intake (Schjolden et al., 2009). These results suggest that the melanocortin system may be involved in the control of food intake of fish, although the lack of hypothalamic response to fasting leads to reservations concerning how the system is regulated. In the absence of a POMC hypothalamic response, the system can be regulated at three different levels at least: i) differential processing of POMC precursor after progressive fasting. Of note is the fact that the intracerebroventricular administration of β -endorphin, which is encoded at the C-terminal end of POMC, has been shown to stimulate appetite in the goldfish (De Pedro et al., 1995). Because of the contrasting actions of POMC peptides on food intake, the effects of nutritional status might manifest themselves at posttranscriptional levels by the differential regulation of the prohormone convertases involved in goldfish POMC processing. In food-restricted goldfish with enhanced feeding drive, POMC cleavage may be directed towards the production of β -endorphin, whereas a positive energy balance may preferentially drive POMC processing to production of the food-intake inhibitor α -MSH. However, no experiments in fish have tested this hypothesis. ii) Adjustment in the number of central melanocortin receptors. The system could also be regulated by alteration of the receptor density in areas controlling food intake. Therefore, during fasting, the availability of receptors could be reduced, thus decreasing sensitivity to endogenous melanocortin peptides or constitutive signalling. Experiments in barfin flounder (*Verasper mosari*) and sea bass have demonstrated that progressive fasting does not modify melanocortin MC₄ receptor mRNA expression levels in the hypothalamus (Kobayashi et al., 2008; Sánchez et al., 2009a) but no experiments have been carried out to evaluate the availability of the receptor at the plasma membrane iii) Counter regulation of receptor activity by the central melanocortin antagonists or inverse agonists. It is plausible that the activity of the receptor is basically regulated by the action of an inverse agonist, whereas proper agonists only work under specific physiological conditions. Experiments in goldfish (Cerdá-Reverter and Peter, 2003) and zebrafish (Song et al., 2003) demonstrated that hypothalamic AGRP expression is dramatically increased during fasting at the same time intervals in which POMC expression remains constant. Our pharmacological experiments in goldfish and sea bass melanocortin receptors have demonstrated that zebrafish AGRP (83–127) is a potent competitive antagonist at sea bass melanocortin MC₄ receptor (Sánchez et al., 2009a) but not at sea bass melanocortin MC₅ receptor (Sánchez et al., 2009b). The C-terminal fragment of the zebrafish AGRP can also work as an inverse agonist, inhibiting both basal and forskolin-induced melanocortin MC₄ receptor activity (Sánchez et al., 2009a; Sánchez et al., 2010). Suggestively, sea bass melanocortin MC₄ receptor constitutive activity and/or AGRP inverse agonism, but not competitive antagonism, depends on inhibition of the intracellular phosphodiesterase system when the receptor is overexpressed in human embryonic kidney (HEK)-293 cells. This suggests that both AGRP agonism and antagonism in the sea bass melanocortin MC₄ receptor system are

mediated through different intracellular signalling pathways with differential sensitivities to phosphodiesterase inhibitors. To the best of our knowledge, such phosphodiesterase dependence has been only studied in the sea bass melanocortin MC₁ and MC₄ receptors and this opens new targets for investigation of melanocortin-induced metabolic syndrome.

Both HS024 and SHU9119 have been demonstrated to work as functional competitive antagonists at sea bass melanocortin MC₄ receptor (Cerdá-Reverter et al., 2003b; Sánchez et al., 2009a; Sánchez et al., 2010). However, both compounds are potent agonists in goldfish melanocortin MC₅ receptor (Cerdá-Reverter et al., 2003c) and sea bass melanocortin MC₅ receptor (Sánchez et al., 2009b), suggesting that the central melanocortin effects on food intake are mediated via melanocortin MC₄ receptor. *In situ* hybridization studies in goldfish (Cerdá-Reverter et al., 2003b) and sea bass (Sánchez et al., 2009a) have demonstrated that this receptor is profusely expressed within the CNS. In fact, sea bass melanocortin MC₄ receptor expression is basically restricted to the CNS, while goldfish melanocortin MC₄ receptor is also expressed in the peripheral tissues. Sea bass receptor transcripts are restricted to the telencephalon, preoptic area, ventral thalamus, tuberal and lobular hypothalamus, optic tectum and rombencephalon. The distribution of the receptor in goldfish brain is similar to that reported in sea bass, with transcripts within the rostral telencephalon, preoptic area and tuberal hypothalamus. Experiments in zebrafish (Forlano and Cone, 2007) and seabass (Ríos, Agulleiro, Leal, Cerdá-Reverter, unpublished results) suggest that melanocortin MC₄ receptor distribution is consistent with the localization of MSH/AGRP immunoreactive terminals. In the zebrafish brain, AGRP terminals overlap the denser MSH innervation but both AGRP and MSH neurons also independently project into several areas. The highest innervation is found within the rostral and magnocellular preoptic area and caudal tuberal hypothalamus where we have described profuse expression of the sea bass melanocortin MC₄ receptor. The phenotype of the neurons expressing melanocortin MC₄ receptor is unknown and deserves further investigation in the future but it is known that MSH terminals lie in close apposition to corticotrophin-releasing hormone-producing neurons in the posterior preoptic area of the goldfish (Matsuda et al., 2008). The preoptic and hypothalamic regions vary greatly among vertebrates. As described above, the lateral tuberal nucleus is thought to be the teleostean homologue of the mammalian arcuate nucleus, while the parvo- and magnocellular neurons of the preoptic nucleus seem to be homologues of the mammalian supraoptic and paraventricular nuclei, all of them pivotal points in the control of the energy balance in mammalian species (Berthoud, 2002). Neuronal pathways involved in the control of food intake in fish are not well known. However, the tuberal hypothalamus, lateral torus and the inferior hypothalamic lobe have been suggested as being involved in the integration of the viscerosensory information and elaboration of coordinated responses modifying the energy balance in fish (Rink and Wullimann, 1998).

In summary, the results obtained in several fish species suggest that the melanocortin system may induce an inhibitory tone on food intake via constitutive activation of the central melanocortin MC₄ receptor. This constitutive tone is mainly regulated by the binding of AGRP within the tuberal hypothalamus and preoptic area. Agonist binding would increase melanocortin tone under particular physiological conditions. In support of this hypothesis, it has been reported that the overexpression of AGRP in zebrafish results in increased linear growth and total body weight. Transgenic AGRP fish also exhibit visceral adipocyte hypertrophy and increased total triglyceride levels suggesting that these fish are obese (Song and Cone, 2007). The absence of systemic α -MSH also results in hyperphagia and obesity in rainbow trout. Therefore, the “cobalt” phenotype, so-named because of its “cobalt-blue” body colour, has been attributed to the absence of most of the *pars intermedia* of the pituitary, where α -MSH is synthesized. This variant of trout is hyperphagic and, typically, also

has an enlarged liver and shows fat accumulation in the abdominal cavity, reflecting the absence of α -MSH-mediated lipolytic activity (Yada et al., 2002). This suggests that the melanocortin system plays a dual role in the control of the energy balance by activating peripheral lipolytic activity, thus enhancing energy expenditure, and through the central inhibition of food intake. Experiments in sea bass have demonstrated that MSH can stimulate hepatic lipolysis via sea bass melanocortin MC₅ receptor (Sánchez et al., 2009b).

3. Melanocortins and pigmentation

Although more than 100 loci have been identified as being involved in vertebrate pigmentation, the melanocortin system is consistently a key determinant of the pigment phenotype. In mammals, the colour pattern is determined by the distribution of two pigments, pheomelanin (red/yellow) and eumelanin (brown/black). Both pigments are synthesized from a common precursor, dopaquinone, in response to respectively low or high levels of tyrosinase, the rate-limiting enzyme in the synthesis of melanins. Two main loci are responsible for the pheomelanin/eumelanin synthesis rate in the melanocyte—*extension* (Robbins et al. 1993) and regulates the synthesis of melanin via activation of tyrosinase. Briefly, α -MSH activation of melanocortin MC₁ receptor leads to increased intracellular levels of cAMP, which, in turn, lead to phosphorylation of the cAMP responsive-element-binding protein (CREB). CREB transcriptionally regulates several genes, including the transcription factor microphthalmia (MITF). MITF is pivotal in the expression of several pigment-synthesizing enzymes, including tyrosinase, and differentiation factors (Lin and Fisher, 2007). Therefore, melanocortin MC₁ receptor activation, results in high levels of tyrosinase, which leads to eumelanogenesis and, by extension, to dark (brown/black) pigmentation (Sakai et al., 1997). Accordingly, recessive yellow mice (*e/e*) which exhibit an almost yellow fur have been demonstrated to carry a frameshift mutation between the fourth and fifth transmembrane domains of melanocortin MC₁ receptor, while a sombre phenotype (*E^{so}/E^{so}*) that has almost entirely black fur exhibits a point mutation that confers constitutive activity to the melanocortin MC₁ receptor (Robbins et al. 1993).

Agouti-signalling protein fills up the *agouti* locus (Bultman et al., 1992). This paracrine factor is synthesized in the hair bulbs and is efficiently released to melanocytes and hair matrix cells to regulate melanin production (Matsunaga et al., 2000). Agouti-signalling protein binding to melanocortin MC₁ receptor reduces the constitutive or α MSH-induced signalling, reducing MITF expression and resulting in lower tyrosinase levels than in the presence of high levels of cysteine switches eumelanogenesis to pheomelanogenesis and by extension darker to paler (yellow/red) colour patterns (Sakai et al., 1997; Aberdam et al., 1998). A high degree of polymorphism in the ASP (Miltenberger et al., 2002) and epigenetic modifications (Cropley et al., 2006) result in a high variety of mutant phenotypes, ranging from all black (*a/a*) to a uniform yellow (*Ay/a*) hair coat. Non agouti (*a/a*) extreme phenotypes carrying inactivating mutations in ASP display black fur (Bultman et al., 1992). The unusual allele, *Ay*, consists of a large deletion in the non-coding regions that places ASP gene under the control of the *Raly* promoter, an ubiquitously expressed RNA binding protein. The associated phenotype is characterized by ubiquitous expression of AS gene, resulting in yellow fur, hyperphagia, hyperinsulinemia, increased linear growth, an increased propensity for developing tumors, premature infertility and maturity-onset obesity (Miller et al., 1993; Michaud et al., 1993). This metabolic syndrome is mediated by antagonizing α -MSH signalling at the central melanocortin MC₄ receptor that arbitrates the negative effects of melanocortin peptides on the energy balance (Lu et al., 1994). The ectopic expression of ASP in the brain mimics the action of AGRP, a central antagonist that regulates the inhibitory tone imposed by

melanocortins on food intake at melanocortin MC₃ and MC₄ receptors (Cone, 2006). The regional and temporal expression of different ASP isoforms, produced by the usage of alternative promoters, is responsible for the dorsal–ventral pigment pattern in mouse but also for the subapical yellow banding in the agouti coloration (Vrieling et al., 1994). Agouti-signalling protein not only modifies the switch from eumelanogenesis to pheomelanogenesis but also inhibits the differentiation of neural crest precursors into melanocytes (Aberdam et al., 1998; Sviderskaya et al., 2001). Microarray analyses of *in vitro* cultured melan-a melanocytes have suggested that cell treatment with ASP reproduces dedifferentiation stages by reducing the number of melanogenic proteins and increasing cell migration (Le Pape et al., 2009).

Typical fish colour patterns are obtained as a result of the combination of several types of chromatophores commonly found in the dermis. Pigment cells are divided into light-absorbing (melanophores, xanthophores, erythrophores and cyanophores) and light-reflecting (leucophores and iridophores) chromatophores. Fish melanophores are known to contain only eumelanins (black-brown pigments), whereas xanthophores and erythrophores contain carotenoids and/or pteridines, which contribute to reddish and yellowish components of skin coloration. Iridophores are commonly localized in whitish and silvery areas of the skin, predominantly on the belly surface. They contain crystalline platelets composed of purines, mainly guanine, responsible for the reflection of the light. The interaction of light with the patterned distribution of pigmented cells creates the colour pattern in fish (Fujii, 1993). A common morphological pattern in many non-mammalian vertebrates is a sharp demarcation in the coloration between dorsal and ventral areas. In teleosts, this countershading is achieved by a patterned distribution of the pigment cells, with the light-absorbing and light reflecting chromatophores mostly distributed in the dorsal and ventral areas, respectively (Fujii, 1993). Early experiments in amphibian and fish species suggested that the dorsal–ventral pigment pattern is achieved through a putative diffusible melanization inhibition factor (MIF), mainly produced by cells in the ventral skin, which inhibits melanoblast differentiation and stimulates or supports iridophore proliferation in the ventrum (Fukuzawa and Ide, 1988; Bagnara and Fukuzawa, 1990; Zuasti et al., 1992; Zuasti, 2002). Amphibian MIF was reported to block stimulation of the melanization provoked by α -MSH in neural explants of *Xenopus* (Fukuzawa and Bagnara, 1989). Subsequently, amphibian MIF was also shown to block α -MSH-induced tyrosine hydroxylase and dopa oxydase activity in mouse malignant melanocytes, both key enzymes in the melanogenic pathway (López-Contreras et al., 1996). Although MIF has been partially purified and MIF-antibodies have localized its expression in the ventral skin of leopard frog (*Rana forreri*), the molecular structure remains unidentified (Fukuzawa et al., 1995). Similar experiments in catfish demonstrated that conditioned medium from catfish ventral skin and semi-purified amphibian MIF inhibits melanoblast differentiation but stimulates or supports iridophore proliferation (Zuasti, 2002).

Recent studies have demonstrated that the melanocortin system is also a key player in the establishment of the adult pigment pattern in fish. Frameshift mutations introducing a premature stop codon in melanocortin MC₁ receptor or inactivating mutations in blind Mexican cave tetra (*Astyanax mexicanus*) are responsible for a decrease in the number of melanophores and in the melanin content. This phenotype was recapitulated by melanocortin MC₁ receptor morpholino knockdown experiments in zebrafish (Gross et al., 2009). Our experiments in sea bass have demonstrated that sea bass melanocortin MC₁ receptor is constitutively activated and expressed in both dorsal and ventral skin (Sánchez et al., 2010), suggesting that the ligand-independent activity of the receptor may be responsible for melanization in fish. If melanocortin MC₁ receptor is constitutively activated and expressed in both dorsal and ventral surfaces, it would

be difficult for fish to take on a dorsal ventral colour pattern. Therefore, the melanocortin MC₁ receptor function is probably inhibited in the ventrum to help establish the pigment pattern. This model goes along with the presence of non-diffusible MIFs in the ventral area but not in the dorsal region (see above). Our recent studies in goldfish strongly suggest that ASP fish orthologue is non-mammalian MIF. Experiments in goldfish (Cerdá-Reverter et al., 2005) and pufferfish (Kurokawa et al., 2006) showed that ASPs are mainly expressed in the ventral skin with very low levels in the dorsal skin. The identity of ASP-expression cells is still unknown. Recombinant goldfish ASP interacts with the melanocortin system, working as a competitive antagonist and probably as an inverse agonist to decrease both the NDP-MSH-induced and constitutive intracellular accumulation of cAMP in pufferfish melanocortin MC₁ receptor and goldfish melanocortin MC₄ receptor expressing cell lines. Goldfish ASP also inhibits NDP-MSH-induced melanosome movements (Cerdá-Reverter et al., 2005) and injection of capped goldfish ASP mRNA in the dorsal skin of flatfish, turbot (*Scophthalmus maximus*) and Senegalese sole (*Solea senegalensis*) dramatically inhibits melanization (Ceinos R, Guillot R, Rotllant P and Cerdá-Reverter JM, unpublished results). Therefore, our hypothesis is that melanocortin MC₁ receptor constitutively drives melanization in fish skin but the inverse agonism of ASP in the ventral skin reduces melanocortin MC₁ receptor activity, thus inhibiting both melanization and melanoblast differentiation but supporting iridophore proliferation. This hypothesis could be extended not only to melanization since red/orange goldfish lacking melanocytes also display a dorsal–ventral pigment polarity. ASP is mainly expressed within the ventral skin of xanthic goldfish, suggesting that ASP inhibits the differentiation of light absorbing chromatophores (Cerdá-Reverter et al., 2005). We also hypothesized that the site of MITF expression inhibition lies downstream of ASP effects. Zebrafish exhibits two subfunctionalized copies of MITF (Lister et al., 2001). MITFa is coexpressed with MITFb in the retinal pigment epithelium but not in the neural crest melanoblast, where only MITFa is expressed. A single base mutation in the MITFa results in the almost complete absence of melanophores throughout embryonic and larval development, an absence that persists during adulthood in the *nacre* phenotype but pigmented retinal epithelium.

In contrast to the absence of melanophores, *nacre* mutants show an approximate 40% increase in the number of iridophores (Lister et al., 1999). MITF has also been reported to be sufficient to direct the differentiation of medaka embryonic stem-like cells into melanocytes (Bejar et al., 2002).

Nacre phenotype (inhibition of melanoblast differentiation and iridophore proliferation) resembles those inferred properties of poikilotherm MIF that we have hypothesized to be ASP.

Our experiments overexpressing ASP in transgenic zebrafish have demonstrated a dramatic reduction in the number of melanophores within the dark stripes and an increased number of iridophore, leading to a severe disruption of the stripe pattern that result into spotted “cheetahfish” (Guillot R, Ceinos R, Rotllant J and Cerdá-Reverter JM, unpublished results).

The involvement of components of the melanocortin system other than melanocortin MC₁ receptor and ASP in the colour pattern regulation in fish should be also considered. We have demonstrated that melanocortin MC₅ receptor is highly expressed within the ventral skin but not in the dorsal skin of the sea bass (Sánchez et al., 2009b). Similarly, goldfish melanocortin MC₅ receptor is also expressed in the skin despite the lack of differentiation between dorsal and ventral areas (Cerdá-Reverter et al., 2003b). Sea bass melanocortin MC₄ receptor is not expressed in the skin (Sánchez et al. 2009a) but a low level of sea bass melanocortin MC₂ receptor expression was detected in both dorsal and ventral areas (Agulleiro M, Sánchez E and Cerdá-Reverter JM, unpublished results). In contrast, goldfish melanocortin MC₄ receptor is expressed within the dorsal skin but not in the ventral

area (Cerdá-Reverter et al., 2003b). AGRP could be also involved in the regulation of the colour pattern in fish since expression levels in the dorsal skin were higher than those observed in the dorsal skin of the goldfish (Cerdá-Reverter and Peter, 2003). High levels of AGRP₁ expression in the skin have been also reported in Atlantic salmon (Murashita et al., 2009) even though low levels were detected in pufferfish (Kurokawa et al., 2006) and sea bass (Agulleiro M, Sánchez E and Cerdá-Reverter JM unpublished results).

4. Melanocortins and stress response

As in other vertebrates, activation of the hypothalamus–pituitary–interrenal axis plays an essential role in the stress response in fish. Following stressor exposure, the hypothalamic neurons release corticotrophin-releasing hormone (CRF) to the anterior pituitary (rostral *pars distalis*) where the corticotropes are located. The processing of POMC in the corticotrophs leads to the production of ACTH, which activates melanocortin MC₂ receptor in the interregal tissue (analogous to the adrenal cortex in tetrapods), which, in turn, controls cortisol synthesis. Cortisol, the main corticosteroid in fish, is released to the blood to regulate a wide array of systems in both stressed and non-stressed animals (Wendelaar Bonga, 1997; Flik et al., 2006; Alsop and Vijayan 2009).

Studies on HPI development have demonstrated that the stress response is organized early in the ontogenetic process. Maternal POMC mRNA is already present in fertilized eggs but endogenous expression begins 18 h postfertilization and POMC pituitary cells are visible by whole-mount *in situ* hybridization as early as 24 h postfertilization within the anterior domain (Hansen et al., 2003). These early corticotroph cells already respond to dexamethasone treatment (negative feedback) at 48 h postfertilization but interrenal development, as assessed by the expression of key steroidogenic enzymes, is only sensitive after 5 days post fertilization. Interrenal tissue development seems to be independent of pituitary development on the first two days post fertilization. In fact, the expression of steroidogenic enzymes (22–28 h postfertilization) precedes melanocortin MC₂ receptor expression (32 h postfertilization), and the expression pattern of the studied steroidogenic and chromafin genes is similar in mutants lacking pituitary cells (including corticotrophs). However, these mutants exhibit severe impairment of the interrenal function at 5 days postfertilization suggesting that proliferation and full functional differentiation of the interregal organ depends on ACTH/melanocortin MC₂ receptor interaction (To et al., 2007).

A cortisol stress response is obtained at 4 days postfertilization in zebrafish but early exposure to stressors seems to influence normal hypothalamus–pituitary–interrenal axis development; in particular, the magnitude of cortisol response to stressors is reduced during adulthood (Alsop and Vijayan, 2009).

In adult rainbow trout, acute stressors rapidly elicit a significant increase in plasma ACTH (1 h post-stress) and melanocortin MC₂ receptor expression (4 h post-stressor). This up-regulation of melanocortin MC₂ receptor expression is clearly induced by the ACTH since *in vitro* incubation of interregal slides with the hormone leads to an increase in the receptor expression. Using this *in vitro* system in rainbow trout, ACTH was able to induce an increase in cortisol synthesis that was not blocked by melanocortin MC₄ receptor antagonist (SHU9119), but NDP-MSH was incapable of inducing cortisol release to the cell culture medium. This suggests that α -MSH is not involved in the cortisol response to stress (Aluru and Vijayan, 2008) despite reports that melanocortin MC₅ receptor is profusely expressed in the head kidney of the rainbow trout (Haitina et al., 2004). Similar results were also reported in carp even though α -MSH levels were increased after 1 and 7 days of restraint stress. The superfusion of interrenal tissue with homogenate from the *pars intermedia* elicited an increase in cortisol release, suggesting the

presence of unknown corticotrops in the above pituitary domain (Metz et al., 2005). In summary, it seems that, as in other vertebrates, melanocortin MC₂ receptor is the main player in the cortisol response to stress.

The study of melanocortin MC₂ receptor activation has been limited because of the lack of a readily transfectable heterologous expression system. Only adrenocortical-derived cell lines (Y6 or OS3), from which endogenous melanocortin MC receptor expression is absent, have rendered appropriate data on melanocortin MC₂ receptor pharmacology (Schimmer et al., 1995). Expression experiments in a range of non-adrenal cells suggested that melanocortin MC₂ receptor cannot reach the plasma membrane and is retarded in the endoplasmic reticulum, thus compromising its functional expression. This justification suggested the presence of an accessory factor that works as a melanocortin MC₂ receptor-specific transport system in the adrenocortical cells (Noon et al., 2002). One gene candidate that encodes for a small single transmembrane domain protein with high expression in the adrenal cortex was identified in humans, showing familial glucocorticoid deficiency (FGD). This syndrome is characterized by resistance to ACTH, i.e. high plasma ACTH levels but lacking adrenal glucocorticoids. The protein is known as melanocortin MC₂ receptor accessory protein (MRAP). The alternative splicing of the last two exons gives rise to two isoforms that differ in the C-terminal region (MRAP α and MRAP β ; Metherell et al., 2005). Knockdown of endogenous mouse MRAP in Y1 adrenocortical cells, which express a functional endogenous melanocortin MC₂ receptor, leads to insensitivity to ACTH, demonstrating that MRAP is essential for producing an ACTH responsive melanocortin MC₂ receptor (Cooray et al., 2008). MRAP interacts with the melanocortin MC₂ receptor to facilitate correct folding, and subsequent glycosylation and receptor cell surface expression (Metherell et al., 2005) but they are also essential for ACTH binding and ACTH-induced cAMP production (Sebag and Hinkle, 2009a; Roy et al., 2007). Recent experiments have identified a second form of MRAP in the human genome called MRAP2. This protein shares the structural characteristics of MRAP but is expressed only in the human brain and adrenal gland. Similar to MRAP₁, MRAP₂ assists melanocortin MC₂ receptor cell surface expression and allows the ACTH-induced cAMP production (Sebag and Hinkle, 2009; Chan et al., 2009). Our preliminary experiments expressing sea bass melanocortin MC₂ receptor in HEK-293 cells failed to generate an ACTH-stimulated cAMP increase. However, when human MRAPs were cotransfected, the sea bass melanocortin MC₂ receptor was able to mediate ACTH-induced cAMP production, suggesting the presence of a conserved melanocortin MC₂ receptor trafficking system in non-mammalian vertebrates.

In silico searches using available public databases rendered three different MRAPs in zebrafish, i.e. MRAP₁, MRAP_{2a} and MRAP_{2b}. Subsequently, we identified orthologue sequences in several teleosts fish. MRAP₁ is mainly expressed in the anterior kidney of the zebrafish presumably in the interregal tissue but the cell type has not been identified. In contrast, both MRAP_{2a} and MRAP_{2b} expression was found in every tissue tested. Cell trafficking studies demonstrated that zebrafish melanocortin MC₂ receptor cannot reach plasma membrane when overexpressed in HEK-293 cells. When zebrafish or human MRAP₁ (Agulleiro MJ, Roy S, Gallo-Payet N and Cerdá-Reverter JM, unpublished results) was coexpressed together with zebrafish melanocortin MC₂ receptor in the above cell system, both proteins were colocalized in the cell surface. However, when zebrafish melanocortin MC₂ receptor was coexpressed with MRAP_{2a} or MRAP_{2b}, both receptor and accessory protein labelling fluorochromes were colocalized but never in the plasma membrane. Both zebrafish melanocortin MC₂ receptor and MRAP_{2a} were mainly found in the cytoplasm, while zebrafish melanocortin MC₂ receptor and MRAP_{2b} were colocalized in the cytoplasm and also in the nuclear membrane (Agulleiro et al., 2010). Interestingly, it has been reported that mouse melanocortin MC₂ receptor is complexed with nucleoporin 50 expressed in Y6 adrenal

cells and translocated from the membrane to the nucleus after ACTH stimulation (Doufexis et al., 2007). Therefore, it is possible that MRAP₂ may be involved in this novel signalling pathway. According to trafficking studies, zebrafish melanocortin MC₂ receptor responds to ACTH stimulation only when co-expressed with zebrafish MRAP₁ or human MRAP₁, but not with zebrafish MRAP_{2a} or MRAP_{2b}, as seen from increases in the intracellular cAMP levels in HEK cells. The receptor did not become functional in the absence of MRAP₁ but when MRAP_{2a} or MRAP_{2b} were coexpressed together with MRAP₁ in cells stably expressing zebrafish melanocortin MC₂ receptor, the magnitude of the response was increased. This suggests that MRAP₁ cooperates with the MRAP₂ subtypes to increase the ACTH response (Agulleiro et al., 2010).

Developmental studies in zebrafish demonstrated that MRAP₁ expression is exclusively detected by whole-mount *in situ* hybridization in the interregal tissue at 4 days post fertilization; however, studies revealing MRAP_{2a} and MRAP_{2b} expression consistently detected diffuse expression within the brain at 2 days post fertilization. After 2 days, pituitary corticotrops are already responsive to dexamethasone but ACTH-dependent interregal tissue development was noted at 5 days post fertilization (see above), suggesting that interregal MRAP₁ expression could be a crucial point in ACTH-dependent interregal tissue development (Agulleiro MJ, Guillot R, Sanchez and Cerdá-Reverter, unpublished results). Incipient studies on MRAP₁ promoter revealed putative recognition sites for peroxisome proliferator-activated receptor (PPAR), estrogen receptor (ER) and glucocorticoid receptors (GR), which suggests that MRAP₁ is not only an accessory protein but a key point in the regulation of ACTH-induced glucocorticoid synthesis or, by extension, in the stress response in vertebrates.

Acknowledgements

This work was partially supported by grants from the Ministry of Science and Innovation (MICINN) AGL2007-65744-C03-02, CSD 2007-00002 and AGL2010-22247-C03-01 to JM C-R. MJA is recipient of a "Juan de la Cierva" research contract (2009) from the Spanish Science and Innovation Ministry.

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