Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (Dicentrarchus labrax)

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Sánchez E, Rubio VC, Thompson D, Metz J, Flik G, Millhauser GL, Cerdá-Reverter JM. Phosphodiesterase inhibitor-dependent inverse agonism of agouti-related protein on melanocortin 4 receptor in sea bass (Dicentrarchus labrax). Am J Physiol Regul Integr Comp Physiol 296: R1293–R1306, 2009. —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, whereas inactivating mutations are a genetic cause of obesity. The binding of agouti-related protein (AGRP) reduces not only 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 ortholog. 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 signaling pathways, a view that opens up new targets for the treatment of melanocortin-induced metabolic syndrome.

melanocyte-stimulating hormone; proopiomelanocortin; constitutive activity; 3-isobutyl-1-methylxanthine; obesity

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; Ref. 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 signaling 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, such as naturally occurring agonists, agouti and agouti-related proteins (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 signaling at MC3R and MC4R (56).

A body of evidence substantiates an essential role for central signaling 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 overexpressing 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 coadministration of AGRP (53), which is endogenously released from the neuropeptide Y neurons in the arcuate nucleus (32). Recent experiments have demonstrated that AGRP is processed intracellularly by proprotein convertases into the active form AGRP33–123 (24). AGRP and MSH terminals colocalize 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 a 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, since 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 (22). Experiments in zebrafish have further demonstrated that AGRP acts as a competitive antagonist at MC4R, whereas 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 nonmammalian 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. Last, we provide physiological evidence supporting the constitutive activity of the sea bass MC4R.

**EXPERIMENTAL PROCEDURES**

**Animals and reagents.** Male and females sea bass (Dicentrarchus labrax) were kindly supplied by Tinamenor (Santander, Spain). Before the experiments, the animals were maintained in 500-liter tanks supplied with continuously aerated running sea water for 2 mo. Fish were hand fed at 9:00 AM with a commercial diet (Proaqu Nutrición). Animals were anesthetized in 2-phenoxy-ethanol (0.1%) for 2 min before any manipulation and were killed by rapid decapitation. All experiments were approved by the bioethics committee of the Scientific Investigation Superior Council and carried out in a registered installation (code 36271-42-A) of the Agricultural, Fisheries and Alimentation Ministry, Institute of Aquaculture from Torre de la Sal, in accordance with the principles published in the European animal directive (86/609/CEE) concerning the protection of experimental animals. Melanocortin peptides were obtained from Bachem (Germany). Unless otherwise indicated, all reagents were purchased from Sigma (St. Louis, MO).

**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 5’ and 3’ primers, 1× Taq DNA polymerase buffer, 1.5 mM MgCl2, and 0.5 units of Taq DNA polymerase, where the 5’ primer (FlwFish) was a 20-mer with the sequence 5’-TAYATACACATMTYTATGC-3’ and the 3’ primer (RevFish) had the sequence 5’-TGATGTTGATGATGATGTCG-3’ (Fig. 1). PCR products of ~300 base pairs (bp) were isolated from low melting point (LMP) Nuseive GTG agarose gel (FMC) ligated into pGEM-T easy vector (Promega) and subsequently transformed into XLI-Blue Escherichia coli. One clone that contained an inserted of expected size was sequenced.

To resolve the 3’ end sequence of the sea bass (sb)MC4R cDNA, 3’ rapid amplification of cDNA ends (RACE) PCR was performed. For 3’ RACE PCR, cDNA was synthesized using 5’-adapter primer [5’-CAGTCGAGCTGACATCGA(T)3-3’. Two rounds of PCR amplified the 3’ end with adapter primer (5’-CAGTCGAGCTGACATCGA(T)3-3’ and sbMC4R_3’RACE_1 primer (5’-CAGTCGAGCTGACATCGA(T)3-3’) and then adapter primer and sbMC4R_3’RACE_1 primer (5’-GCTATCGGTATCATGACAT-3’). 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) following the manufacturer’s instructions. Specific primers for genome walking were sbMC4R_5Walker_1 primer (5’-GCAATGCTCTGAGGTTGATG-3’) for the first PCR and sbMC4R_5Walker_2 primer (5’-GAGATGATGATGATGATG-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’-ATAAGCTTGAACACCACAGGCTC-3’) and Xhoi-MC4R-reverse (5’-TATCTTCTGTTCG-3’) (Fig. 1). A 1,036-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 the European Molecular Biology Laboratory Nucleotide Sequence Database (accession no. 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the Taq DNA polymerase (Invitrogen) and specific primers. The 5’ promoter was sbMC4R_3’RACE_1 (see above), and the 3’ primer (sbMC4R cDNA_synthesis) had the sequence 5’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the 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’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the Taq DNA polymerase (Invitrogen) and specific primers. The 5’ promoter was sbMC4R_3’RACE_1 (see above), and the 3’ primer (sbMC4R cDNA_synthesis) had the sequence 5’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the Taq DNA polymerase (Invitrogen) and specific primers. The 5’ promoter was sbMC4R_3’RACE_1 (see above), and the 3’ primer (sbMC4R cDNA_synthesis) had the sequence 5’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the Taq DNA polymerase (Invitrogen) and specific primers. The 5’ promoter was sbMC4R_3’RACE_1 (see above), and the 3’ primer (sbMC4R cDNA_synthesis) had the sequence 5’-CAAGT-GATCATGAGGATGA-3’. The cDNA was subsequently amplified with the Taq DNA polymerase (Invitrogen) and specific primers. The 5’ promoter was sbMC4R_3’RACE_1 (see above), and the 3’ primer (sbMC4R cDNA_synthesis) had the sequence 5’-CAAGT-GATCATGAGGATGA-3’.
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, postfixed 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-aminopropyltriethoxylane-treated slides and then air-dried at room temperature overnight. Three consecutive series covering the length of the sea bass brain were made: two series were used for hybridization with the sense and antisense probes, and 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 1 wk.

Before hybridization, sections were deparaffinized, rehydrated, and postfixed in 4% PAF for 20 min. Slides were then rinsed twice in PB for 5 min and treated with a Proteinase-K solution (20 μg/ml in 50 mM Tris-HCl and 5 mM EDTA, pH 8) for 5 min at room temperature. Slides were then washed in PB and postfixed again in PAF for 5 min, 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 room temperature.

The entire coding region of the sea bass MC4R receptor sequence was cloned into pGEM-T easy vector (Promega). Antisense and sense RNA probes were synthesized in vitro by linearizing the plasmid with NcoI and SalI (Takara). In vitro transcription was carried out with SP6 or T7 RNA polymerase, respectively. Both sense and antisense probes were labeled with 10 μl of 35S-labeled 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 min at 37°C in the presence of 50 units of RNAsin (Promega) and then incubated at −20°C for 3 h with 10 μg/ml 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 labeled probes were then stored at −20°C and used within 1 wk.

The 35S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM DTT to obtain 2 × 10^5 cpm/ml. After 5 min of incubation at 80°C, 35S-UTP riboprobes were diluted 1:10 (final concentration of probes: 10 mM DTT and 2 × 10^4 cpm/ml) in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris·HCl (pH 8), 5 mM EDTA (pH 8), 10% Dextran sulfate, 1× Denhardt’s solution, and 0.5 μg/ml yeast RNA type III. Subsequently, 100 μl of hybridization solution were added to each pretreated slide (see above), which were coveredslipped and incubated in a humidified chamber at 55°C overnight. On the following day, coverslips were removed by incubating slides in a solution containing 5× standard saline citrate buffer (SSC; with 1× SSC containing 150 mM NaCl and 15 mM sodium citrate, pH 7) and 10 mM DTT for 30 min at 55°C. The slides were then rinsed in 2× SSC, 50% formamide, and 10 mM DTT for 30 min at 65°C and immersed three times in NTE buffer (500 mM NaCl, 10 mM Tris·HCl, and 5 mM EDTA; pH 7.5) for 10 min at 37°C. After RNase treatment (20 μg/ml RNase in NTE) for 30 min

Fig. 1. Nucleotide and deduced amino acid sequence of sea bass melanocortin receptor 4 (MC4R). Nucleotide and amino acid sequence numbers are indicated at left and right, respectively. Amino acids in shaded boxes indicate putative transmembrane domains predicted using Split 4.0 Server (http://split.pmfst.hr/split/). 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 is FM253127.
Embryonic kidney (HEK) cells were transfected using a modified atlas of sea bass (12, 16, 17). Anatomical locations were confirmed by reference to a brain atlas of sea bass (12, 16, 17). 

Protein synthesis, purification, and folding. Zebrafish AGP (Ac-83-127-NH₂) was synthesized using Fmoc synthesis on an Applied Biosystems (Foster City, CA) 433A peptide Synthesizer on a 0.25-mmol scale. The synthesis was monitored using the Synthesis version 2.0 software package. The peptide was assembled on a Rink-amide-MBHA resin, and preactivated Fmoc-Cys(Trt)-OPfp was used. All amino acids and resins were purchased through NovaBiochem (San Diego, CA). 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HBTU) was obtained from Advanced Chemtech (Louisville, KY). Fmoc deprotection was achieved using a 1% hexamethylenemine and 1% 1,8-diazabicyclo[4.5.0]undec-7-ene solution in dimethylformamide (DMF). Deprotection was monitored by conductivity and continued until the conductivity level returned to the baseline, and then synthesis resumed. Deprotection time ranged from 2.5 to 7 min. Coupling used four equivalents of Fmoc-amino acid in HBTU/dissopropylethylamine (DIEA) for all amino acids with the exception of preactivated cysteine. A threefold excess of Fmoc-Cys(Trt)-OPfp was dissolved in 1.5 ml of 0.5 M HOAc/DMF with no DIEA for coupling. The peptides were NH₂-terminal acetylated by reacting with 0.5 M acetic anhydride in DMF for 5 min. Fully synthesized peptide resin was split into three reaction vessels, washed with dichloromethane, and dried. A solution of 8 ml of trifluoroacetic acid (TFA) containing 200 µl each of triisopropylsilane/1,2-ethanedithiol/liquified phenol (TIS/EDT/phenol, as scavengers) was added to each reaction vessel of dry peptide resin for 1.5 h. The resin was filtered and washed with 1 ml of TFA, and the combined filtrate and wash was then added to 90 ml of cold, dry diethyl ether for precipitation. The precipitate was collected by centrifugation, and the ether was discarded. The pellet was dissolved in 40 ml of 1:1 H₂O-acetonitrile (0.1% TFA) and then lyophilized.

The crude peptide was purified by reversed-phase (RP)-HPLC on C4 Vydac (Hesperia, CA) preparative columns. Fractions were collected and analyzed by electrospore ionization-mass spectrometry (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 (2.0 M guanidine HCl/0.1 M Tris, 3 mM GSH, and 0.4 mM GSSG, pH 8, at a peptide concentration of 0.1 mg/ml) and stirring for 14 h. Folding was monitored by 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 atomic mass units (amu). The folded product was purified by RP-HPLC on a C18 preparative column, and its identity was confirmed as the fully oxidized product by ESI-MS (AGRP Ac-83-127-NH₂; 5,287.1 calculated average amu isotopes, 5,288 amu observed). 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 the University of California, Davis.

Cell culture and transfection. If not specifically indicated, human embryonic kidney (HEK) cells were transfected using a modified calcium phosphate transfection method (20) and grown in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), penicillin (100 U/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, 10 µl of the lysate were preserved for protein quantification. Forty microliters of phosphate saline buffer containing 0.5% BSA and 60 µl of substrate buffer (1 mM MgCl₂, 10 mM KCl, 5 mM β-mercaptoethanol, and 200 mg/ml o-nitrophenyl-β-D-galactopyranoside) were added to the remaining lysate volume. The plate was incubated at 37°C for 5 h, 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 a vasoactive intestinal peptide promoter placed downstream of tandem repetitions of cAMP-responsive elements (CRE; Ref. 21), was generated by cotransfection (50:1) of pCRE/β-galactosidase plasmid (kindly supplied by Dr. R. Cone, Vanderbilt University Medical Center) and the pcMV/HyTK plasmid, which harbors a hygromycin resistance gene (65). Cells were selected in medium containing 400 µg/ml hygromycin B (Invitrogen).

β-Galactosidase activity was tested after incubating resistant clones in 96-well plates (15,000 cells/well) with assay medium [DMEM +0.1 mg/ml BSA + 0.1 mM 3-isobutyl-1-methylxanthine (IBMX)] containing 10⁻⁶ M forskolin for 6 h. 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 vector 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 the MCR agonist 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, ztAGRP, SHU9119, and HS024) in the absence of IBMX. The effect of ztAGRP 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-labeled [Nle₄, D-Phe⁷]α-MSH (NDP-MSH). Nonspecific binding was defined as the amount of radioactivity remaining bound to the intact cells after incubation in the presence of 10 µM unlabeled NDP-MSH.

Effects of progressive fasting on melanocortin system. To evaluate the effects of fasting on hypothalamic and pituitary gene expression, 10 groups of 10 fish each (body weight 117 ± 1.54 g) were adapted for 1 wk to individual 500-liter aquaria and fed ad libitum at 9:00 AM. After this acclimation period, five groups were fed a same diet whereas five others were fasted. One fish each from the fed or fasted groups was sampled at 12:00 PM (3 h postfeeding for fed groups) at 1, 4, 8, and 15, and 29 days. Anesthetized fish were weighed, and pituitary dissected for immediate total RNA extraction. RNA samples were kept at −80°C in 75% ethanol until cDNA synthesis for quantitative PCR (see Real-time quantitative PCRs). Plasma was stored at −80°C until assayed.

Hormone measurements. Plasma α-MSH was measured by radioimmunoassay according to Arends et al. (3).
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 using one-way analysis of the variance followed by Tukey’s multiple range test (P < 0.05).

RESULTS

Molecular cloning of sea bass MC4R. By means of RT-PCR and using degenerate primers designed against conserved regions of fish MCR sequences, we cloned a 301-bp fragment showing high identity to the MC4Rs reported in other vertebrate species. The 3’ extremity 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; Figs. 1 and 2). Similar to other melanocortin receptors, the sea bass MCR ortholog exhibits short extracellular (ECL) and intracellular (ICL) loops and shares cysteine residues at positions 258, 272, and 278 (sea bass numbering) that are fully conserved in all MCRs (Fig. 2 and http://www.gpcr.org). The deduced amino acid sequence displays potential N-glycosylation sites within the NH2-terminal domain at positions 2 and 15 and one additional site within the first ECL (position 97; Fig. 1). This predicted ECL is considerably longer than the second and third ECLs, which are only four amino acids long. Similarly, the third 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 MCRs. 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 NH2-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 orthologs resides within the NH2-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 TMD2 and lowest in TMD4 (<57%). The overall identity ranged between 80 and 86% within the TMD3, TMD6 and TMD7 and between 75 and 77% within 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 ~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

Fig. 2. Alignment of melanocortin receptor (MCR) amino acid sequences from human (Homo sapiens; top). Sea bass MCR sequence is highlighted in bold type. Dots indicate amino acids identical to the top sequence. Dashes were introduced to improve alignment. Shaded boxes frame indicate putative transmembrane domains predicted with Split 4.0 Server (see Fig. 1). Arrowheads indicate fully conserved cysteine residues in all melanocortin receptors.
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 before 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 (Vv; 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 the 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 Fig. 5, A and B). Slightly more caudally, profuse MC4R mRNA expression was found in the magnocellular neurons of the preoptic nucleus (PM; Fig. 4C and Fig. 5, C and 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 suprahypothalamic nucleus (NSC; Fig. 4C and Fig. 5, C and 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 Fig. 5, E and 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 Fig. 5, E–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. 4, E and F, and Fig. 5, G and 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 rostrocaudal extension of the lateral recess forming the lateral part
of the lateral recess nucleus (NRLl; Fig. 4, F and G, and Fig. 5, G and H).

In the thalamus, MC4R mRNA-expressing neurons were restricted to the ventromedial nucleus of the ventral thalamus (VM; Fig. 4D and Fig. 5, I and J). A profuse expression was further found in the outer layer of the periventricular gray zone (PGZ) of the mesencephalic tectum (Fig. 4, D–G). Finally, in the rhombencephalon, MC4R-producing cells were placed in the superior reticular nucleus of the reticular formation (RS; Fig. 4G and Fig. 5, K and 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 (Fig. 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 CREs. Saturation experiments displayed a single saturable site for 125I-NDP-MSH (Fig. 7A) and showed that the receptor binds this agonist in a manner similar to that of the human, zebrafish, and goldfish MC4R (14). The sea bass MC4R was 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 (EC50) of 0.094 nM. Sea bass MC4R activation by /H9251/MSH and monkey /H9252/MSH showed an EC50 of 0.822 and 3.333 nM, respectively, whereas the EC50 increased to 18.8 and 15.13 nM when cells were incubated with human ACTH or desacetyl-MSH (Fig. 7). Sea bass MC4R was also activated by the nonselective melanocortin agonist MTII (EC50 /H11005/0.31 nM). However, MTII-stimulated cAMP intracellular accumulation decreased by coincubation with 1 /H9262/MHS024 (EC50 /H11005/17 nM), SHU9119 (EC50 /H11005/120 nM), and zfAGRP (EC50 /H11005/85 nM) (Fig. 8). When a phosphodiesterase inhibitor (IBMX) was added to the medium, the response of the reporter gene to the MTII incubation increased (EC50 /H11005/0.01 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 (IC50 /H11005/1.14 nM). Under these conditions, AGRP also decreased MTII-stimulated cAMP production (EC50 /H11005/9.9 nM) as a competitive antagonist would. Table 1 summarizes data for IC50 and EC50 of the sea bass MC4R.

**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 ortholog (data not shown). In rodents, MC4R expression is restricted to the CNS (47). Studies in chicken (60) and goldfish (14) have demonstrated that MC4R is also expressed in the peripheral tissues, 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 for by hypothalamic contamination of the hypophys-
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 αMSH/AGRP distribution in the sea bass have been 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 (NAPv and 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 compared with 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; Ref. 1), which is thought to be a mediator of the appetite-suppressing effect.
effects of stress in fish (6). A recent report demonstrated that the central anorectic effects of α-MSH in goldfish are mediated, in part, via CRF and described 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, AGRP was also detected in the dorsal (Vd) and ventral (Vv) parts of the ventral telencephalon in zebrafish. All these areas have been demonstrated to be MC4R-producing areas of the sea bass brain. In addition, 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 MC4R expression within 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

effects of stress in fish (6). A recent report demonstrated that the central anorectic effects of α-MSH in goldfish are mediated, in part, via CRF and described 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, AGRP was also detected in the dorsal (Vd) and ventral (Vv) parts of the ventral telencephalon in zebrafish. All these areas have been demonstrated to be MC4R-producing areas of the sea bass brain. In addition, 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 neuropeptide Y (10, 11), suggesting the telencephalic interaction between both peptidergic systems. Only α-MSH-ir, and 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 also have been described in the PGZ and reticular formation of zebrafish (30), where we presently 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 toward 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 rats. 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 rats, 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 homolog of the mammalian arcuate nucleus. In addition, the parvo- and magnocellular neurons of the preoptic nucleus seem to be homologs 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 viscerosensory 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 overexpression of the endogenous antagonists agouti and AGRP (22). Our previous studies 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 stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fasting has no effects on hypothalamic POMC expression but dramatically stimulates food intake in fed animals. Progressive fast...
switch from Gi to Gs proteins. This exchange could decrease -adrenergic receptor regulating the efficiency of the receptor (31). These authors defended the view that the measurement of cAMP after pretreatment of cells with IBMX does not truly reflect the melanocortin receptor signaling. Therefore, we thought initially that the inverse agonism of AGRP on melanocortin receptors could be an artifact 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, AMP-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 (Sánchez E, Rubio VC, and Cerdá-Reverter JM, unpublished results). 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 signaling from G protein-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 also has been 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 signaling pathways through which melanocortin receptors exert their effects are not well understood. Activated melanocortin receptor binds to Gi, 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 signaling pathways, including mitogen-activated protein (MAP) kinase (53) and inositol/Ca²⁺ (49) and probably AMP-activated-protein kinase (AMPK) pathways (45) also have been reported to be involved in MC4R intracellular signaling. A very recent study 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 downregulation 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 signaling pathways with differential sensitivities to phosphodiesterase inhibitors. More experiments on intracellular signaling 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 already has been demonstrated in vitro and in vivo (see Introduction for references). It has been proposed that the NH₂-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 NH₂-terminal domain 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 NH₂-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 in the current study 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 signaling in the absence of a decrease of agonist production, through AGRP binding. In this report, we further explore two additional levels that can potentially regulate the melanocortin signaling in the absence of variations of hypothalamic POMC expression. These levels included the downregulation of the hypothalamic receptor expression and the peripheral production of melanocortins. Similar to that reported in barfin flounder (Verasper moseri; Ref. 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 signaling via brain receptors during progressive fasting. Differential regulation of POMC processing or N-acetylation of MSH during fasting, potential regulatory levels of the melanocortin signaling 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 that 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 systems permits speculation that the mammalian AGRP-MC4R interaction also is dependent on the intracellular phosphodiesterase system. This view opens up new targets for the treatment of obesity in humans. Our future research must focus the study of activity of the different intracellular pathways after AGRP stimulation of the HEK cells stably expressing sea bass MC4R in the presence or absence of phosphodiesterase inhibitors.

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