

# Endogenous Melanocortin Antagonist in Fish: Structure, Brain Mapping, and Regulation by Fasting of the Goldfish Agouti-Related Protein Gene

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**Agouti-related protein (AGRP) is a naturally occurring antagonist of melanocortin. In mammals, central AGRP expression is restricted to the arcuate nucleus in which it plays a key role in the control of energy balance by antagonizing melanocortin effects at melanocortin 4 receptors. In goldfish, melanocortin 4 receptor is profusely expressed within the main brain areas for the control of energy balance, and central administration of agonist or antagonist analogs inhibits or stimulates food intake, respectively. Here we demonstrate that the goldfish genome has a homologous gene to mammalian AGRP. Detailed brain mapping by *in situ* hybridization shows that AGRP is exclusively expressed in the ventrobasal**

**hypothalamic lateral tuberal nucleus, the teleostean homolog of the arcuate nucleus. Fasting up-regulates its mRNA levels in the lateral tuberal nucleus. In the periphery, AGRP is expressed in several tissues including ovary, muscle, and ventral skin, suggesting that AGRP might regulate peripheral actions of melanocortin peptides. The results provide the first evidence for an endogenous melanocortin antagonist in non-tetrapod species and suggest that hypothalamic overexpression during fasting might regulate the inhibitory effects of melanocortin peptides on food intake in goldfish. (*Endocrinology* 144: 4552–4561, 2003)**

VARIOUS EVIDENCE INDICATES that central melanocortin signaling is a nodal point in the control of energy balance in mammals by exerting an inhibitory tone on food intake and stimulating energy expenditure (1, 2). Melanocortin signaling is mediated by binding to a family of specific G protein-coupled receptors that positively couple to adenyl cyclase. Five melanocortin receptors (MC1R–MC5R) have been characterized by molecular cloning in mammals and chicken. Peptides derived from proopiomelanocortin (POMC), namely ACTH and melanocyte-stimulating hormones (MSH),  $\alpha$ -,  $\beta$ -, and  $\gamma$ -MSH, are the main known agonists for MCRs, whereas agouti and agouti-related protein (AGRP) act as endogenous antagonists (3). Agouti protein is a potent melanocortin antagonist at MC1R and MC4R (4), whereas AGRP is inactive at MC1R but equally potent in inhibiting melanocortin signaling at MC3R and MC4R (5).

Agouti gene was first cloned in mice (6) and found to encode a 131 amino acid protein with structural characteristics of a secreted protein having a hydrophobic signal sequence and lacking any transmembrane domain. Agouti protein shows a highly basic N-terminal region with a high proportion of arginine and lysine residues. A proline-rich area immediately precedes the cysteine rich C-terminal domain that resembles the conotoxins and plectoxins of snails and spiders, respectively (7). In mice, agouti protein is

mainly produced by dermal papillae cells, adjacent to melanocytes, and acts within the hair follicle microenvironment to control the switch between production of eumelanin (black-brown pigment) and pheomelanin (yellow-red pigment) by antagonizing  $\alpha$ -MSH effects on MC1R in the follicle melanocytes (8). The unusual allele of the agouti locus,  $A^y$ , consists of a large deletion in the noncoding regions that places agouti gene under the control of the *Raly* promoter, a ubiquitously expressed RNA binding protein. The associated phenotype is characterized by yellow fur and ubiquitous expression of agouti gene, resulting in hyperphagia, hyperinsulinemia, increased linear growth, increased propensity for developing tumors, premature infertility, and maturity-onset obesity (9, 10). This metabolic syndrome is mediated by antagonizing  $\alpha$ -MSH signaling at central MC3R and MC4R that arbitrate negative effects of melanocortin peptides on energy balance (11–13).

The antagonist of peripherally synthesized agouti protein on central MCRs suggests the existence of a homologous pathway in the brain to the agouti-signaling pathway in the skin. The AGRP-encoding cDNA was first cloned in mice and human by similarity screening of expressing sequence tags based on the pattern of cysteine in the C-terminal region of agouti. AGRP protein lacks the highly basic N-terminal and proline-rich regions, but it shares strong homology to agouti protein within the polycysteine domain (5, 14). Agouti and AGRP contains 10 cysteine residues, nine of them spatially conserved, that form five disulfide bridges essential for the conformational stability and biological functions (15). These characteristics are also conserved in chicken AGRP, the only nonmammalian sequence reported (16). In mice, AGRP is mainly produced within the adrenal gland and the hypothalamic arcuate nucleus (5, 14) in which it is coexpressed

Abbreviations: AGRP, Agouti-related protein; DTT, dithiothreitol; MCR, melanocortin receptor; MSH, melanocyte-stimulating hormone; NLTl, lateral part of the lateral tuberal nucleus; NLTp, posterior part of the lateral tuberal nucleus; NPY, neuropeptide Y; NRL, lateral recess nucleus; PAF, paraformaldehyde; PB, phosphate buffer; POMC, proopiomelanocortin; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; SSC, saline citrate buffer; SSPE, sodium chloride/sodium phosphate/EDTA.

with neuropeptide Y (NPY) (17). AGRP projections are widely spread within the rat brain and overlap POMC projections, reaching main areas for neuroendocrine and energy homeostasis control (18). Fasting severely increases AGRP expression in the arcuate nucleus and AGRP transcripts are elevated in the hypothalamus of *ob/ob* and *db/db* obese mice (5, 14). Central injections of the C-terminal fragment (83–131) of AGRP have been demonstrated to stimulate food intake in rat for 72 h (19). Chronic infusion increases body weight in both *ad libitum* and pair-fed rats, suggesting additional effects on energy expenditure (20). Transgenic mice overexpressing AGRP display a similar metabolic syndrome as that of agouti yellow mice (5) and MC4R knockout mice (11), suggesting that AGRP modulates the melanocortin inhibitory tone on energy balance by acting at central MC3R and MC4R.

Previous results in the goldfish have demonstrated that the central melanocortin system may have a physiological role in the control of food intake. Intracerebroventricular administration of the melanocortin agonist analogs, NDP-MSH and MTII, significantly decrease cumulative food intake levels in 24-h fasted animals (21, 22). On the contrary, central administration of HS024, a specific goldfish MCR4 antagonist analog, stimulates cumulative food intake in previously fed animals. POMC, the endogenous precursor of the melanocortin agonists, is centrally expressed within the lateral tuberal nucleus, the teleost homolog of the mammalian arcuate nucleus (22). Similarly, MCR4 is widely expressed within the goldfish forebrain, showing profuse expression levels in the preoptic area and the whole rostrocaudal extension of the lateral tuberal nucleus (21). However, hypothalamic POMC-mRNA levels remain unchanged during progressive fasting up to 7 d (22), suggesting the existence of an endogenous melanocortin antagonist. In this article we explore the existence of an endogenous melanocortin antagonist in fish. The aim of this study was to identify the structure of AGRP gene in goldfish, map and detect AGRP mRNA expression in the brain and peripheral tissues, and determine the effects of changes in energy status on AGRP mRNA expression.

## Materials and Methods

### Animals and reagents

Male and female goldfish (*Carassius auratus*) were purchased from Mount Parnell Fisheries (Mercersburg, PA). Before experiments, fish were acclimated to constant photoperiod 16 h light/8 h darkness in 65-liter tanks receiving a constant flow of aerated water at 20°C for at least 1 wk. Fish were fed a 2% body weight ration once a day with trout pellets (Moore Clark, St. Andrews, New Brunswick, Canada). Animals were anesthetized in 0.02% tricaine methanesulfonate (MS-222, Syndel Laboratories Vancouver, British Columbia, Canada) for 2 min before any manipulation. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals. Unless otherwise indicated, all reagents were purchased from Sigma (St. Louis, MO).

### Molecular cloning, gene structure, and DNA sequencing

Filters from a goldfish genomic library made in the vector  $\lambda$ -GEM-11 (23), kindly supported by Dr. K. L. Yu (Department of Zoology, University of Hong Kong, Hong Kong), containing approximately  $10^6$  clones were screened with a mouse AGRP probe consisting of the exon 4 (5), kindly supported by Dr. G. S. Barsh (Howard Hughes Medical

Institute, Stanford University School of Medicine). Membranes were hybridized in hybridization solution [35% formamide, 6× sodium chloride/sodium phosphate/EDTA (SSPE), 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution and 10  $\mu$ g/ml yeast tRNA type III, 1× SSPE containing 150 mM NaCl, 1 mM EDTA, 9 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.4)] containing  $6 \times 10^5$  cpm/ml dCTP  $\alpha$ - $^{32}\text{P}$  at 42°C. Final washes were performed in 1× SSPE at 42°C. Two clones named 1.1.1.1 and 5.2.4.1 were isolated. A fragment expanding 5.7 kb from clone 5.2.4.1 was sequenced on both strands and found to contain a translated region showing a high homology level to mice AGRP exon 4. Unfortunately, this homology region occupied the 3' end of the clone 5.2.4.1 resulting in a truncated goldfish AGRP sequence.

To resolve both 5' and 3' ends of goldfish AGRP cDNA, 5' and 3' rapid amplification of cDNA ends (RACE)-PCR was performed as described earlier (24). For 3' RACE PCR cDNA was synthesized using dT-adaptor primer (5'-GGC CAC GCG TCG ACT AGT AC(T)<sub>17</sub>-3'). Two rounds of PCR amplified the 3' end with adapter (5'-GGC CAC GCG TCG ACT AGT AC-3') and SP1 (5'-ATG ATT ATG ATG CTG AAC AT-3') primers and then adapter primer and E2-2 primers (5'-ATC ACA TCC AAA CCT GAG-3'). After low melting point purification, a 529-bp fragment was subcloned into pGEM-T easy vector and sequenced. For 5' RACE PCR, cDNA synthesis was primed with ARPE4RV primer (5'-GAT CCA TGC TAC GGC AGT-3') and cDNAs were polyadenylated at 3' end using terminal dideoxy transferase (Promega, Madison, WI). The polyadenylated cDNAs were then amplified by two rounds of PCR first using dT-JAP-adaptor (5'-CAG TCG AGT CGA CAT CGA (T)<sub>12</sub>-3'), JAP-adaptor (5'-CAG TCG AGT CGA CAT CGA-3') and 5AGRP1 primer (5'-CGA CAG TAA CAC GTA TCA C-3'). Nested PCR was done by using JAP-adaptor and 5AGRP2B primer (5'-GAC TGC TGA TGA GGG ATG). A 529-bp fragment was subcloned and sequenced. Finally, full goldfish cDNA amplification was done by RT-PCR with primers AGRPFULL-F (5'-TGG ACA CAG ACC CCT GC) and AGRPFULL-R (5'-CCT ATG GAT AGT GTG AGG-3'). A 673-bp DNA fragment was subcloned into pGEM-T easy vector and sequenced on both strands. The nucleotide sequence of goldfish AGRP has been deposited with EMBL Nucleotide Sequence Database under accession numbers AJ555492 and AJ555493.

### RT-PCR and Southern blot analysis

Total RNA was purified with Trizol (Invitrogen, Carlsbad, CA) and treated with RQ1-Dnase (Promega). Superscript II reverse transcriptase was used for cDNA syntheses by priming total RNA from testis, ovary, intestine, fat, liver, muscle, spleen, kidney, gill, dorsal skin, ventral skin, retina, heart, pituitary, and brain with oligo (dT)<sub>12–18</sub> (Invitrogen). PCR amplification was carried out with the primers E2-2 and ARPE4RV, thus expecting an amplification band of 299 bp. Subsequently, PCR fragments were separated onto 1.2% agarose gel, transferred to Hybond-N nylon membrane (Amersham, Piscataway, NJ). Hybridizations were carried out in hybridization solution (50% formamide, 6× SSPE, 0.5% SDS, 5× Denhardt's solution and 10 mg/ml yeast tRNA type III) using full-length AGRP cDNA as probe. Final washes were performed in 0.1× SSPE at 65°C. Hybridization signals were scanned using PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics). As internal control of the reverse transcription step, PCR for  $\beta$ -actin cDNA amplification was carried out as previously reported (21).

### Probe specificity in Northern blot

Full-length AGRP inserted into pGEM-T easy vector was used to prepare probe, and its specificity was assessed by Northern blot analysis. Total RNA from five discrete brain areas, *i.e.* telencephalon-preoptic region, hypothalamus, optic tectum-thalamus, medulla, cerebellum, and pituitary, were extracted with Trizol and its concentration estimated based on absorbance at 260 nm. About 15  $\mu$ g total RNA from discrete brain areas and pituitary were electrophoresed onto 1.5% agarose gel containing 2.2 M formaldehyde and transferred by capillarity to Hybond-N nylon membranes (Amersham). Membranes were prehybridized for at least 3 h in hybridization solution (50% formamide, 5× SSPE, 1% SDS, 5× Denhardt's solution, and 20  $\mu$ g/ml yeast tRNA type III). Full coding sequence of goldfish AGRP was used as probe. Hybridization was carried out overnight in fresh hybridization solution containing 2 ×

$10^6$  cpm/ml dCTP [ $\alpha$ - $^{32}$ P] at 42 C. Final washes were performed in  $0.1 \times$ SSPE /0.1% SDS at 65 C.

### *In situ hybridization*

*In situ* hybridization experiments were carried out as described previously (25). Animals were anesthetized then transcardially perfused with 20 ml of physiological saline solution (NaCl 0.65%) and subsequently 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). Transverse serial sections were cut at 6  $\mu$ m using a rotary microtome. One section every 60  $\mu$ m was mounted on 3-aminopropyltriethoxylane-treated slides and then air dried at room temperature overnight. Three consecutive series covering the entire extension of the goldfish brain were done. Two series were used for hybridization with the sense and antisense 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 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  $\mu$ g/ml in 50 mM Tris-HCl, 5 mM EDTA (pH 8)] for 5 min at room temperature. Slides were next washed in PB and post fixed 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.

Full-length AGRP inserted into pGEM-T easy vector was used to prepare riboprobes. Antisense and sense RNA probes were synthesized *in vitro* by linearizing the plasmids with *Nco*I or *Sal*I (Invitrogen), and *in vitro* transcription was carried out with SP6 or T7 RNA polymerase, respectively. Both sense and antisense probes were labeled with 10  $\mu$ l  $^{35}$ S-UTP (10 mCi/ml) using the riboprobe synthesis kit (Amersham) as described by the manufacturer. After *in vitro* RNA synthesis, samples were treated with RQ1-Dnase (Promega) for 15 min at 37 C in presence of 50 U RNasin (Promega) and then incubated at  $-20$  C for 3 h with 10  $\mu$ g/ml yeast RNA type III in an 8% formamide solution. Probes were subsequently purified on Sephadex G50 columns. The two fractions containing the highest radioactivity 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  $^{35}$ S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM dithiothreitol (DTT) to obtain  $2 \times 10^5$  cpm/ $\mu$ l. After 5 min incubation at 80 C,  $^{35}$ S-UTP riboprobes were diluted 1/10 [final concentration of probes, 10 mM DTT and  $2 \times 10^4$  cpm/ $\mu$ l] in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 10% dextran sulfate, 1 $\times$  Denhardt's solution, and 0.5  $\mu$ g/ml yeast RNA type III. Subsequently, 100  $\mu$ l of hybridization solution was added to each pretreated slide (see above), which were coverslipped and incubated in a humidified chamber at 55 C overnight. The following day coverslips were removed by incubating slides into a solution containing 5 $\times$  standard saline citrate buffer (SSC) [1 $\times$  SSC contains 150 mM NaCl, 15 mM sodium citrate (pH 7)] and 10 mM DTT for 30 min at 55 C. The slides were then rinsed in 2 $\times$  SSC, 50% formamide, and 10 mM DTT for 30 min at 65 C and three times immersed into NTE buffer [500 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA (pH 7.5)] for 10 min at 37 C. After ribonuclease treatment (20  $\mu$ g/ml ribonuclease in NTE) for 30 min at 37 C, slides were rinsed three times in NTE buffer for 10 min at 37 C, once in 2 $\times$  SSC, 50% formamide, and 10 mM DTT for 30 min at 65 C, once in 2 $\times$  SSC for 15 min at room temperature and twice in 0.1 $\times$  SSC for 15 min at room temperature. Slides were finally dehydrated in increasing graded ethanol solutions containing 0.3 M ammonium acetate and dried at room temperature. After the hybridization process, slides were dipped in photographic emulsion (Amersham) and exposed under dry conditions at 4 C for 5–7 d, developed in Kodak D-19 and counterstained with toluidine blue 0.02%. Anatomical locations were confirmed by reference to a brain atlas of goldfish (26).

### *Effects of fasting on hypothalamic AGRP gene expression*

To evaluate the effects of fasting on hypothalamic AGRP expression, eight groups of six fish each (body weight =  $25.9 \pm 0.2$  g) were adapted

for a 1-wk period to individual 65-liter aquaria and fed a 4% body weight ration at 1000 h. After this acclimation period, four groups were continued to be fed the same ration, whereas four others were fasted. One each fed and fasted group were sampled at 1300 h (3 h post feeding for fed groups) at 1, 3, 5, and 7 d, respectively. After deep anesthesia fish were weighed, decapitated, and whole hypothalamus dissected for immediate total RNA extraction. Samples were kept at  $-80$  C in ethanol 75% until assayed. To evaluate AGRP mRNA levels, 7  $\mu$ g total RNA from each individual hypothalamus were denatured in 42% formamide, 1.6% 3[N-morholino]propanesulfonic acid, and 2 M formaldehyde for 15 min at 65 C. Samples were subsequently blotted onto Hybond-N nylon membrane (Amersham) by vacuum suction using a slot blot apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). RNA was fixed for 2 h at 80 C and membranes were prehybridized in hybridization solution (50% formamide, 5 $\times$ SSPE, 1% SDS, 5 $\times$  Denhardt's solution, 50 mg/ml yeast tRNA type III) for 3 h. Hybridization was carried out overnight in fresh hybridization solution containing  $1.5 \times 10^6$  cpm/ml [ $\alpha$ - $^{32}$ P] dCTP at 42 C. Full-length AGRP probe was labeled as before. Final washes were performed in 0.1 $\times$ SSPE/0.1% SDS at 65 C. Subsequently, membranes were stripped and reprobed with [ $\alpha$ - $^{32}$ P] dCTP labeled probe for  $\beta$ -actin as well-loading control. *In situ* hybridization experiments on five fed or 5-d fasted fish each tested the potential involvement of the lateral part of the lateral tuberal nucleus (NLTI) in the hypothalamic AGRP expression after fasting.

### *Data analysis and statistics*

Sequences were compiled in GenTool software package (BioTools Inc., Edmonton, CA) and compared with known agouti and AGRP sequences from the National Center for Biotechnology Information database by using BlastX. Sequence alignments were performed using Pileup from the GCG package and ClustalX 1.81 from Canadian Bioinformatics Resources (<http://www.cbr.nrc.ca>). The cleavage site for removal of the hydrophobic signal peptide was predicted using SignalP V1.1 (<http://www.cbs.dtu.dk/services/SignalP/>). In gene expression studies, hybridization signals were scanned using PhosphorImager (Molecular Dynamics) and quantified by ImageQuant software (Molecular Dynamics). The AGRP mRNA levels were normalized as a ratio to  $\beta$ -actin mRNA and then expressed as a percentage of the hypothalamic AGRP mRNA levels from the fed control group at d 1. Statistical analysis was conducted by one-way analysis of the variance followed by Scheffé's multiple range test. Differences in AGRP mRNA levels between the fed and unfed animals during the same day were tested by *t* test. Statistical significance was considered at  $P < 0.05$ .

## Results

### *Cloning and characterization of the goldfish AGRP gene*

By homology screening of a goldfish genomic DNA library with a mouse AGRP probe consisting of the exon 4 that encodes C-terminal poly-cysteine domain (mouse AGRP 86–131), two positive clones were isolated. The 3' end fragment expanding 5.7 kb from clone 5.2.4.1 was sequenced and found to contain a truncated fragment corresponding to the fourth exon of mammalian AGRP genes. Figure 1 shows the nucleotide sequence of the goldfish AGRP gene. Analysis of the hydropathy profiles of the possible translations of the clone 5.2.4.1 enabled us to estimate the translation initiation site for AGRP within the goldfish genomic sequence and assign primers for PCR. Previous RT-PCR experiments using specific primers that flank the putative goldfish AGRP exon 4 and cDNAs from discrete parts of the goldfish brain were made to obtain a source of AGRP mRNA. RT-PCR using hypothalamic cDNA as template resulted in an expected size band of about 165 bp (data not shown). Subsequently, SP1 and ARPE4RV primers were used to amplify hypothalamic cDNA. RT-PCRs generated a band of about 361 bp (Fig. 1). Sequence analysis provided information on the exon-intron

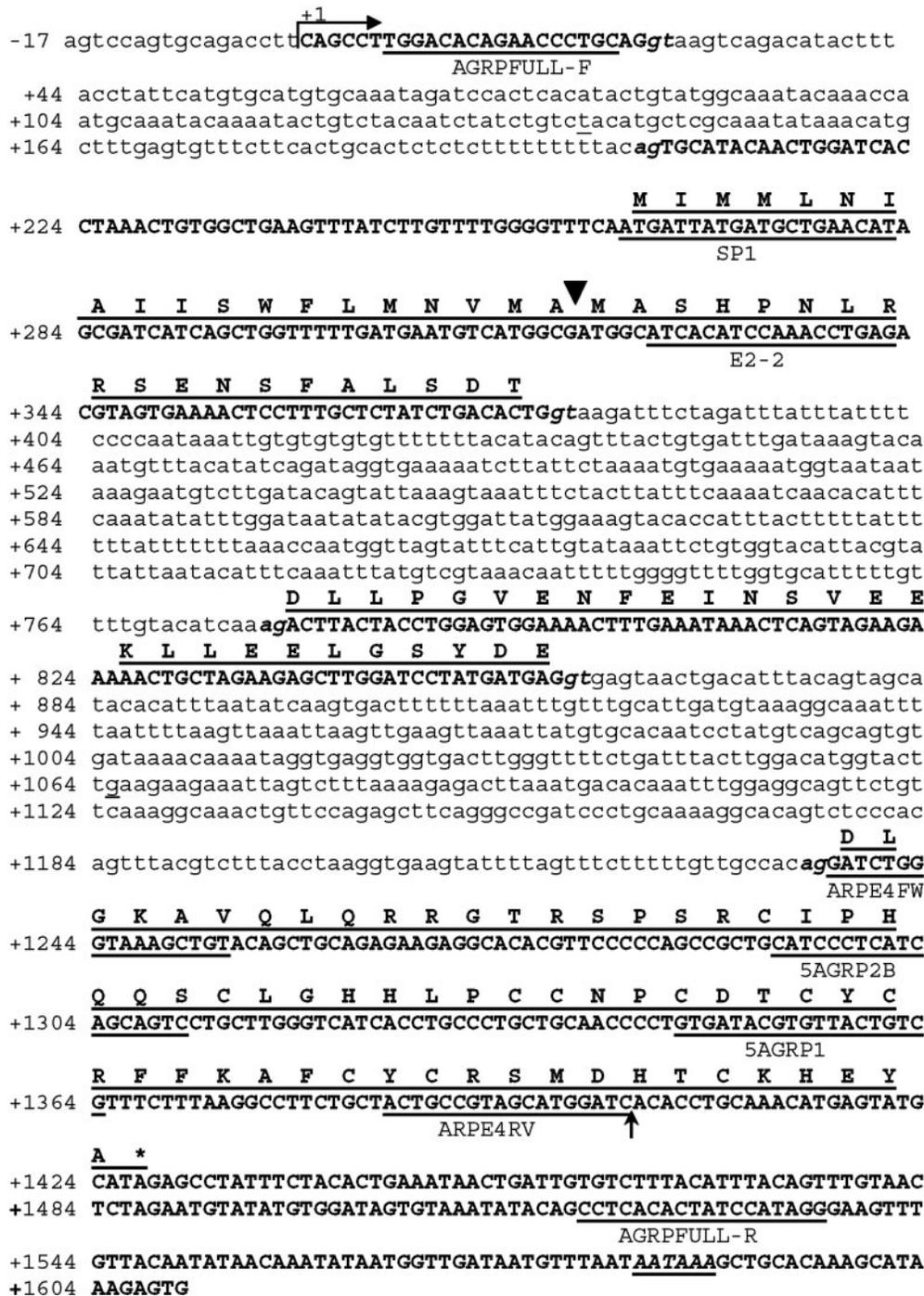


FIG. 1. Nucleotide and deduced amino acid sequence of the goldfish AGRP gene. Nucleotide numbers are indicated on the left side. Introns are shown in lowercase letters, and exons are shown in bold uppercase letters. Exon-intron splice junctions are in italic, bold letters. The stop codon is marked (\*). Nucleotides of the consensus polyadenylation site (AATAAA) are in bold, italic uppercase letters and are underlined. Arrowhead indicates the predicted cleavage site for removal of the hydrophobic signal peptide, whereas an arrow displays the 3' end of the genomic clone 5241. Nucleotide sequences of primers used in 5', 3', and RT-PCR amplification are underlined. Goldfish AGRP mRNA sequence accession no. AJ555492.

boundaries of the goldfish AGRP gene. To obtain the sequence of the complete peptide precursor as well as determine the major transcription initiation site, RACE-PCR was performed in the 3' and 5' directions. The 3' RACE-PCR generated fragments of about 530 bp and provided information about the end region of the exon 4 and the 3'-

untranslated region. The 5' RACE-PCR resulted in a single band of about 360 bp, which provided information about the 5' untranslated region, the transcription initiation site, and the exon-intron boundaries of the goldfish AGRP gene (Fig. 1). To corroborate the sequences generated by 3' and 5' RACE, specific primers (AGRPFULL-F and AGRPFULL-R)

targeting the respective untranslated regions were designed and used in RT-PCR with hypothalamic cDNA. RT-PCR resulted in an expected size band of 571 bp, which was subcloned and sequenced. No sequence discrepancies suggesting allelic differences or gene duplication were found in any sequence comparison.

The cDNA sequence obtained for goldfish AGRP is 651 bp long and has an open reading frame encoding pre-AGRP with 128 amino acids. The 5' and 3' untranslated regions are 82 and 185 and bp long, respectively, with the latter containing a consensus polyadenylation signal AAUAAA. The peptide precursor has the same organization as that of other species. The first 19 amino acids are estimated to constitute the signal peptide, which is followed by the 109 amino acids of the mature peptide. Goldfish AGRP lacks a highly basic N-terminal and proline-rich region but displays a polycysteine C-terminal domain. The latter region contains 10 cysteine residues with identical spatial pattern to that of agouti proteins, but similar to AGRP molecules exhibits a short amino acid extension following the tenth cysteine residue (Fig. 2). Goldfish AGRP displays low identity level to both tetrapod agouti or AGRP molecules, but the identity level is higher when compared with AGRPs than to the agouti proteins. Goldfish AGRP is 17% and 30% identical with human agouti and AGRP, respectively. When considering equivalent taxons for agouti and AGRP sequences (human, mouse, and pig), goldfish AGRP displays 24 and 37 constant positions, respectively. The identity level seems to be constrained to the polycysteine domain, in which goldfish AGRP is 35% and 50% identical with human agouti and AGRP, respectively. Within the latter domain goldfish AGRP displays 14 and 9 unique substitutions when compared with mammalian agouti or AGRP sequences, thus further supporting its homology to AGRP molecules.

Comparison between cDNA and genomic sequences displayed four alignment blocks, denoting that goldfish AGRP

gene is organized into four exons separated by three introns. Goldfish AGRP gene is a relatively small gene spanning 1610 bp from the start of the noncoding exon to the end of the 3' untranslated region, including the polyadenylation site. Exon 1 is 25 bp long and contains part of the 5' untranslated region. Exon 2 (172 bp) includes the remaining part of the 5' untranslated region, the region encoding the signal peptide and the first 19 amino acids of the putative mature peptide. Exon 2 ends with the nucleotide base G that constitutes the first base of an aspartic codon after splicing. Exon 3 (80 bp) starts with the last two nucleotide bases AC of the latter aspartic codon and encodes the following 27 amino acids of the mature peptide. Exon 4 (374 bp) encodes the remaining portion of the mature peptide, including the polycysteine domain and contains the 3' untranslated region. We cannot rule out the existence of some additional short intron within the 3' untranslated region of the goldfish AGRP gene; however, PCR with AGRPFULL-F and AGRPFULL-R on genomic DNA as template generated a single band of the expected size of about 577 bp (data not sh, and all three intron/exon junctions conform to the GT/AG rule of splicing.

*Peripheral and central distribution of AGRP mRNA*

RT-PCR with specific primers E2–2 and ARPE4RV resulted in a band of the expected size of about 0.3 kb (Fig. 3A). The identity of the band was confirmed by Southern blot hybridization with a goldfish AGRP probe including the full coding region (Fig. 3B). Goldfish AGRP mRNA was detected in the pituitary, brain, ventral skin, muscle, testis, and ovary and minor levels in eye, dorsal skin, heart, kidney, spleen, and intestine (Fig. 3, A and B). No bands or hybridization signal for goldfish AGRP were obtained in PCR reactions using gill, liver, and fat cDNA or water (control) as template (Fig. 3, A and B). Inverse transcriptions and cDNA quality

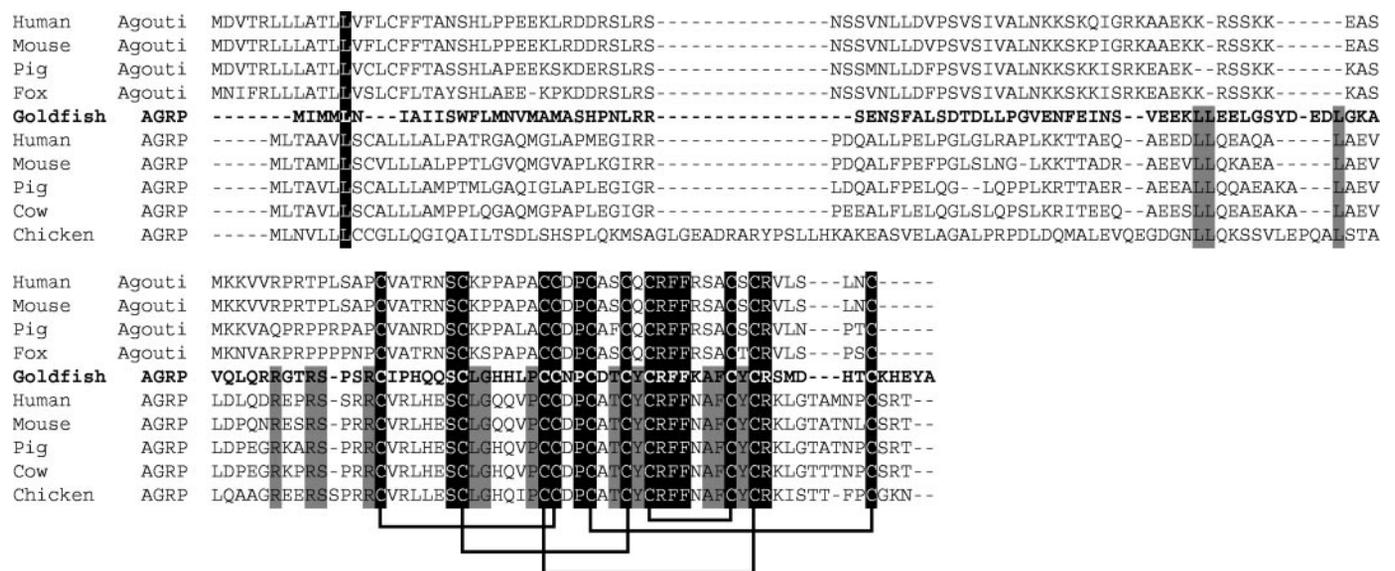


FIG. 2. Alignment of agouti [human (NM.001672), mouse (L06451), pig (AJ251837), fox (Y09877)], and AGRP [human (NM.001138), mouse (NM.007427), pig (AF177762), cow (NM.173983), chicken (AB029443)] amino acid sequences. Goldfish AGRP sequence is highlighted in bold letters. Dashes were introduced to improve alignment. Black boxes show amino acid residues conserved in all sequences. Gray boxes show residues conserved only in AGRP sequences. Lines joining cysteine residues indicate disulfide bonds.

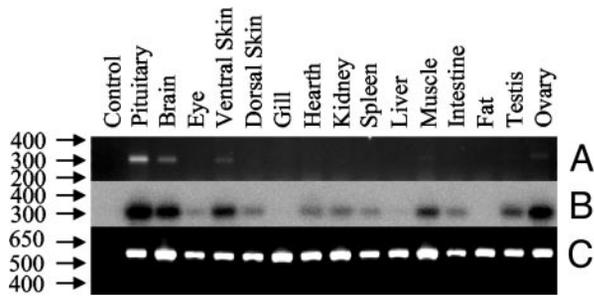


FIG. 3. Distribution of goldfish AGRP mRNA expression as revealed by RT-PCR assay followed by Southern blot hybridization. A, Ethidium bromide-stained agarose gels of RT-PCR amplifications carried out with primers set E2–2 and ARPE4RV (Fig. 1). B, PhosphorImaging screen of Southern blot analysis with a probe including the full coding region of the goldfish AGRP following RT-PCR assays. C, Ethidium bromide-stained agarose gels of RT-PCR amplifications carried out with primer sets actin 1 and actin 2 (21). Control, No cDNA.

were corroborated by PCR amplification of  $\beta$ -actin cDNA that yielded bands of expected size in all reactions (Fig. 3C).

Probe specificity and central distribution of AGRP mRNA were initially assessed by Northern blot on total RNA from several discrete brain areas and pituitary. Two different bands about 0.7 and 0.8 kb were detected in hypothalamus, but no expression levels were observed in telencephalon-preoptic area, optic tectum-thalamus, cerebellum, medulla, and pituitary (Fig. 4). These are reasonable sizes considering the addition of a poly-A tail. Although only one cDNA type was isolated during the cloning process, given the tetraploid nature of goldfish, two separate mRNAs may be expected for any given gene (27). In addition, *in situ* hybridization with sense AGRP-cRNA probes never generated specific signals in the goldfish brain, further supporting the probe specificity (data not shown). Cell groups expressing AGRP mRNA were exclusively found in the ventral portion of the tuberal hypothalamus of the goldfish brain. Highest expression levels were found within the caudal area of the tuberal hypothalamus. The first positive perikarya were localized within the ventral area of the posterior part of the lateral tuberal nucleus (NLTP). This cell population exhibited few positive perikarya in each section (Fig. 5A). The most profuse cell group was detected slightly more caudally coinciding with the first manifestation of the medial part of the lateral recess nucleus (NRL, Fig. 5B). At this level, AGRP-expressing neurons are located in a ventral and periventricular region of the nucleus. Some of these periventricular neurons appear to make contact with the cerebrospinal fluid (data not shown). In only one case of 20 animals processed for *in situ* hybridization, AGRP-mRNA expression was detected within the NLTI (Fig. 5A). In this particular specimen, expression levels within NLTI were much higher than those observed in the periventricular area of the lateral tuberal nucleus.

#### Effects of fasting on hypothalamic AGRP gene expression

As revealed by slot blot analysis, hypothalamic AGRP expression levels in fed animals were unchanged during the whole sampling period (Fig. 6). One-day fasting did not affect hypothalamic expression levels, but progressive fasting induced a significant increase in the hypothalamic

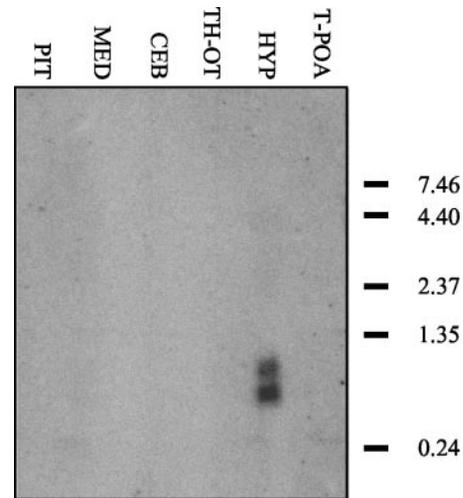


FIG. 4. Film autoradiogram of Northern-blotted total RNA from pituitary (PIT), medulla (MED), cerebellum (CEB), thalamus-optic tectum (TH-OT), hypothalamus (Hyp), and telencephalon-preoptic area (T-POA). A total of 15  $\mu$ g RNA were electrophoresed on a 1.5% agarose gel, containing 2.2 M formaldehyde, transferred onto a nylon membrane filter, and prehybridized during 3 h in 50% formamide, 5 $\times$  SSPE, 1% SDS, 5 $\times$  Denhardt's solution, containing 20  $\mu$ g/ml yeast tRNA type III at 42 C. The  $^{32}$ P labeled goldfish AGRP probe was then added to fresh solution and hybridization was carried out for 16 h under the same conditions. Final washes were performed in 0.1 $\times$  SSPE/0.1% SDS at 65 C. The numbers on the right side of the blot correspond to the localization of size marker RNAs (in kb). Two RNA bands of about 0.7 and 0.8 kb were detected in the hypothalamic tissue.

AGRP-mRNA levels, which were maximal after 5 d. *In situ* hybridization studies never detected AGRP expression within the NLTI, suggesting that the latter nucleus is not involved in the AGRP-increased expression after fasting (data not shown).

## Discussion

In this article, we demonstrated that the goldfish genome has a homolog gene to mammalian AGRP, which has been described as a potent endogenous antagonist of mammalian MC3R and MC4R that may regulate the melanocortin inhibitory tone on feeding (5). In the goldfish brain, AGRP is mainly expressed in the caudal portion of the hypothalamic lateral tuberal nucleus, in which fasting up-regulates its mRNA levels. We also report the goldfish AGRP gene structure and its tissue expression pattern.

By homology screening of a goldfish genomic library, 5', 3' RACE, and RT-PCR, the nucleotide and deduced amino acid sequence of goldfish AGRP was determined. Both gene and precursor have a comparable organization to tetrapod counterparts. Goldfish AGRP gene is organized into four exons separated by three small introns. Gene transcription generates a 651-bp-long mRNA that encodes a 128 amino acid precursor. In human, AGRP gene presents two alternative transcriptions. Gene transcription in the arcuate nucleus generates a larger mRNA with a long 5' untranslated region. The smaller transcript, expressed in the peripheral tissues (mainly adrenal gland), seems to be attributable to the absence of the first exon containing 5' untranslated region (14). Goldfish AGRP-mRNA characterization by 5' and 3'

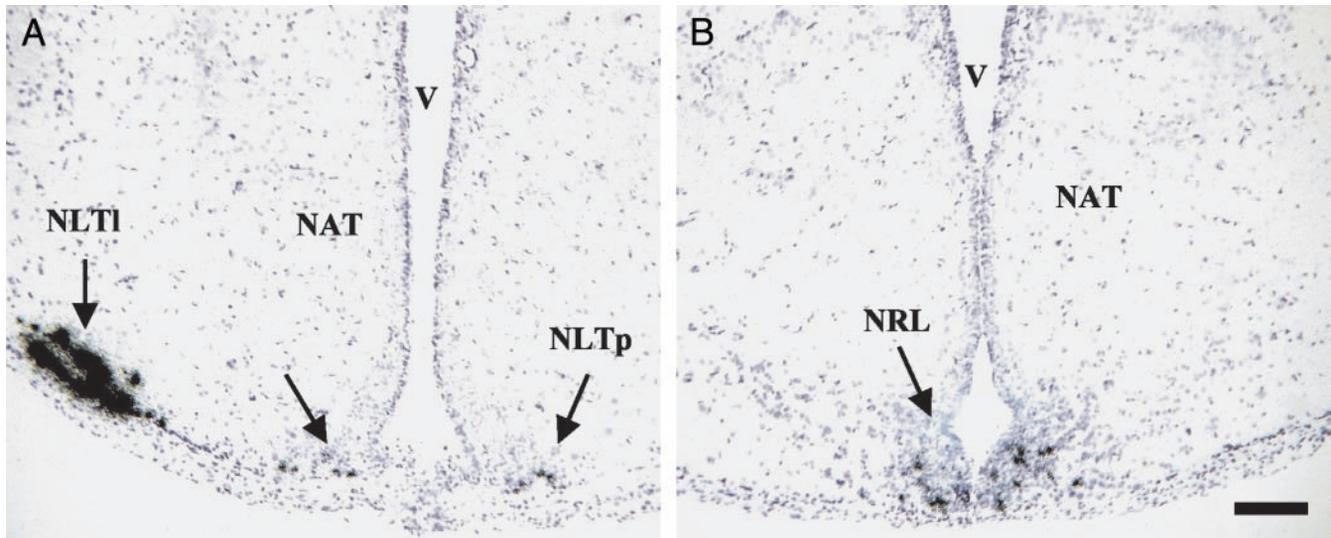


FIG. 5. Bright-field photomicrographs of transverse sections of the goldfish brain from rostral (A) to caudal (B) mediobasal hypothalamus. A, Positive AGRP neurons placed in the rostral-most aspect of the NLTp. In this section, high expression levels were found within the NLTI; however, AGRP expression was found within the latter nucleus in only 1 of 20 animals tested. B, AGRP-expressing cell bodies in the periventricular area of the NRL. AGRP expression was found within NLTp and NRL of all tested animals. NAT, Anterior tuberal nucleus; V, third ventricle. Scale bar, 100  $\mu$ m.

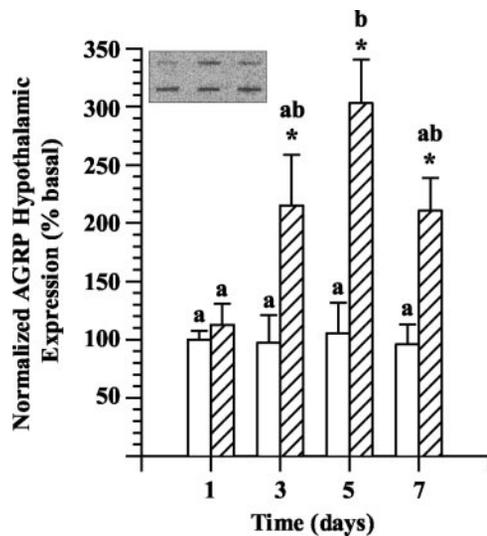


FIG. 6. Effects of progressive fasting on hypothalamic AGRP expression. Levels of AGRP mRNA were expressed as the ratio of AGRP mRNA/ $\beta$ -actin mRNA and normalized as percentage of the fed group at d 1. Data are mean  $\pm$  SEM ( $n = 6$ ). Dissimilar superscripts indicate significant differences (one-way ANOVA,  $P < 0.05$ ). Asterisks indicate significant differences ( $P < 0.05$ ) after  $t$  test of fasted animals, compared with food intake values of fed animals at the same day. Inset shows representative slot blots of 5-d fed (upper panel) and fasted animals (lower panel).

RACE-PCR was performed from hypothalamic RNA. Results demonstrate that goldfish AGRP gene transcription in the hypothalamus involves four exons and, similar to human AGRP, the first exon is untranslated. Expression studies cannot discriminate the existence of alternative transcripts in peripheral tissues because primers targeting specific sequences in exons 2 and 4 were used. However, two different-size hybridization bands were detected in total RNA from hypothalamus by using full-cDNA goldfish AGRP as a probe

in Northern blot assays. RACE-PCR in direction 5' performed by using primers designed against the exon 4 exclusively showed a single-size band when using hypothalamic cDNA as template. The sequenced 5' RACE clones displayed identical sequences that perfectly aligned to the AGRP genomic sequence. Parity of both 5' and 3' extremes was further corroborated by PCR amplification with primers targeting respective untranslated regions. Given the tetraploid nature of goldfish (27), we concluded that the results observed in Northern blot correspond to an uncharacterized second copy of AGRP gene present in the goldfish genome.

The putative goldfish AGRP precursor has structural characteristics of a secreted protein displaying a hydrophobic signal. Processing of the putative signal peptide produces a 109 amino acid mature protein that includes a characteristic C-terminal polycysteine domain. The C-terminal domain of goldfish AGRP exhibits 10 cysteine residues that restrain identical spacing to that of agouti proteins, but similar to AGRP molecules, it shows a short amino acid C-terminal extension following the 10th cysteine residue. Studies focused to characterize the structure and the active core of AGRP molecule have demonstrated that mature human AGRP shows disulfide bonds among cysteine residues, C87–C102, C94–C108, C101–119, C105–129, and C110–C117 (28). Eight of the 10 cysteine residues are functionally essential in the homologous agouti protein and mutations in the pair of cysteine residues that anchor the final seven residues of the agouti protein result in only partial loss of the *in vivo* activity (29). The C-terminal fragment of human AGRP 87–132 retains the same disulfide pattern adopting the inhibitor cysteine knot fold previously identified for invertebrate toxins (15, 30–32).

The engineered protein human AGRP (87–120, C105A) exhibits MCR binding and activity almost identical with that of AGRP (87–132), thus suggesting that antagonistic activity and receptor selectivity reside within the first 34 amino acids

of human AGRP (87–32) that form the inhibitor cysteine knot domain of AGRP (15, 32). These first 34 residues of human AGRP (87–132) are consistently ordered and contain a three-stranded antiparallel  $\beta$ -sheet with two of the strands forming a  $\beta$  hairpin. On the contrary, the final 12 amino acids exhibit conformational heterogeneity (15, 31), and mutations within the human AGRP fragment (120–127) do not significantly affect binding properties to both MC3R and MC4R (33). On the contrary, mutations in the triplet RFF, contained inside the shortest predicted loop of the human AGRP, dramatically decrease AGRP binding to both MC3R and MC4R (31, 33). This small motif shows properties resembling the general chemical properties of the core sequence HFRW in MSHs (12) and has been proposed to form a primary contact point for the ligand-receptor interaction (15, 33). Structural analysis has suggested that this protruding short loop projects the RFF triplet to the solvent-exposed region of the  $\beta$ -hairpin (15, 31, 32). This RFF triplet is fully conserved in agouti and AGRP sequences including goldfish AGRP. The highest identity to tetrapod AGRP molecules is observed within the goldfish AGRP fragment (97–117), in which 18 of 21 residues (85%) are identical in all reported AGRP sequences. The identity is much lower within the previous 13 amino acids, in which only five residues are identical (38%) and minimal (8%) in the final 12 amino acids, in which only the 10th cysteine residue is common to all sequences.

McNulty *et al.* (15) proposed that the differential MCR selectivity of agouti and AGRP might reside in the residues between the first and third cysteine residue in which low identity of agouti *vs.* AGRP is observed. The N-terminal loop might directly interact with the second and/or third ex-loops of MC3R and MC4R. However, full-length human AGRP with glutamine residues (Q) at positions 97 and 98 both replaced by alanine (A) exhibit binding at MC3R and MC4R equivalent to wild-type AGRP (33). Within this proposed loop only three residues, *i.e.* leucine (L92), glycine (G93), and proline (P97) of goldfish AGRP sequence, are fully conserved in all reported AGRP sequences. Except for position 101 of fox agouti that shows a P/S replacement, the equivalent positions are constant in agouti sequences, *i.e.* arginine (K101), proline (P102), and alanine (A106) residues (human agouti numbering). Overall this suggests that these constant positions in AGRP sequences that differ from those constant positions in agouti sequences might be critical in the interaction with MCRs as well as in determining differential MCR selectivity of agouti and AGRP. Although additional studies are needed, the goldfish AGRP sequence may bring light to the AGRP interactions and selectivity by mammalian MCRs.

Goldfish AGRP is expressed in a variety of tissues including brain, pituitary, ventral skin, muscle, testis, and ovary. Minor levels were also detected in eye, dorsal skin, heart, kidney, spleen, and intestine. Within the goldfish brain, studies by Northern blot demonstrated AGRP-mRNA expression in the hypothalamus, but no hybridization signal was detected in the pituitary. The discrepancy between tissue expression profiles obtained by PCR and Northern blot in the goldfish brain may be accounted for by differences in the sensitivity of the techniques. Shutter *et al.* (14) localized AGRP expression in the brain, adrenal gland, lung, and testis

in human and mice by RT-PCR and Northern blot. On the other hand, Ollmann *et al.* (5) detected AGRP expression in the brain, adrenal gland, kidney, lung, ovary, and muscle in mice by Southern blotting of RT-PCR products. The expression levels varied with the tissue type reaching maximal levels in the brain and adrenal gland. In contrast in White Leghorn chicken, AGRP is evenly expressed in all tissues examined including brain, heart, kidney, liver, spleen adrenal gland, ovary, testis, uropygial gland, skeletal muscle, and adipose tissue as revealed by RT-PCR (16). It has been suggested that the ubiquitous AGRP expression in this chicken strain might result from an unusual mutation analogous to the  $A^y$  allele in mice. In fact, White Leghorn chickens display metabolic alterations similar to those of agouti mice including hyperinsulinemia and hyperglycemia (16).

RT-PCR experiments in the goldfish demonstrated expression of AGRP in the ventral skin, but only marginal levels were found in the dorsal skin. Similar to many other vertebrates, common goldfish have a specific pigment pattern revealing red color in the dorsal area and a progressive gradation toward white-yellow tones in the abdominal skin. In mice,  $\alpha$ -MSH is the primary hormonal regulator of pigmentation and acts by binding to MC1R and elevating intracellular cAMP, thus inducing tyrosinase activity, the rate-limiting enzyme of the melanogenesis (8). Differences in dorsal and ventral pigmentation are caused by variation in action of agouti gene (34) that induces melanocytes to switch from the syntheses of black pigment (eumelanin) to yellow-red pigment (phaeomelanin) by antagonizing  $\alpha$ -MSH effects at MC1R. Thus, the light-bellied agouti phenotype ( $A^W$ ) results from a pulse of agouti expression during the hair growth cycle in the dorsum (leading to agouti-colored coat hairs) and continuous expression during the hair growth cycle in the ventrum (resulting in a yellow to white coat hairs). Differential AGRP expression in the ventral skin supports a role in pigment synthesis and suggests that AGRP may be involved in the color patterning of the goldfish. A possible role of AGRP in the control of color patterning was previously suggested in chicken (16).

*In situ* hybridization studies further characterized neuronal expression of AGRP gene in the goldfish brain. Results demonstrated that the restriction of the central AGRP-mRNA expression to the infundibular hypothalamus has been conserved throughout vertebrate evolution. In the goldfish brain, AGRP transcripts were exclusively localized within the posterior part of the lateral tuberal nucleus as well as in the periventricular area of the lateral recess nucleus. The lateral tuberal nucleus has been proposed to be the teleostean homolog of the mammalian arcuate nucleus (21), and our results further support this homology. In rodents AGRP-mRNA expression is confined to the arcuate nucleus, particularly in the same set of neurons that produce NPY. AGRP-NPY colocalization has also been corroborated in Japanese quail (35). In goldfish, data on central expression of NPY mRNA are inconsistent. *In situ* hybridization studies failed to detect NPY-mRNA in the infundibular hypothalamus, and only a weak signal was reported within the hypothalamic inferior lobe (36, 37), but studies by Northern blot have reported strong NPY expression levels within the hypothalamic area. In addition, hypothalamic NPY mRNA lev-

els are markedly up-regulated by fasting in goldfish (38, 39). Similarly, NPY-like immunoreactivity has been reported within the lateral tuberal nucleus of the goldfish infundibular hypothalamus (40). Therefore, more experiments are required to corroborate the evolutionary conservation of hypothalamic NPY-AGRP colocalization.

Interestingly, 1 of 30 animals assayed by *in situ* hybridization displayed high AGRP-mRNA levels in the NLTI. The latter nucleus is known to be the main melanin-concentrating hormone expression site in the fish brain, including goldfish (Cerdá-Reverter, J. M., unpublished results). This neurohormone is released to the bloodstream following white background adaptation, provoking concentration of melanin granules in the melanocytes, inducing skin palloring (41). We hypothesize that if AGRP-mRNA is expressed within NLTI of some animals, the peptide could be released to the bloodstream as a neurohormone in a similar way to that of melanin-concentrating hormone, resulting in a morphological color alteration analogous to that of agouti yellow mice. However, screening of five pure white and yellow color animals each by *in situ* hybridization failed to detect AGRP expression within the NLTI (data not shown). In mammals, arcuate AGRP expression is strongly up-regulated by fasting (42). Our results demonstrate that goldfish hypothalamic AGRP transcription is up-regulated after 3 d fasting. AGRP levels remain elevated up to 7 d fasting, reaching maximum values at 5 d. *In situ* hybridization studies on 5-d fasted animals demonstrated that this increased AGRP expression occurred only within the goldfish periventricular area of the NLT (data not shown).

Our previous results suggested that the central melanocortin system exerts an inhibitory tone on goldfish food intake through MC4R signaling. Involvement of the melanocortin system in the control of energy balance of fish is corroborated by the cobalt phenotype of rainbow trout, so named because of their cobalt-blue body color. This phenomenon has been attributed to the absence of most of the pars intermedia of the pituitary, in which  $\alpha$ -MSH is synthesized. This variant of trout is hyperphagic and typically also has an enlarged liver and fat accumulation in the abdominal cavity, reflecting the absence of  $\alpha$ -MSH lipolytic activity (43). The regulatory mechanisms of the melanocortin inhibitory tone are unknown because hypothalamic POMC-mRNA levels remain unchanged during progressive fasting up to 7 d (22). Data reported here show that the melanocortin inhibitory tone might be regulated by AGRP gene overexpression during fasting. Proteolytic cleavage of POMC in the brain mainly produces  $\alpha$ -MSH that tonically inhibits food intake by acting at central MC4R. During progressive fasting and energy depletion, hypothalamic AGRP production could antagonize  $\alpha$ -MSH effects at MC4R decreasing melanocortin inhibition, thus enhancing feeding drive. Experiments designed to evaluate *in vitro* AGRP antagonist on central MCRs as well as *in vivo* effects of centrally administered goldfish AGRP on food intake are required to corroborate this hypothesis. Recently cloned goldfish MC4R (21) and MC5R (Cerdá-Reverter, J. M., unpublished results) together with production of recombinant goldfish AGRP will help to elucidate the physiological significance of AGRP overexpression during fasting in goldfish.

In conclusion, we demonstrate that the goldfish genome has a homolog gene to mammalian AGRP, which is both centrally and peripherally expressed. Central expression, restricted to the infundibular hypothalamus, is up-regulated by fasting, thus suggesting that goldfish AGRP might display a functional role in the melanocortin inhibition of food intake observed in goldfish.

## Acknowledgments

Received April 10, 2003. Accepted June 26, 2003.

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This work was supported by Grant A6371 (to R.E.P.) from the Natural Sciences and Engineering Research Council of Canada. During this work J.M.C.-R. was recipient of a Killam Postdoctoral Fellowship from Killam Trust Association.

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