PHOSPHODIESTERASE INHIBITOR-DEPENDENT INVERSE AGONISM OF AGOUTI-RELATED PROTEIN (AGRP) ON MELANOCORTIN 4 RECEPTOR IN SEA BASS (*Dicentrarchus labrax*)

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Abbreviated title: AGRP inverse agonism

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ABSTRACT

The melanocortin 4 receptor (MC4R) is a G-protein coupled receptor mainly expressed in the central nervous system of vertebrates. Activation of the MC4R leads to a decrease in food intake, while inactivating mutations are a genetic cause of obesity. The binding of agouti-related protein (AGRP) reduces agonist-stimulated cAMP production (competitive antagonist) but also the basal activity of the receptor, as an inverse agonist. Transgenic zebrafish overexpressing AGRP display increased food intake and linear growth, indicative of a physiological role for the melanocortin system in the control of the energy balance in fish. We report on the cloning, pharmacological characterization, tissue distribution and detailed brain mapping of a sea bass (Dicentrarchus labrax) MC4R orthologue. Sea bass MC4R is profusely expressed within food-intake controlling pathways of the fish brain. However, the activity of the melanocortin system during progressive fasting does not depend on the hypothalamic/pituitary proopiomelanocortin (POMC) and MC4R expression, which suggests that sea bass MC4R is constitutively activated and regulated by AGRP binding. We demonstrate that AGRP acts as competitive antagonist and reduces MTII-induced cAMP production. AGRP also decreases the basal activity of the receptor as an inverse agonist. This observation suggests that MC4R is constitutively active and supports the evolutionary conservation of the AGRP/MC4R interactions. The inverse agonism, but not the competitive antagonism, depends on the presence of a phosphodiesterase inhibitor (IBMX). This suggests that inverse agonism and competitive antagonism operate through different intracellular signalling pathways, a view that open up new targets for the treatment of melanocortin-induced metabolic syndrome.

Keywords: Melanocyte-stimulating hormone (MSH), Proopiomelanocortin (POMC), MC4R, Constitutive Activity, IBMX, Obesity.
INTRODUCTION

Melanocortins are posttranscriptional products of a complex precursor named proopiomelanocortin (POMC). They are mainly comprised of adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormones (α-, β-, γ- and δ-MSH; 26). POMC is produced in the pituitary but two discrete groups of neurons in the hypothalamus and the medulla of the central nervous system (CNS) of rodent also produce this precursor (22). Melanocortin signalling is mediated by binding to a family of specific G protein-coupled receptors that stimulate adenylyl cyclase activity. In vertebrates, five melanocortin receptors (MC1R-MC5R) have been characterized by molecular cloning. Subtype 2 receptor is ACTH specific, whereas the other four MCRs distinctively recognize MSHs (55). Expression studies in mammalian species have demonstrated that only MC3R and MC4R are significantly expressed within the CNS (22). The melanocortin system is not exclusively regulated by the binding of endogenous agonists, since naturally occurring antagonists, agouti (AG) and agouti related protein (AGRP), compete with melanocortin peptides for MCRs. Agouti protein is a potent antagonist at MC1R and MC4R (44), whereas AGRP is inactive at MC1R but equally potent in inhibiting melanocortin signalling at MC3R and MC4R (56).

A body of evidences substantiates an essential role for central signalling through MC3R and MC4R in the regulation of energy intake and expenditure in mammalian species (22). Mice lacking MC4R exhibit hyperphagia, hyperinsulinemia, increased linear growth and obesity (35). A similar phenotype is also observed in mice over-expressing agouti or AGRP genes (38, 42, 52). Central administration of the MCR agonist, MTII, produces a dose-dependent reduction in food intake in mice (28) but not in MC4R-deficient mice (43). These anorexic effects are blocked by the co-administration of AGRP (53), which is endogenously released from the NPY neurons in the arcuate nucleus (32). Recent experiments have demonstrated
that AGRP is processed intracellularly by proprotein convertases into the active form AGRP 83-123 (24). AGRP and MSH terminals co-localize in the MC4R-expressing neurons (4). However, the presence of AGRP terminals in MC4R-expressing neurons lacking MSH innervation (29) suggests that AGRP has effects on MC4R not only as competitive antagonist. *In vivo* experiments with mutant mice that exhibit a neural selective POMC deficiency have demonstrated that AGRP modulates the energy balance via a mechanism independent of MSH and MC3/4R competitive antagonism (61). MC4R has been reported to display constitutive activity, as it increases basal cAMP production in the absence of ligand (50) by spontaneously mimicking an agonist-occupied state (59). Several *in vitro* experiments have demonstrated that AGRP binding to MC4R decreases basal cAMP production (18, 20, 33, 36, 50, 59), to suppress constitutive activity of the receptor. This suggests that AGRP not only blocks the agonist activity, but also stabilizes the inactive conformation of the receptor, and thus either suppresses or reduces the constitutive activity as an inverse agonist does. This constitutive activity may support a tonic satiety signal required for the long-term energy homeostasis that is ultimately regulated by AGRP (22, 59).

We previously demonstrated that the melanocortin system may play a physiological role in the control of food intake in teleost fish. The central administration of melanocortin agonist inhibits food intake, whereas MC4R antagonist increases intake levels in fed animals (14, 15). Short- or long-term fasting does not induce changes in hypothalamic POMC expression (15) but sharply increases hypothalamic AGRP production (13). Experiments in zebrafish have further demonstrated that AGRP acts as a competitive antagonist at MC4R, while gene overexpression in transgenic models results in obesity and increased linear growth (57). Immunocytochemical studies revealed differential densities of AGRP and α-MSH terminals in discrete brain areas, in line with an α-MSH-independent AGRP activity (30). The evolutionary conservation of the AGRP inverse agonism and the MC4R constitutive activity
remains unexplored. We first approached this question by molecular cloning and pharmacological characterization of sea bass MC4R. We also studied the distribution of the MC4R-mRNA in the brain and the response of the central and peripheral (pituitary) melanocortin system to progressive fasting. Our results provide the first evidence demonstrating that AGRP acts as an inverse agonist at MC4R in non-mammalian species and suggest that the sea bass receptor is constitutively active. Furthermore, we show that the inverse agonism of AGRP, but not the competitive antagonism, depends on phosphodiesterase inhibition. Lastly, we provide physiological evidence supporting the constitutive activity of the sea bass MC4R.

EXPERIMENTAL METHODS

Animals and reagents
Male and females sea bass (*Dicentrarchus labrax*) were kindly supplied by Tinamenor (Santander, Spain). Prior to the experiments, the animals were maintained in 500-litre tanks supplied with continuously aerated running sea water for two months. Fish were hand fed at 9.00 am with a commercial diet (Proaqua Nutrición). Animals were anesthetized in 2-phenoxy-ethanol (0.1%) for 2 min before any manipulation and sacrificed by rapid decapitation. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/CEE) concerning the protection of experimental animals. Melanocortin peptides were from Bachem (Germany). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis MO, USA).

Molecular Cloning of Sea Bass MC4R
Genomic DNA isolated from blood was used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and degenerate primers designed against conserved regions of the known MC4R sequences. The following reaction conditions applied: 0.2 mM dNTP, 0.4 μM FwFish and RevFish primers, 1 X Taq DNA polymerase buffer, 1.5 mM MgCl2 and 0.5 units Taq DNA polymerase. The 5’ primer (FwFish) was a 20-mer with the sequence: 5’ TAYATCACCATMTTYTACGC 3’. The 3’ primer (RevFish) had the sequence 5’ TSAGVGTGATGGCKCCCTT 3’ (Fig.1). PCR products of about 300 base pair (bp) were isolated from low melting point (LMP) Nusieve GTG agarose gel (FMC) ligated into pGEM-T easy vector (Promega) and subsequently transformed into XLI-Blue E. coli. One clone that contained an insert of expected size was sequenced.

To resolve the 3’ end sequence of the sea bass MC4R cDNA, 3’ RACE-PCR was performed. For 3’ RACE PCR cDNA was synthesized using dT-adapter primer (5’ CAGTCGAGTCGACATCGA (T)17 3’). Two rounds of PCR amplified the 3’ end with adapter (5’ CAGTCGAGTCGACATCGA 3’) and sbMC4R_3’RACE_1 (5’ GTTGGTCTACATCAGCAT 3’) primers and then adapter primer and sbMC4R_3’RACE_1 (5’ GCATCCTGTTTATCATCTAC 3’, Fig. 1). After LMP purification a 532-bp fragment was subcloned into pGEM-T easy vector and sequenced. The 5’ region was cloned using the Genome Walker Kit (Clontech) and following manufacturer’s instruction. Specific primers for genome walking were sbMC4R_5Walker_1 primer (5’ GCAATGCTGCGATGCGCTTCATGTGCA 3’) for the first PCR and sbMC4R_5Walker_2 primer (5’ GTCCAGATGCTGCTGATGACCAACATTGC 3’) for the nested PCR (Fig.1). A 547-bp fragment was subcloned into pGEM-T easy vector and sequenced. Finally, the full coding region was amplified by PCR, using genomic DNA as template and the primers Hind-MC4R-Forward (5’ TATAAGCTTATGAACACCAGAGGCTC 3’) and XhoI-MC4R-Reverse (5’ TATCTCGAGGAGTCGCTGCTGCTC 3’, Fig.1). A 1036-bp DNA
fragment was subcloned into pGEM-T easy vector and sequenced on both strands. The nucleotide sequence of sea bass MC4R has been deposited with EMBL Nucleotide Sequence Database under accession numbers FM253127.

**RT-PCR and Southern blot analysis**

Total RNA was purified from fresh tissues (testis, ovary, intestine, fat, liver, white and red muscle, spleen, head kidney and kidney body proper, gill, dorsal skin, ventral skin, retina, heart, pituitary and brain) and treated with RQ1-DNase (Promega). Superscript II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming with oligo (dT)$_{12-18}$ (Invitrogen). The cDNA was subsequently used as template for touchdown PCR reactions with Taq DNA polymerase (Invitrogen) and specific primers. The 5’ primer was sbMC4R$_{3’}$RACE$_1$ (see above) and the 3’ primer (sbMC4R$_{cDNA}$$_{synthesis}$) had the sequence 5’ CAAGTGATCATGAGGATGA 3’ (Fig.1). Subsequently, PCR fragments were separated onto 1.2 % agarose gel, transferred by capillarity to Hybond-N nylon membrane (Amersham). Membranes were prehybridized for at least 3 hours in hybridization solution (50 % formamide, 6X SSPE, 0.5 % SDS, 5 X Denhardt’s solution and 10 mg/ml yeast tRNA type III, 1 X SSPE containing 150 mM NaCl, 1 mM EDTA, 9 mM NaH$_2$PO$_4$, pH = 7.4). Hybridization was carried out overnight in fresh hybridization solution containing 0.5 X 10$^6$ cpm/ml dCTP [α-$^{32}$P] at 42 °C. A 301-bp probe containing the central region of the sea bass MC4R coding region was used. Final washes were performed in 0.1 X SSPE at 65 °C. After 2 hours and 3 days of exposure at -80 °C films were developed and scanned. As internal control of the reverse transcription step, touchdown PCR for 18S RNA was carried out. Primer sequences were 18S$_{Forw}$ 5’ GCATGCCGGAGTCTCGTT 3’ and 5’ 18S$_{Rev}$ 5’ TGCATG GCCGTTCTTAGTTG 3’.
**Real time quantitative PCRs**

To evaluate gene mRNA levels, total RNA from individual hypothalamus or pituitary was treated with RQ1-DNase. One microgram was used as template for cDNA synthesis, which was primed with random hexaprimers (Invitrogen). Two microliters of cDNA (POMC and MC4R) or diluted cDNA (18S RNA) and primers (70 nM) were added to 7.5 μl of Sybr green PCR master mix (ABgene, Thermo Scientific, Spain) and the volume was adjusted to 15 μl with water. PCRs were carried out on an iCycler (Bio-Rad, Madrid, Spain). Data were analyzed with the ΔΔCt (cycle threshold) method. As internal control a fragment of the sea bass 18S RNA gene was amplified, using primers 18S_Forw and 18S_Rev primers (see above). Specific gene primers were as follow: sbMC4R_For_a: 5´ATGAACACCACACAGGGCTCA3´, sbMC4R_Rev_a 5’ ATAGCATCC TGTGGACGAGT 3´ for sea bass MC4R (Fig.1) and sbPOMC_Forw_1 5’ ATGTGTCTCTGTGTGGTTATTG 3’, sbPOMC_Rev1 5’ GCGACAGAGCTGGATACA 3’ for sea bass POMC (62).

**In situ hybridization**

In situ hybridization experiments were carried out as described previously. Animals were anaestheticized, then transcardially perfused with 50 ml of physiological saline solution (NaCl 0.65%) and subsequently perfused with the same volume of fixative containing paraformaldehyde (PAF, 4%) in phosphate buffer (PB, 0.1 M pH 7.4). After decapitation, the brains were removed, post-fixed overnight in the same fixative at 4°C, dehydrated, and embedded in Paraplast (Sherwood, St Louis, MO). Serial 6 μm cross sections were cut using a rotary microtome. One section every 200 μm was mounted on 3-aminopropyltriethoxysilane (TESPA)-treated slides and then air-dried at room temperature (RT) overnight. Three consecutive series covering the length of the sea bass brain were made. Two series were used
for hybridization with the sense and anti-sense probes. The last series was stained with cresyl-violet 0.1% for detailed identification of brain nuclei. Sections were stored at 4°C under dry conditions and used for hybridization within one week.

Before hybridization, sections were deparaffinized, rehydrated and post-fixed in 4% PAF for 20 minutes. Slides were then rinsed twice in PB for 5 minutes and treated with a Proteinase-K solution (20 μg/ml in 50 mM Tris-HCl, 5mM EDTA, pH = 8) for 5 minutes at RT. Slides were then washed in PB and post-fixed again in PAF for 5 minutes, subsequently rinsed in sterile water and acetylated in a triethanolamine (0.1 M, pH 8) / acetic anhydride solution. Sections were then dehydrated and dried at RT.

The entire coding region of the seabass MC4R receptor sequence was cloned into pGEM-T easy vector (Promega). Anti-sense and sense RNA probes were synthesized in vitro by linearizing the plasmid with NcoI and Sal I (Takara). In vitro transcription was carried out with SP6 or T7 RNA polymerase, respectively. Both sense and anti-sense probes were labelled with 10 μl of 35S-UTP (10 mCi / ml) using a riboprobe synthesis kit (Promega, Barcelona, Spain) as described by the manufacturer. After in vitro RNA synthesis, samples were treated with RQ1-DNase for 15 minutes at 37°C in presence of 50 units of RNAsin (Promega) and then incubated at -20°C for 3 hours with 10 μg/ml of yeast RNA type III in an 8% formamide solution. Probes were subsequently purified using Sephadex G50 columns. The two fractions containing the highest radioactivity level were pooled and precipitated in ethanol-sodium chloride at -20°C. The labelled probes were then stored at -20°C and used within one week.

The 35S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM DTT to obtain 2x10^5 cpm/μl. After 5 minutes incubation at 80°C, 35S-UTP riboprobes were diluted 1/10 (final concentration of probes, 10 mM DTT and 2x10^4 cpm/μl) in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA
(pH 8), 10% Dextran sulphate, 1x Denhardt’s solution and 0.5 μg/ml yeast RNA type III. Subsequently, 100 μl of hybridization solution was added to each pre-treated slide (see above), which were cover-slipped and incubated in a humidified chamber at 55°C overnight. The following day coverslips were removed by incubating slides in a solution containing 5x standard saline citrate buffer (SSC, 1x SSC containing 150 mM NaCl, 15 mM sodium citrate, pH 7), 10 mM DTT, for 30 minutes at 55°C. The slides were then rinsed in 2xSSC, 50% formamide, 10 mM DTT for 30 minutes at 65°C and three times immersed in NTE buffer (500 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5) for 10 minutes at 37°C. After RNAse treatment (20 μg / ml RNAse in NTE) for 30 minutes at 37°C, slides were rinsed three times in NTE buffer for 10 minutes at 37°C, once in 2x SSC, 50% formamide, 10 mM DTT for 30 minutes at 65°C, once in 2xSSC for 15 minutes at RT and twice in 0.1x SSC for 15 minutes at RT. Slides were finally dehydrated in increasing degrees of ethanol solutions containing 0.3 M ammonium acetate and dried at RT. After the hybridization process, slides were dipped in photographic emulsion (Amersham) and exposed under dry conditions at 4°C for 5 to 7 days, developed in Kodak D-19 and counter-stained with toluidine blue 0.02%. Anatomical locations were confirmed by reference to a brain atlas of sea bass (12, 16, 17).

**Peptide synthesis, purification and folding**

Zebrafish AGRP (Ac-83-127-NH2) was synthesized using Fmoc synthesis on an Applied Biosystems (Foster City, CA) 433A Peptide Synthesizer on a 0.25mmol scale. The synthesis was monitored using the SynthAssist version 2.0 software package. The peptide was assembled on a Rink-amide-MBHA resin and pre-activated Fmoc-Cys(trt)-OPfp was used. All amino acids and resins were purchased through NovaBiochem (San Diego, CA). HBTU was obtained from Advanced Chemtech (Louisville, KY). Fmoc deprotection was achieved using a 1% hexamethylenimine and 1% 1,8-Diazabicyclo[4.5.0]-undec-7-ene solution in
DMF. Deprotection was monitored by conductivity and continued until the conductivity level returned to the baseline, then synthesis resumed. Deprotection time ranged from 2.5-7 minutes. Coupling used 4 equivalents Fmoc-amino acid in HBTU/DIEA for all amino acids with the exception of pre-activated Cysteine. A 3-fold excess of Fmoc-Cys(Trt)-OPfp was dissolved in 1.5mL 0.5M HOAt/DMF with no DIEA for coupling. The peptides were N-terminal acetylated by reacting with 0.5M acetic anhydride in DMF for 5 minutes. Fully synthesized peptide resin was split into 3 reaction vessels, washed with DCM and dried. A solution of 8mL TFA containing 200μL each of TIS/EDT/liquefied Phenol (as scavengers) was added to each reaction vessel of dry peptide resin for 1.5h. The resin was filtered and washed with 1mL TFA and the combined filtrate and wash was then added to 90mL cold dry diethyl ether for precipitation. The precipitate was collected by centrifugation and the ether was discarded. The pellet was dissolved in 40mL 1:1 H₂O:Acetonitrile (0.1% TFA) and then lyophilized. The crude peptide was purified by RP-HPLC on C4 Vyda (Hesperia, CA) preparative columns. Fractions were collected and analyzed by ESI-MS on a Micromass (Wythenshawe, UK) ZMD mass spectrometer to confirm the correct molecular weight. Fractions containing the peptide as a major constituent were combined and lyophilized.

Air oxidative folding of zAgRP was accomplished by dissolving the unfolded peptide into folding buffer (2.0M GuHCl/0.1M Tris, 3mM GSH, 4 mM GSSG, pH 8) at a peptide concentration of .1mg/mL) and stirring for 14h. Folding was monitored by reversed phase (RP)-HPLC on a C18 analytical column, which revealed a single peak for the folded material that shifted to an earlier retention time than the fully reduced peptide and ESI-MS indicated a difference of 10 amu. The folded product was purified by RP-HPLC on a C18 preparative column and its identity confirmed as the fully oxidized product by ESI-MS (AGRP Ac-83-127-NH₂: 5287.1 calc. ave. isotopes 5288 amu obs.). Reinjecting a small sample of the
purified peptide on an analytical RP-HPLC column assessed purity of the peptide. Quantitative analysis of the peptide concentration was carried out by amino acid analysis at the molecular structure facility at UC Davis.

_Cell culture and transfection_

If not specifically indicated, HEK cells were transfected using a modified calcium phosphate transfection method (20) and grown in DMEM (Invitrogen) containing 10% foetal bovine serum (Invitrogen), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂ at 37°C.

_Galactosidase enzyme assay_

Galactosidase enzyme assays were performed as previously described (21). Briefly the medium was removed and 50 μl of lysis buffer containing 250 mM Tris-HCl pH=8 and 0.1 % Triton X-100 were added. After one round of freezing (-80 °C) and thawing, ten microlitres of the lysate were preserved for protein assays. Forty microliters of phosphate saline buffer containing 0.5 % BSA and 60 μl of substrate buffer (1mM MgCl₂, 10mM KCl, 5mM β-mercaptoethanol and 200 mg/ml o-nitrophenyl-β-D-galactopyranoside, ONPG) were added to the remaining lysate volume. The plate was incubated at 37 °C for 5h and the absorbance was read at 405 nm in a 96-well plate reader (Tecan). Measurements were normalized by the protein content determined using the BCA protein assay kit (Pierce).

_Pharmacological experiments_

A HEK-293 cell clone, stably expressing β-galactosidase under the control of vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP responsive elements (CRE, 21), was generated by co-transfection (50:1) of pCRE/β-galactosidase
plasmid (kindly supplied by Dr. R Cone, Vanderbilt University Medical Center) and the 
tgCMV/HyTK plasmid, which harbours a hygromycin resistance gene (65). Cells were 
selected in medium containing 400 μg/ml of hygromycin B (Invitrogen). β-galactosidase 
activity was tested after incubating resistant clones in 96-well plates (15,000 cells/well) 
with assay medium (DMEM medium + 0.1mg/ml bovine serum albumin, BSA + 0.1 mM 
isobutylmethylxanthine, IBMX) containing 10⁻⁶ M forskolin during 6 hours. The clone 
showing highest response to forskolin (Clon-Q) was selected for subsequent experiments. 
The full coding region of the sea bass MC4R was released from pGEM-T easy vector (see 
above) and subcloned into pcDNA3 (Invitrogen). Double stable clones expressing β-
galactosidase and sea bass MC4R were made by transfecting Clon-Q with the latter 
construct using G-418 selection (800 μg/ml). Clones were tested by incubating cells with 
MTII 10⁻⁶ M in the assay medium. The clone Q/9 was selected for the characterization of 
the activation profiles in response to several melanocortins (α-MSH, diacetyl-MSH, 
desacetyl-MSH, human ACTH, monkey β-MSH, zfAGRP, SHU9119 and HS024) in the 
absence of IBMX. The effect of zfAGRP on basal and MTII-stimulated MC4R activity 
was studied in both the presence and absence of the phosphodiesterase inhibitor. MC4R 
activation assays were performed in quadruplicate wells and repeated at least three times 
independently.

For saturation experiments, intact Q/9 cells were incubated in a final volume of 75 μl for 
2.5 h at 37 °C and carried out with serial dilutions of [¹²⁵I][Nle⁴, D-Phe⁷]α-MSH (NDP-
MSH). Non-specific binding was defined as the amount of radioactivity remaining bound 
to the intact cells after incubation in the presence of 10 μM unlabelled NDP-MSH.

*Effects of progressive fasting on melanocortin system*
To evaluate the effects of fasting on hypothalamic and pituitary gene expression, ten groups of 10 fish each [body weight (BW) = 117 ± 1.54 g] were adapted for one-week to individual 500-liter aquaria and fed *ad libitum* at 9.00 a.m. After this acclimation period, five groups were fed a same ratio whereas five others were fasted. One each fed or fasted groups was sampled at 12.00 (3 hours post-feeding for fed groups) at 1, 4, 8 and 15 and 29 days, respectively. Anesthetized fish were weighed and blood samples were obtained by puncture of the caudal vessels. Subsequently the fish were decapitated and the whole hypothalamus and pituitary dissected for immediate total RNA extraction. RNA samples were kept at –80°C in 75% ethanol until cDNA synthesis for quantitative PCRs (see above). Plasma was stored at -80 until assayed.

**Hormone measurements**

Plasma α-MSH was measured by radioimmunoassay according to (3).

**Data analysis and statistics**

Sequence comparisons and alignments were performed using ClustalX. A phylogenetic tree was derived using public domain CulstalX, which uses the Neighbor-Joining method on a matrix of distances. The membrane protein secondary structure was predicted using the Split 4.0 Server (http://split.pmfst.hr/split/4/). Receptor activation data were fitted using SigmaPlot software. In gene expression studies, specific mRNA levels were normalized as a ratio to 18S RNA. Statistical analysis was conducted by one-way analysis of the variance followed by Tukey’s multiple range test (p<0.05).

**RESULTS**
Molecular cloning of seabass MC4R

By means of RT-PCR and using degenerate primers designed against conserved regions of fish melanocortin receptor sequences, we cloned a 301 bp fragment showing high identity to the MC4Rs reported in other vertebrate species. The 3’extreme was resolved by 3’RACE PCR, whereas the sequence of the 5’ end was obtained by PCR screening of a partial genomic library. The full cDNA sequence was obtained by PCR amplification of genomic DNA. As with most MCRs, the coding region of the putative sea bass receptor does not contain introns. The cloned fragment contains an open reading frame of 981 bp that encodes a putative 327-amino acid protein with seven putative hydrophobic transmembrane domains (TMDs, Fig. 1, 2). Similar to other melanocortin receptors, the sea bass MC4R orthologue exhibits short extracellular (ECL) and intracellular (ICL) loops and shares cysteine residues at positions 258, 272 and 278 (sea bass numbering) which are fully conserved in all melanocortin receptors (Fig. 2 and http://www.gpcr.org). The deduced amino acid sequence displays potential N-glycosylation sites within the N-terminal domain at positions 2 and 15 and one additional site within the first extracellular loop (ECL, position 97, Fig. 1). This predicted ECL is considerably longer than the second and third ECLs that are only four amino acids long. Similarly, the third intracellular loop (ICL) is more extensive than the first and second predicted ICLs, which are only six amino acids long. In addition, sea bass MC4R shares the PMY motif in the second TMD that is conserved in most melanocortin receptors. The deduced amino acid sequence is 67% and 86% identical to human and pufferfish MC4R, but only 48, 40, and 63% identical to pufferfish MC1R, MC2R and MC5, respectively and 60 % identical to zebrafish MC3R. The identity is unequally distributed and the N-terminal extracellular domain displays the lowest identity level to other MC4Rs, including pufferfish MC4R. More than 30% of the divergence between sea bass and human MC4R orthologues
resides within the N-terminal domain, encoding only 14% of total protein length. A more
detailed comparison shows that the overall identity level of sea bass receptor to other MC4Rs
is highest (>94%) within the TMD2 and lowest in the TMD4 (<57%). The overall identity
ranged between 80 and 86% within the TMD3, TMD6 and TMD7 and between 75 and 77%
within the TMD1 and TMD5.

Peripheral and central distribution of MC4R mRNA

RT-PCR with specific primers targeting sequences within TMD4 and TMD6 of the sea bass
MC4R (Fig. 1) resulted in a band of the expected size of about 321 bp. The identity of the
band was confirmed by Southern blot hybridization with a sea bass MC4R probe including
the full coding region. Sea bass MC4R mRNA was easily detected in the retina, brain and
pituitary gland but very low levels were also distinguished in the liver, fat tissue, testis and
white muscle (Fig. 3A). No bands or hybridization signal for goldfish MC4R were obtained
in PCR reactions using spleen, gill, intestine, dorsal and ventral skin, red muscle, heart and
ovary cDNAs or water (control) as template (Fig. 3A). Inverse transcriptions and cDNA
quality were corroborated by PCR amplification of 18S RNA that yielded bands of expected
size in all reactions (Fig. 3B).

To further characterize neuronal expression of MC4R mRNA, we used the in situ
hybridization technique. Hybridization with sense MC4R-cRNA probes never generated
specific signals in the sea bass brain (data not shown), in support of the probe specificity.
Figure 4 schematically represents the distribution of MC4R mRNA within sea bass brain.
Cell groups expressing MC4R mRNA were detected in the following areas: telencephalon,
preoptic area, hypothalamus, ventral thalamus, tectum mesencephalic and rombencephalon.
The first MC4R-expressing neurons were localized in the caudal and rostral regions of the
dorsal (Vd) and ventral (Vv) part of the ventral telencephalon, respectively (Fig. 4A). At the
same level, some large cells expressing MC4R could be detected in the central region of the
dorsolateral part of the ventral telencephalon (Dlv, Fig. 4A). Just prior to the opening of the
preoptic recess, a conspicuous population of large MC4R-expressing cell bodies was found in
the central part of the ventral telencephalon (Vc, Fig. 4B). At this level, some scattered
MC4R-expressing perikarya were observed in the posterior part of the ventral telencephalon
(Vp Fig. 4B). Within the dorsal telencephalon, some MC4R-expressing cell bodies were
placed in the caudal area of the medial part (Dm, Fig. 4A).

Positive MC4R-labeled neurons were evident in several parts of the preoptic area. The most
rostral positive cells were those of the rostral pole of the parvocellular preoptic nucleus
(NPOpc), also named as anterior periventricular nucleus in other species, located at the level
of the preoptic recess entrance. MC4R-mRNA expressing neurons of the NPOpc are found at
periventricular positions where almost all neurons seem to be melanocortin dependent (Fig.
4B and 5A,B). Slightly more caudally, profuse MC4R mRNA expression was found in the
magnocellular neurons of the preoptic nucleus (PM, Fig. 4C and 5C,D). Progressively caudal,
MC4R positive perikarya were found in the periventricular anterior (NAPV) and in the
ventral pole of the preoptic area that coincides with the suprachiasmatic nucleus (NSC, Fig.
4C and 5C,D).

Within the tuberal hypothalamus, almost all divisions were found to profusely produce
MC4R mRNA. MC4R mRNA-expressing cell bodies were found in the dorsal (NLTd) and
ventral (NLTv) parts of the lateral tuberal nucleus (Fig. 4D and 5E,F). In the NLT, MC4R
neurons lined the third ventricle and most periventricular neurons appeared to make contact
with the ventricular wall (data not shown). More caudally, a profuse population of MC4R-
mRNA expressing cell bodies were located in the dorsal (NRLd) and ventral (NRLv) parts of
the lateral recess nucleus (Fig. 4E and 5E, F, G, H). At this level, a distinct population of
MC4R-mRNA expressing cells is placed in the medial part of the diffuse nucleus of the
inferior lobe (NDLIm, Fig. 4E, F and 5G, H). Some MC4R producing cells were also observed in the medial area of the lateral tours (TLa, Fig. 4F). MC4R-mRNA expressing cells also coat the entire rostro-caudal extension of the lateral recess forming the lateral part of the lateral recess nucleus (NRLl, Fig. 4F, G and 5G, H).

In the thalamus, MC4R mRNA-expressing neurons were restricted to the ventromedial nucleus of the ventral thalamus (Fig. 4D, 5I-J). A profuse expression was further found in the outer layer of the periventricular gray zone (PGZ) of the mesencephalic tectum (Fig. 4D-G). Finally, in the rombencephalon, MC4R producing cells were placed in the superior reticular nucleus of the reticular formation (RS, Fig 4G, 5K-L). No MCR4-mRNA expression was detected in the pituitary by in situ hybridization.

**Fasting effects on melanocortin system of the sea bass**

Quantitative PCR did not yield significant differences in hypothalamic POMC- and MC4R-mRNA expression levels when fed and fasted animals were compared after 1, 4, 8, 15 and 29 days of fasting (Fig. 6A). Similarly, no differences in pituitary POMC expression (Fig. 6A) or plasma MSH levels (Figure 6B) were detected after progressive fasting.

**Binding and activation by melanocortin analogs**

For pharmacological and functional characterization of the sea bass MC4R, the coding region was ligated into pcDNA3 and stably expressed in HEK 293 cells already producing β-galactosidase under the control of cAMP responsive elements. Saturation experiments displayed a single saturable site for [125I]-NDP-MSH (Fig. 7A) and that the receptor binds this agonist in a similar manner to that of the human, zebrafish abnd goldfish MC4R (14). The sea bass MC4R is not activated by potential melanocortin antagonists such as SHU9119, HS024 or zfAGRP. However, sea bass MC4R is positively coupled to the cAMP-signaling
pathway in response to diacetyl-MSH with a half-maximal effective concentration (EC\textsubscript{50}) of 0.094 nM. Sea bass MC4R activation by α-MSH and monkey β-MSH showed an EC\textsubscript{50} of 0.822 nM and 3.333 nM, respectively, whereas effective concentration increased to 18.8 nM and 15.13 nM when cells were incubated with human ACTH or desacetyl-MSH (Fig 7). Sea bass MC4R was also activated by the non-selective melanocortin agonist MTII (EC\textsubscript{50}= 0.31 nM). However, MTII-stimulated cAMP intracellular accumulation decreased by co-incubation with 1 μM of HS024 (EC\textsubscript{50}=17 nM), SHU9119 (EC\textsubscript{50}=120 nM) and zfAGRP (EC\textsubscript{50}=85 nM; Fig. 8). When a phosphodiesterase inhibitor (IBMX) was added to the medium, the response of the reporter gene to the MTII incubation increased (EC\textsubscript{50}=0.01 nM, Fig. 9). However, the incubation of sbMC4R-expressing HEK cells with AGRP in the presence of IBMX sharply decreased the basal activity of the receptor as an inverse agonist would (IC\textsubscript{50}= 1.14 nM). Under these conditions, AGRP also decreased MTII-stimulated cAMP production (EC\textsubscript{50}=9.9 nM) as a competitive antagonist would.

**DISCUSSION**

The present study demonstrates that AGRP acts as an inverse agonist at sea bass MC4R. However, the inverse agonism, but not the competitive antagonism, is dependent on the presence of a phosphodiesterase inhibitor in the culture medium. Our results demonstrate that sea bass MC4R is profusely expressed within food-intake controlling pathways of the fish brain. However, the activity of the melanocortin system during progressive fasting does not depend on the hypothalamic/pituitary POMC and MC4R expression, which suggests that sea bass MC4R is constitutively activated and regulated by AGRP binding. Sequence comparisons show that the cloned receptor in the sea bass displays higher identity to pufferfish MC4R than to other fish MCR. Both pufferfish and zebrafish MC4Rs have
previously been shown to display strong phylogenetic relationships with their respective mammalian counterparts (40, 56). Our phylogenetic analysis supports that this new gene is an MC4R orthologue (data not shown). In rodents, MC4R expression is restricted to the central nervous system (47). Studies in chicken (60) and goldfish (14) have demonstrated that MC4R is also expressed in the peripheral tissues, and this suggesting a participation of the receptor in the peripheral function of the melanocortin peptides (14). In the sea bass, MC4R expression was limited to the neural tissue, as occurs in mammalian models, with some residual expression in the adipose tissue, white muscle and testis. A high expression level of sea bass MC4R was also detected in the pituitary gland by RT-PCR, although in situ hybridization studies were unable to demonstrate MC4R-mRNA producing cells in the gland. This discrepancy may be accounted by hypothalamic contamination of the hypophyseal tissue during the pituitary dissection or by differences in technique sensitivity.

To further understand the regulation of the central melanocortin signaling and its involvement in the circuits controlling food intake, we studied MC4R-mRNA distribution in sea bass brain by in situ hybridization. Receptor transcripts were restricted to the telencephalon, preoptic area, ventral thalamus, hypothalamus, optic tectum and rombencephalon. So far, no studies on central α-MSH/AGRP distribution in the sea bass were published but MC4R-mRNA distribution is in perfect agreement with previous studies on the AGRP (30) and α-MSH-like immunoreactive (ir) fiber distribution in the teleost brain (2, 30, 51). In this group of fish, POMC and AGRP are expressed in different populations of the lateral tuberal nucleus (13, 15, 30). In zebrafish, both AGRP and POMC neurons project dorsally to innervate several thalamic areas, including the ventromedial nucleus where we have demonstrated MC4R expression (30). The main α-MSH/AGRP fiber aggregation passes through the caudal region of the preoptic area (anterior periventricular nucleus, NAPv and suprachiasmatic nucleus, NSC) to densely accumulate at the lateral aspect of the magnocellular preoptic nucleus (PM).
All nuclei are melanocortin responsive areas expressing MC4R in sea bass brain. The PM is the only nucleus of the zebrafish brain where denser AGRP innervation was detected when compared to α-MSH-ir fibers. Interestingly, MC4R receptors in the goldfish PM are found in the lateral aspect of the nucleus and this perfectly matches the lateral aggregation (14) of α-MSH/AGRP fibers reported for zebrafish (30). Our results suggest that most of the magnocellular cells i.e. lateral and periventricular cells, will be responsive to melanocortin peptides, via MC4R in the sea bass. Some of these cells in the preoptic nucleus of teleost fish also produce corticotrophin-releasing hormone (CRF, 1), which is thought to be a mediator of the appetite-suppressing effects of stress in fish (6). A recent report has demonstrated that the central anorectic effects of α-MSH in goldfish are mediated, in part, via CRF and describes the presence of melanocortin terminals in CRF-producing neurons (44). Both AGRP and α-MSH neuronal systems also display an abundance of fibers within the rostral preoptic area that extend around the anterior commissure into the postcommissural (Vp) and supracommissural (Vs) nucleus of the zebrafish forebrain. Our present results demonstrate that MC4R is highly expressed within the rostral preoptic area including the parvocellular (NPOpc) and anteroventral (NPOav) pars of the parvocellular preoptic nucleus. A heavy innervation of α-MSH and, to a lesser extent, for AGRP was also detected in the dorsal (Vd), ventral (Vv) of the ventral telencephalon in zebrafish. All these areas have been demonstrated to be MC4R-producing areas of the sea bass brain. Additionally, we have demonstrated the presence of a conspicuous MC4R-expressing population within the caudal area of the central part of the ventral telencephalon (Vc). Previous studies in sea bass have demonstrated that cells in the Vv and Vc distinctly produce NPY (10, 11) suggesting the telencephalic interaction between both peptidergic systems. Only α-MSH-ir, but no AGRP-ir, fibers are found in abundance in the medial zone of the dorsal area of the zebrafish telencephalon (Dm),
where we have described MC4R expression in the sea bass. Ascending α-MSH- and AGRP-projections have also been described in the periventricular gray zone and reticular formation of zebrafish (30), where we here report MC4R expression in the sea bass brain. Descending α-MSH/AGRP pathways in zebrafish display a high and moderate immunoreactivity density of α-MSH and AGRP, respectively, just caudally to the ventral hypothalamus and projecting dorsally through the caudal hypothalamus into the semicircular torus. Some positive terminals are also evident in the dorsal hypothalamus and inferior lobe. Similar results were reported for goldfish, in which the density of the ACTH-like fiber network is maximal in the ventral hypothalamus, particularly in the median part of the lateral tuberal nucleus towards areas around the lateral and posterior hypothalamic recess (51). In the sea bass, MC4R is profusely expressed within the whole extension of the lateral tuberal nucleus as well as in the inferior lobe including the nucleus of the lateral recess and semicircular torus. In summary, the localization of the sea bass MC4R is in good agreement with the reported distribution of α-MSH and AGRP terminals in other teleosts. Morphological comparisons between fish and mammalian brain are complex. Detailed distribution of MC4R expression has only been described in the murine species (4, 37, 41, 47) and goldfish (14). The distribution of MCR in sea bass brain is similar to the pattern reported in goldfish but the number of discrete structures expressing MC4R seems to be lower than that reported in rat. This is especially patent within the posterior and midbrain, suggesting the acquisition of new central functions for MC4R in the mammalian lineage. Within the forebrain, the MC4R expression distribution seems to be well conserved. In rat, MC4R is profusely expressed in the hypothalamus including anteroventral periventricular, ventromedial preoptic, median preoptic, paraventricular, dorsomedial and arcuate nuclei. The preoptic and hypothalamic regions are highly variable among vertebrates, but the lateral tuberal nucleus is thought to be the teleostean homologue of the mammalian arcuate nucleus.
In addition, 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 (7). 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 to be involved in the integration of the viscero-sensory information and elaboration of coordinated responses modifying the energy balance in fish (64). Therefore, our results support a role for MC4R in the main areas controlling food intake in fish.

In mammals, the melanocortin system is a key regulator of body weight. Inactivation of the central MC4R leads to profound obesity, as does the hypothalamic over-expression of the endogenous antagonists, agouti and AGRP (22). Our previous studies have also demonstrated the participation of the melanocortin system in the regulation of food intake in fish, via central MC4R. Therefore, MTII inhibits feeding in fasted animals, whereas the selective MC4R antagonist, HS024, stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulated AGRP production in the lateral tuberal nucleus (13-15). This strongly suggests that AGRP has α-MSH-independent functions at fish MC4R, as reported in the mammalian systems (33, 50, 59). To test this hypothesis we developed a HEK-293 cell line stably expressing sea bass MC4R and a reporter gene under the control of a promoter carrying tandem repetitions of cAMP-responsive elements. We also synthesized zebrafish AGRP (83-127) using Fmoc synthesis. It has been previously demonstrated that AGRP 83-132 is the active form in the mammalian central nervous system (24). Cell incubation with mimetic (MTII) and endogenous (α-MSH) melanocortin peptides stimulated galactosidase activity within the nanomolar range. Unlike in mammals, the α-MSH-induced activation was greater than that produced by β-MSH. The sea bass MC4R was stimulated with monkey β-MSH, which is only 55% identical to sea
bass β-MSH, so no definitive conclusion can be drawn. Diacetyl-MSH was the most potent melanocortin to stimulate sea bass MC4R-induced galactosidase activity, showing an EC$_{50}$ value about 8 times lower than found for α-MSH. However, the EC$_{50}$ value of the cAMP mediated-galactosidase activity increased 160 and 200 times when cells were incubated with desacetyl-MSH and ACTH, respectively. This demonstrates that sea bass MC4R is activated with lower efficiency by human ACTH than MSHs and suggests that the acetylation level is critical for the activation of the sea bass receptor. Acetylation plays a crucial role in the biological activity of neuropeptides. In mammals, desacetyl-MSH is more abundant than α-MSH in the brain (9) but, in contrast to our results, it activates mouse MC4R more efficiently than the acetylated peptide (48). However, it has also been reported that α-MSH is more efficient than desacetyl-MSH when activating MC4R in absence of IBMX, suggesting that pretreatment of cells with this phosphodiesterase inhibitor masks the signalling differences between both peptides (31). Accordingly, the intracerebroventricular administration of α-MSH more potently inhibits food intake than desacetyl-MSH (31). Our experiments support the masking effect of the IBMX treatment since they were performed in the absence of the inhibitor. All three MSH isoforms have been described in fish pituitary (27) and the regulation of MSH acetylation seems to be an important mechanism for the background adaptation (3). There is no information about the regulation of isoforms acetylation in the fish hypothalamus but our results support a possible regulation of MSH acetylation in the same organ.

None of the synthetic (HS024 and SHU9119) or endogenous (AGRP) MC4R antagonists had any effect on sea bass MC4R-mediated galactosidase activity but all three peptides acted as competitive antagonists at the sea bass receptor activity in the absence of IBMX. Both SHU9119 and AGRP were more efficient than HS024 when MTII-stimulated galactosidase activity decreased. The data show that all three antagonists are suitable for physiological
studies in sea bass. As expected, when IBMX was added to the incubation medium, the EC_{50} value of the MTII-induced galactosidase activity decreased. Therefore, the use of IBMX increases the sensitivity of the assays to evaluate the activation of positively cAMP-coupled receptors. Under the above conditions, AGRP competitively antagonized the effects of MTII on sea bass MC4R activity in a similar manner to that observed without IBMX but severely decreased the basal activity of the sea bass MC4R, just as an inverse agonist should do. These results were corroborated by incubation of cells with MTII and AGRP in the presence of IBMX. At the lowest doses of MTII (10^{-10} - 10^{-9} M) the addition of 10^{-6} M AGRP kept the sea bass MC4R activity at around 30% of the basal level. Competitive antagonism by AGRP on fish MC4R was reported earlier (57) but the agonistic action is newly reported here. However, we have demonstrated that the AGRP inverse agonism depends on treatment of the cells with IBMX. To the best of our knowledge, all assays reporting AGRP effects on melanocortin receptors involved IBMX-pretreated cells. Only one study has reported the effect of phosphodiesterase inhibitor on α-MSH-induced receptor activation, showing IBMX-dependent differences in the intracellular signalling (31). These authors defended the view that the measurement of cAMP after pre-treatment of cells with IBMX does not truly reflect the melanocortin receptor signalling. Therefore, we thought initially that the inverse agonism of AGRP on melanocortin receptors could be an artefact of the use of IBMX in the incubation medium. IBMX modifies the intracellular levels of cAMP through degradation but also interferes with their binding to the target enzyme, cAMP-dependent protein kinase A (PKA). Therefore, IBMX increases the basal activity of PKA and inhibits the activation promoted by cAMP (62). However, we developed the same experiment using HEK cells stably overexpressing sea bass MC5R and no effects of AGRP by itself or reducing MTII-induced galactosidase activation (competitive antagonism) were observed with or without IBMX treatment (unpublished results, Sánchez E, Rubio VC and Cerdá-Reverter JM). These results
demonstrate that the AGRP agonism is specific for the MC4R and strongly depends on the phosphodiesterase inhibition in vitro. A possible explanation for these results would involve an AGRP-induced conformational change of the MC4R, leading to the activation of the intracellular phosphodiesterase system. This activation would reduce the cAMP intracellular levels imposed by the constitutive activity of the MC4R, thus decreasing the activation of the PKA. Overexpression in vitro of the MC4R, but not MC5R, could dramatically increase adenylyl cyclase activity because of its constitutive nature. Therefore, this over-activation of adenylyl cyclase could induce high levels of phosphodiesterase activity and this could explain why the effects of AGRP in vitro can only be observed after proper inhibition of phosphodiesterase activity. Alternatively, phosphodiesterase was shown to interact with arrestins to regulate the branching of signalling from G-coupled receptors (23). Phosphodiesterases bind arrestins and are recruited to the occupied receptors, limiting the cAMP accumulation in localized domains. The phosphodiesterase binding to arrestins has been also proposed to control the PKA-phosphorylation of the β-adrenergic receptor regulating the efficiency of the receptor switch from Gs- to Gi-proteins. This exchange could decrease the rate of cAMP generation because Gi activation inhibits adenylyl cyclase and couples the receptor to Gi-linked pathways (5). Interestingly, it has been reported that AGRP induces arrestin-mediated endocytosis of the human MC4R (8) which exhibits a constitutive traffic in hypothalamic neurons (46). Intracellular signalling pathways through which melanocortin receptors exert their effects are not well understood. Activated melanocortin receptor binds to Gs and this leads to stimulation of adenylyl cyclase while increased concentrations of intracellular cAMP activate PKA. Active PKA initiates the transcription of new genes by phosphorylation and activation of cAMP-responsive element binding protein (CREB). However, other intracellular signalling pathways, including mitogenic activated protein (MAP) kinase (53) and inositol/Ca2+ (49) and probably adenosine monophosphate
activated-protein kinase (AMPK) pathways (45) have also been reported to be involved in MC4R intracellular signalling. A very recent paper has demonstrated that the disruption of regulatory subunit RIIβ of PKA in agouti lethal yellow mice partially reverses obesity, possibly by increasing kinase activity (25). This suggests that the agouti-induced metabolic syndrome is mediated by down-regulation of the PKA, probably mediated by reduction of cAMP levels.

Our results demonstrate that the competitive antagonism of AGRP is not dependent on the presence of IBMX, suggesting that both AGRP agonism and antagonism in the sea bass MC4R system are mediated through different intracellular signalling pathways with differential sensitivities to phosphodiesterase inhibitors. More experiments on intracellular signalling pathways of the MC4R must be made to corroborate this hypothesis, which opens up new targets for the treatment of melanocortin-induced metabolic syndrome.

The AGRP-mediated decrease of basal galactosidase activity in HEK cells expressing sea bass MC4R suggests that the receptor may be constitutively activated. The constitutive activity of the mammalian MC4R has already been demonstrated in vitro and in vivo (see introduction for references). It has been proposed that the N-terminal domain functions as a tethered intramolecular ligand preserving the constitutive activity of the receptor. This constitutive activity of the MC4R is supposed to impose an inhibitory tone on food intake that is regulated by AGRP binding (22). Therefore mutations like R18C within the N-terminal domain that do not impair the binding of agonist but can drastically reduce the constitutive activity which leads to obesity in humans. Interestingly, sea bass MC4R exhibits an arginine (R) residue N-terminally flanked by an asparagine residue (N) in a similar position to that observed in humans. We do not know if the putative constitutive activity of the sea bass MC4R can operate in vivo but the physiological data reported here support the idea. Progressive fasting does not increase hypothalamic POMC expression in the sea bass as
previously observed in the goldfish (15) and zebrafish (58), suggesting a limited production of agonist during negative energy balance states. In the latter species, fasting dramatically induces hypothalamic AGRP expression (58), suggesting a downregulation of MC4R signalling in the absence of a decrease of agonist production, through AGRP binding. In this paper, we further explore two additional levels that can potentially regulate the melanocortin signalling in the absence of variations of hypothalamic POMC expression. These levels included the down-regulation of the hypothalamic receptor expression and the peripheral production of melanocortins. Similar to that reported in barfin flounder (*Verasper moseri*, 39), the hypothalamic expression of MC4R does not change during progressive fasting. It has been demonstrated that peripherally administered melanocortins can mediate variations of the central melanocortin activity via MC4R (34). However fasting did not induce any change in the pituitary POMC expression or α-MSH plasma levels in the sea bass, suggesting that peripheral melanocortins are not responsible for the downregulation of melanocortin signalling via brain receptors during progressive fasting. Differential regulation of POMC processing or N-acetylation of MSH during fasting, potential regulatory levels of the melanocortin signalling in the absence of POMC production, remain unexplored and are under study in our laboratory.

PERSPECTIVES AND SIGNIFICANCE

Central melanocortin signaling plays a key role in the regulation of energy balance in mammals by exerting an inhibitory tone on food intake and by stimulating energy expenditure. Most actions on energy balance are mediated via central MC3R and MC4R. The later receptor displays a constitutive activity regulated by AGRP binding which may work as competitive antagonist or inverse agonist. Our results demonstrate that fish MC4R also exhibits constitutive activity and that MSH/AGRP/MC4R interaction works in a similar way
to that reported in mammalian systems. It supports the evolutionary conservation of the
AGRP/MC4R interactions as well as the role of the melanocortin system in the control of
energy balance. The absence of regulation in central and peripheral POMC and MC4R
expression during progressive fasting supports a physiological role for inverse agonism in
fish. In addition, our results show that the inverse agonism, but not the competitive
antagonism, depends on inhibition of the intracellular phosphodiesterase system, which
suggests that both systems operate through different intracellular pathways in the regulation
of CREB protein activation. The functional homology between mammalian and fish
melanocortin system permits speculate that the mammalian AGRP/MC4R interaction is also
dependent on the intracellular phosphodiesterase system. This view opens up new targets for
the treatment of obesity in humans. Our future research will focus the study of activity of the
different intracellular pathways after AGRP stimulation of the HEK cells stably expressing sb
MC4R in presence or absence of phosphodiesterase inhibitors.

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**Figure 1.** Nucleotide and deduced amino acid sequence of sea bass melanocortin receptor 4 (MC4R). Nucleotide and amino acid sequence numbers are indicated on the left and right sides, respectively. Grey boxed amino acids indicate putative transmembrane domains
predicted using Split 4.0 Server (http://split.pmfst.hr/split/4/). Open boxes frame tripeptide sequences that conform with the consensus sequence for N-linked glycosylation sites. Oval frames enclose possible phosphorylation sites. Sequences of primers used in RT-PCR amplification are underlined. Sea bass MC4R sequence accession number FM253127.

**Figure 2.** Alignment of melanocortin receptor amino acid sequences from human. Sea bass MC4R sequence is highlighted in bold letters. Dots indicate amino acids identical to the top sequence. Dashes were introduced to improve alignment. Grey boxes show putative transmembrane domains predicted with Split 4.0 Server (see Fig. 1). Arrows indicate fully conserved cysteine residues in all melanocortin receptors.

**Figure 3.** Distribution of sea bass MCR4 mRNA expression in different tissues as revealed by RT-PCR assay followed by Southern blot hybridization. (A) Phosphoimaging screen showing a Southern blot analysis of the sea bass receptor following RT-PCR assays. (B) Ethidium bromide-stained agarose gels showing RT-PCR amplifications of sea bass 18S RNA.

**Figure 4.** Schematic drawings of the successive rostrocaudal transverse sections of the sea bass brain showing the distribution of MC4R-expressing perikarya (black dots) as revealed by in situ hybridization. Number of dots roughly represents expression density but quantitative studies were not done. Scale bar = 1 mm. Abbreviations: (AC) anterior commissure, (AP) accessory pretectal nucleus, (CCe) corpus of the cerebellum, (CP) central posterior thalamic nucleus, (De2) subdivision 2 of the central part of the dorsal telencephalon, (Dld) laterodorsal part of the dorsal telencephalon, (Dlv2) subdivision 2 of the lateroventral part of the dorsal telencephalon, (Dm2, Dm3 and Dm4) subdivision 2, 3 and 4
of the medial part of the dorsal telencephalon, (Dp) posterior part of the dorsal telencephalon, (DP) dorsal posterior thalamic nucleus, (DT) dorsal tegmental nucleus, (HaCo) habenular commissure, (LC) locus coeruleus, (LT) lateral thalamic nucleus, (nIV) trochlear nerve nucleus, (NAPv) anterior periventricular nucleus, (NAT) anterior tuberal nucleus, (NC) cortical nucleus, (NCLI) central nucleus of the inferior lobe, (NDLII) lateral part of diffuse nucleus of the inferior lobe, (NDLIm) medial part of diffuse nucleus of the inferior lobe, (nFR) nucleus of the fasciculus retroflexus, (NGa and NGp) anterior and posterior parts of the glomerular nucleus, (NGT) tertiary gustatory nucleus, (NHd) dorsal part of the habenular nucleus, (NHv) ventral part of the habenular nucleus, (NLTd) dorsal part of the lateral tuberal nucleus, (NLTi) inferior part of the lateral tuberal nucleus, (NLTl) lateral part of the lateral tuberal nucleus, (NLTm) medial part of the lateral tuberal nucleus, (NLV) lateral valvula nucleus, (nMLF) nucleus of the medial longitudinal fasciculus, (NPC) central pretectal nucleus, (NPGc) commissural part of the preglomerular nucleus, (NPGl) lateral part of the preglomerular nucleus, (NPGm) medial part of the preglomerular nucleus, (NPOav) anteroventral part of the parvocellular preoptic nucleus, (NPOpc) parvocellular part of the parvocellular preoptic nucleus, (NPPv) posterior periventricular nucleus, (NPT) posterior tuberal nucleus, (nPVO) nucleus of the paraventricular organ, (NRLd) dorsal part of the lateral recess nucleus, (NRLl) lateral part of the lateral recess nucleus, (NRLv) ventral part of the lateral recess nucleus, (NRP) posterior recess nucleus, (NSC) suprachiasmatic nucleus, (NSV) nucleus of the vascular saccus, (OT) optic tectum, (P) pituitary, (PC) paracommissural nucleus, (pgd) dorsal periglomerular nucleus, (PGZ) periventricular gray zone of the optic tectum, (PM) magnocellular preoptic nucleus, (PPd) dorsal periventricular pretectal nucleus, (PPv) ventral periventricular pretectal nucleus, (PVO) paraventricular organ, (PSm) magnocellular superficial pretectal nucleus, (PSp) parvocellular superficial pretectal nucleus, (RS) superior reticular nucleus, (SV) saccus
vasculosus, (TLa) nucleus of the lateral torus, (TLo) nucleus of the longitudinal torus, (TPp) periventricular nucleus of the posterior tuberculum, (TS) semicircular torus, (VCe) valvula cerebelli, (Vd) dorsal part of the ventral telencephalon, (Vi) inferior part of the ventral telencephalon, (Vl) lateral part of the ventral telencephalon, (VL) ventrolateral thalamic nucleus, (VM) ventromedial thalamic nucleus, (Vp) postcommissural part of the ventral telencephalon, (Vs) supracommissural part of the ventral telencephalon, (Vv) ventral part of the ventral telencephalon.

**Figure 5.** Brightfield (A, C, E, G, I, K) and darkfield (B, D, F, H, J, L) micrographs of transverse sections of the sea bass brain from rostral preoptic area (A) to rombemcephalon (K). *(A, B)* Positive MCR4 neurons placed within the rostral divisions of the preoptic area, parvocellular (NPOpc) and anteroventral parts of the parvocellular preoptic nucleus (NPOav). *(C, D)* MC4R-expressing neurons at the magnocellular preoptic nucleus (PM) and anterior periventricular nucleus (NAPv). *(E, F)* MC4R neurons at the tuberal hypothalamus; dorsal part of the lateral tuberal nucleus (NLTd), ventral part of the lateral tuberal nucleus (NLTv) and ventral part of the lateral recess nucleus (NRLv). *(G,H)* MC4R expression at the hypothalamic inferior lobe; medial part of the diffuse nucleus of the inferior lobe (NDLIm), dorsal part of the lateral recess nucleus (NRLd) and NRLv ventral part of the lateral recess nucleus. *(I, J)* micrography showing MC4R expression within ventromedial thalamus (VM). *(K, L)* MC4R expression within superior part of the reticular formation (RS) and possibly including neurons from the locus coeruleus. Scale bar = 200 μm.

**Figure 6.** Effects of progressive fasting on pituitary and hypothalamic POMC, pituitary MC4R expression (A) and plasma α-MSH levels (B). Gene expression levels were expressed as ratio specific mRNA/18S RNA. Data are mean ± SEM (n=8).
Figure 7. A) Saturation curve were obtained with $[^{125}I]^{[\text{Nle}^4, \text{D-Phe}^7]}\alpha$-MSH and the figure shows specific binding (†) and binding in the presence of 10 µM cold $[^{125}I]^{[\text{Nle}^4, \text{D-Phe}^7]}\alpha$-MSH (Δ). Lines represent the computer-modelled best fit of the data assuming that ligands bound to one-site. B) Pharmacological properties of melanocortin agonist and antagonist at human embryonic kidney 293 cells (HEK-293) stably expressing both sea bass MC4R and a cAMP-responsive β-galactosidase reporter gene in absence of phosphodiesterase inhibitors (IBMX). Data were normalized to protein levels and expressed as percentage of the basal levels. Experiments were performed using quadruplicate data points and repeated at least three independently.

Figure 8. Effects of synthetic (Shu9119 and HS024) and endogenous antagonist (AGRP) on MTII-stimulated galactosidase activity in HEK-293 cells stably expressing both sea bass MC4R and a cAMP-responsive β-galactosidase reporter gene in absence of phosphodiesterase inhibitors (IBMX). Data were normalized to protein levels and expressed as percentage of the basal levels. Experiments were performed using quadruplicate data points and repeated at least three independent times.

Figure 9. Effects of phosphodiesterase inhibitors (IBMX) on pharmacological properties of agonist (MTII) and antagonist (AGRP) at human embryonic kidney 293 cells (HEK-293) stably expressing both sea bass MC4R and a cAMP-responsive β-galactosidase reporter gene. Data were normalized to protein levels and expressed as percentage of the basal levels. IBMX alone induced an increase in the β-galactosidase activity of 131% ± 8.57 when compared to the basal enzyme activity in absence of the phosphodiesterase inhibitor.
Experiments were performed using quadruplicate data points and repeated at least three independent times.
**Graph A**

- **X-axis**: Time (days)
- **Y-axis**: Normalized mRNA expression (relative units)
- **Legend**:
  - MC4R
  - POMC Hypothalamus
  - POMC Pituitary
- **Data Points**: Fed and Fasted conditions are compared at various time points (1, 4, 8, 15, 29 days).

**Graph B**

- **X-axis**: Time (days)
- **Y-axis**: MSH plasma levels (pg/ml)
- **Legend**: Fed and Fasted conditions are compared at various time points (1, 4, 8, 15, 29 days).
TABLE 1

EC50-IC50 and Kd Values from Respective Gene Reporter Activation and Saturation Curves for Melanocortin Analogs on Seabass

MC4R Expressed in HEK-293 Cells.

<table>
<thead>
<tr>
<th></th>
<th>[125I]-NDP-MSH (nM)</th>
<th>Desacetyl-α-MSH (nM)</th>
<th>Diacetyl-α-MSH (nM)</th>
<th>MTII (nM)</th>
<th>Monkey β-MSH (nM)</th>
<th>Human ACTH (nM)</th>
<th>AGRP (nM)</th>
<th>MTII + HS024(1μM) (nM)</th>
<th>MTII + SHU9119(1μM) (nM)</th>
<th>MTII + AGRP (1μM) (nM)</th>
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