ACTH, α-MSH, and control of cortisol release: cloning, sequencing, and functional expression of the melanocortin-2 and melanocortin-5 receptor in Cyprinus carpio

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Submitted 7 December 2004; accepted in final form 3 May 2005

Metz, Juriaan R., Edwin J. W. Geven, Erwin H. van den Burg, and Gert Flik. ACTH, α-MSH, and control of cortisol release: cloning, sequencing, and functional expression of the melanocortin-2 and melanocortin-5 receptor in Cyprinus carpio. Am J Physiol Regul Integr Comp Physiol 289: R814–R826, 2005. First published May 12, 2005; doi:10.1152/ajpregu.00826.2004.—Cortisol release from fish head kidney during the acute phase of the stress response is controlled by the adrenocorticotropic hormone (ACTH) from the pituitary pars distalis (PD). Alpha-melanocyte-stimulating hormone (α-MSH) and β-endorphin, from the pars intermedia (PI), have been implicated in cortisol release during the chronic phase. The present study addresses the regulation of cortisol release by ACTH and α-MSH in common carp (Cyprinus carpio) and includes characterization of their receptors, namely, the melanocortin-2 and melanocortin-5 receptors (MC2R and MC5R). We could not demonstrate corticotropic activity of α-MSH, β-endorphin, and combinations of these. We do show a corticotropic in the PI, but its identity is as yet uncertain. Carp restrained for 1 and 7 days showed elevated plasma cortisol and α-MSH levels; cortisol is still elevated but lower at day 7 than day 1 of restraint. Interrenal response capacity is unaffected, as estimated by stimulation with a maximum dose ACTH in a superfusion setup. MC2R and MC5R appear phylogenetically well conserved. MC2R is predominantly expressed in head kidney; a low abundance was found in spleen and kidney. MC5R is expressed in brain, pituitary PD, kidney, and skin. Quantitative PCR analysis of MC2R and MC5R expression in the head kidney of restrained fish reveals MC2R mRNA downregulation after 7 days restraint, in line with lower plasma cortisol levels seen. We discuss regulation of corticosteroid production from a phylogenetic perspective. We propose that increased levels of α-MSH exert a positive feedback on hypothalamic corticotropin-releasing hormone release to sustain a mild stress axis activity.

IN FISH, THE HYPOTHALAMO-PITUITARY-INTERRENAL AXIS (HPI-axis) is activated in response to stressors (69). Corticotropin-releasing hormone (CRH), produced in the hypothalamic nucleus preopticus, is projected toward the pars distalis (PD) and pars intermedia (PI) of the pituitary gland (28). There, it stimulates release of proopiomelanocortin (POMC)-derived peptides adrenocorticotropic hormone (ACTH; 45), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin. ACTH stimulates synthesis and release of cortisol from the interrenal cells of the head kidney (35, 52, 69). Cortisol secures energy redistribution in the body, pivotal to overcome the stressor (23, 69). Factors other than ACTH may be involved in the control of cortisol release in fish as well: factors such as sympathetic nerve fibers (1), β-endorphin (4), and diacetyl α-MSH (35, 36) have been suggested to have corticotropic activity. The latter suggestion was based on the observation that in tilapia kept in acidified water, plasma levels of α-MSH and cortisol are elevated (36). Moreover, a HPLC fraction of tilapia pars intermedia homogenate, which contained both diacetyl α-MSH and N-Ac β-endorphin, was shown to have corticotropic activity in vitro (4, 36). These observations led to the suggestion that ACTH is the principal stimulator of cortisol release in the acute phase of the stress response, whereas diacetyl α-MSH is the principal stimulator of cortisol release in the chronic phase (36).

This study focuses on the regulation of cortisol release by POMC-derived peptides ACTH and α-MSH in common carp, Cyprinus carpio. The study includes their receptors, namely, the melanocortin-2 and melanocortin-5 receptor (MC2R and MC5R). So far, no functional melanocortin receptor (MCR) profile of fish head kidney has been presented, and such a profile would definitively establish the cortisol-producing cell as target for ACTH and α-MSH. We believe that the study of MCR expression in fish, the earliest vertebrates, will unravel basic mechanisms in the control of acute- and chronic stress-related cortisol release from a phylogenetic perspective.

With respect to the role of MCRs in steroidogenesis, two remarkable and considerable differences exist between the mammalian adrenal gland and the fish head kidney. 1) In mammals, the adrenal cortex is organized in anatomically and functionally distinct layers, with the zona fasciculata as the main site of glucocorticoid production and the zona glomerulosa, where aldosterone is produced. In fish, no such organization is present: the head kidney of fish consists of groups of cortisol-producing cells, often associated with chromaffin cells, around the posterior cardinal veins and their branches, embedded in hematopoietic tissue (14). 2) Fish lack aldosterone, the mineralocorticoid of mammals. In fish, cortisol combines glucocorticoid, as well as mineralocorticoid functions, and its action is dependent on the receptor profile of the target cell (mineralocorticoid receptor and glucocorticoid receptor; 18, 21, 25, 59, 69). In the rat adrenal zona glomerulosa, MC5R is abundantly expressed (26) and may therefore mediate the observed stimulation of aldosterone production and release by α-MSH (30, 68). These two differences have implications for the regulation of steroidogenesis in fish: the cortisol-producing cell may be targeted by both ACTH and α-MSH, and thus both their receptors, MC2R and MC5R, need evaluation for our understanding of the control of cortisol release.

Our knowledge of the melanocortin receptors (MC1R–MC5R) is mainly based on mammalian studies. MCRs belong
to the superfamily of 7-transmembrane domain G protein-coupled receptors (48). Their natural ligands are peptides derived from the precursor hormone POMC; a special feature is that MCRs have endogenous antagonists, namely, agouti and agouti-related peptide. In addition to the well-known effects of the melanocortin peptides on skin pigmentation (via MC1R; 17) and adrenocortical glucocorticosteroidogenesis (via MC2R; 48), over the years, other functions were elucidated. Among these are the MC3R- and MC4R-mediated effects on food intake and energy balance and modulatory effects on the immune system (23, 42). MC2R in mammals is activated by no other natural ligand known except ACTH (19, 56). Therefore, the MC2R is referred to as the ACTH-receptor. The adrenal gland is the essentially exclusive site of its expression (15). Binding of ACTH to the MC2R induces synthesis and release of cortisol from the adrenal zona fasciculata (56). The mammalian MCSR is found in a variety of tissues, notably the skin, muscle, brain, adrenal zona glomerulosa, esophagus, pancreas, thymus, and circulating lymphocytes (15, 34). Characteristic for the MC5R is its expression in and control of exocrine glands, such as the Harderian, preputial, lacrimal, and sebaceous glands (13, 67).

Publicly available data from fish genome projects have recently led to the identification of the first piscine MCR sequences. For example, MC2R has been identified in zebrafish (Danio rerio; 40), Japanese pufferfish (fugu, Takifugu rubripes), and spotted green pufferfish (Tetraodon nigroviridis; 32). MCSR sequences were recognized in the same three species and in trout and goldfish (9, 27, 32, 51).

We present here the cDNA and deduced amino acid sequences of one MC2R and two MCRs of common carp (Cyprinus carpio L.). We investigated their distribution in several tissues and quantitatively assessed the expression in the head kidney in a restraint stress paradigm by means of real-time quantitative polymerase chain reaction. The expression profile in the head kidney is coupled to interrenal in vitro superfusion responses to ACTH and α-MSH.

MATERIALS AND METHODS

Animals. Common carp (Cyprinus carpio L.) of the all-male E4×R3R8 isogenic strain (7), weighing around 60 g, were raised by the Department of Fish Culture and Fisheries of the Wageningen University and Research Centre, The Netherlands. Fish were held in the Nijmegen facilities in 250-liter tanks at 22°C. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) on a ration of 1.5% of the body wt/day. Before sampling, fish were anesthetized in 0.1% (vol/vol) 2-phenoxyethanol (Sigma, St. Louis, MO). Experimental facilities in 250-liter tanks at 22°C. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) on a ration of 1.5% of the body wt/day. Before sampling, fish were anesthetized in 0.1% (vol/vol) 2-phenoxyethanol (Sigma, St. Louis, MO). Experimental protocols were used according to Dutch legislation and approved by the ethical committee of the Radboud University Nijmegen.

In vitro superfusion. Head kidneys and pituitary glands were removed directly after anesthesia. The pituitary glands were separated by netting for either 24 or 168 h. Time-matched groups of six carp were left undisturbed and served as controls.

RNA extraction and cDNA synthesis. Tissues were isolated and immediately homogenized by ultrasonication in 500 μl TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted according to the instructions of the manufacturer. First-strand cDNA was synthesized in a 1-h incubation at 37°C in 20-μl reaction mix containing 1 μg of total RNA, 300-ng random primers, 0.5 mM dNTPs, 10 units RNase Inhibitor (Invitrogen), 10 mM dithiothreitol and 200 U of superscript II reverse transcriptase (Invitrogen). cDNA was stored at −20°C.

Cloning, transformation and sequencing. For MC2R, PCR was carried out with 1 μl of head kidney cDNA as a template in a 50-μl amplification mixture containing 375 μM dNTPs, 1.5 mM MgCl2, 1 U Taq polymerase (Invitrogen) and 0.5 μM of each primer MC2R-fw and MC2R-rv (Table 1). The primer sequences were designed based on the zebrafish MC2R (accession number NM_180971). For MCSR, PCR was performed with 1 μl of brain cDNA as template and MCSR-fw1 and MCSR-rv1 as primers (Table 1). These primer sequences were based on the bovine MCSR (accession number NM_174109).

For both MC2R and MCSR, PCR was performed after an initial 2-min denaturation at 94°C. Forty cycles consisting of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C were performed in a thermocycler (Mastercycler, Eppendorf). A final 10-μl 72°C-elongation step was added to allow for TA cloning. The PCR products were analyzed on a 1% (wt/vol) agarose/ethidium bromide gel. The 501-bp MC2R and 417-bp MCSR products were cloned and sequenced (see below).
The 5'- and 3'-regions of MC2R were obtained using the GeneRacer protocol (GeneRacer kit, Invitrogen). Briefly, 1 µg of fresh carp head kidney total RNA was submitted to the following treatments: 1) dephosphorylation with calf intestinal phosphatase to eliminate truncated mRNA and non-mRNA, 2) removal of the 5' cap structure from intact full-length mRNA with tobacco acid pyrophosphatase, 3) ligation of a GeneRacer RNA oligo with known priming sites to the 5' end of the mRNA and 4) reverse transcription with a GeneRacer oligo dT (50 µM) containing a tail with known priming sites. 5' and 3’-RACE (rapid amplification of cDNA ends)-PCR were performed using GeneRacer primers and gene-specific primers designed on the basis of the 501-bp product: MC2R-5’-RACE and MC2R-3’-RACE1 (Table 1). Template DNA (1 µl RT mix), 10-pmol gene-specific primer, and 30-pmol GeneRacer primer were used in a 50-µl volume containing 1.75 mM MgCl₂, 350 µM dNTPs, and 3.75 U of a Taq polymerase/Tgo polymerase enzyme mix (Roche, Penzberg, Germany). The PCR profile consisted of 1) an initial denaturation of 2 min at 94°C; 2) 5 cycles of 30 s at 94°C and 2 min at 72°C; 3) 5 cycles of 30 s at 94°C, and 2 min at 70°C; 4) 30 cycles of 30 s at 94°C and 2 min at 68°C; and 5) a final 10-min elongation at 68°C. PCR products were analyzed on 1% (wt/vol) agarose/ethidium bromide gel. For 3’-RACE, 1 µl of the PCR product was used for nested PCR with primer MC2R-3’-RACE2. PCR consisted of 30 cycles of 30 s at 94°C and 2 min at 68°C. The obtained PCR product was analyzed on gel, cloned, and sequenced as described below.

The 5'-region of the MC5R was obtained by the GeneRacer protocol after a PCR using RACE-ready cDNA (see above) of carp brain as template and MC5R-RACE1 and MC5R-RACE2 (Table 1) as primers. We could not obtain the 3' region by RACE. The 3'-region was obtained by PCR using brain cDNA as template and MC5R-fw2, MCSR-rv2, and MCSR-rv3 (Table 1) as primers, based on the goldfish MC5R (accession number CAU576322). Cycling conditions were 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C for 40 cycles. PCR products were analyzed on gel and cloned and sequenced as described below.

All PCR products were ligated into pCR4-TOPO plasmid vector and introduced into chemically competent TOP10 Escherichia coli cells (TOPO TA Cloning kit, Invitrogen). After selection on LB-kanamycin agar, plasmids of transformed cells were isolated (Mini-Prep; Biorad, Hercules, CA) and sequenced by the dideoxynucleotide procedure (54) on an ABI Prism 310 automated sequencer (Applied Biosystems). Both strands of the insert were sequenced using T3 and T7 primers (Table 1).

Phylogenetic tree construction. A phylogenetic tree was constructed on the basis of amino acid difference (p-distance) by the neighbor-joining method (53) with MEGA version 2.1 (33). Sequences were retrieved from the SWISS-PROT, EMBL, and Genbank databases at the SRS mirror site of the Centre of Molecular and Biomolecular Informatics (http://services.ebi.ac.uk/srs71/). Multiple sequence alignments were carried out with the ClustalW program at http://www.ebi.ac.uk/clustalw/. Reliability of the tree was assessed by bootstrapping, using 1,000 bootstrap replications.

Real-time quantitative PCR. Relative expression of MC2R and MC5R was assessed by the use of quantitative RT-PCR. One microgram of total RNA was transcribed with 1 unit DNase I (amplification grade; Invitrogen) for 15 min at room temperature to ensure complete removal of traces of genomic DNA. To inactivate DNase, 1 µl of 25 mM EDTA was added, and the sample was incubated for 10 min at 65°C to simultaneously linearize the RNA. The RNA was reverse transcribed (RT) as described above.

For quantitative PCR analysis, 5 µl of 5 times diluted RT-mix was used as template in 25-µl amplification mixture, containing 12.5 µl SYBR Green Master Mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3.75 µl of each primer (final concentration 300 nM). The primer sets used in the PCR were for MC2R: qMC2R-Fw and qMC2R-Rv; for β-actin qACT-Fw and qACT-Rv; for 40S ribosomal protein S11 q40S-Fw and q40S-Rv (Table 1). After an initial step at 95°C for 10 min, a real-time quantitative PCR of 40 cycles was performed (GeneAmp 5700, Applied Biosystems), each cycle consisting of 15-s denaturation at 95°C and 1 min annealing and extension at 60°C. Cycle threshold (CT) values were determined and expression was calculated as a percentage of β-actin or 40S expression (49). Both housekeeping genes gave similar results. For clarity, results are presented here only relatively to β-actin expression.

Table 1. Primer sequences and corresponding positions in the open reading frames

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Gene Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC2R</td>
<td>MC2R-fw</td>
<td>230-253a</td>
</tr>
<tr>
<td></td>
<td>MC2R-rv</td>
<td>730-707a</td>
</tr>
<tr>
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<td>MC2R-5’RACE</td>
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<td></td>
<td>MC2R-3’RACE2</td>
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<td>qMC2R-fw</td>
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<td>qMC2R-rv</td>
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<tr>
<td>MCSR</td>
<td>MCSR-fw1</td>
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<tr>
<td></td>
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<td>496-515</td>
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<tr>
<td></td>
<td>MCSR-rv1</td>
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</tr>
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<td>MCSR-rv2</td>
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</tr>
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<td>MCSR-rv3</td>
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</tr>
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<td></td>
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<td>qMC5R-rv</td>
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<td>q40S-rv</td>
<td>433-410</td>
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<tr>
<td>Vector</td>
<td>T3</td>
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</tr>
<tr>
<td></td>
<td>T7</td>
<td>TAATACGACTCACTATAGAG</td>
</tr>
</tbody>
</table>

*Zebrafish (NM_180971); *cow (NM_174109); *goldfish (CAU576322, indicated position is outside open reading frame); *carp β-actin (CYIACCTBA); *carp 40S ribosomal protein S11 (AB012087).
Statistics. Differences among groups were assessed by the nonparametric Mann-Whitney U-test. Superfusion data were analyzed by ANOVA and Tukey’s post hoc testing. Significance was accepted at $P < 0.05$. Values are expressed as means ± SD.

RESULTS

Superfusion experiments. To confirm whether pituitary pars intermedia (PI) tissue synthesizes products with corticotropic activity, head kidneys were stimulated with PI homogenates in a superfusion setup (Fig. 1A). A profound stimulation of cortisol release was observed. ACTH tested subsequently on the same tissue also stimulated the release of cortisol. After exposure to synthetic α-MSH, no changes in cortisol release were observed in any head kidney (Fig. 1, B and C). Also, the stress of 1- and 7-day restraint did not induce detectable interrenal responsiveness to α-MSH; nor were combinations of peptides, including diacetyl α-MSH and different forms of β-endorphin, effective. Upon stimulation with ACTH, the same tissues always responded with a consistent and firm rise in cortisol release.

Restraint altered interrenal sensitivity in two ways. 1) Initial cortisol release was higher in stressed fish than in control fish, in line with in vivo plasma cortisol levels; initial release in 7-day confined fish was lower than in 1-day confined fish but still significantly higher than in controls. 2) Although absolute cortisol release (area under the peak) in stressed fish was not significantly different from controls, the kinetics of the ACTH-stimulated cortisol release had changed in 7-day stressed fish; release returned to baseline levels significantly faster after removal of the ACTH stimulus.

Plasma hormones. After both 1 and 7 days of restraint, plasma cortisol levels were significantly elevated (Fig. 2A); after 7 days, cortisol levels had decreased again but were still significantly elevated compared with undisturbed fish. Plasma α-MSH levels had significantly increased following stress (Fig. 2B).

Cloning and sequencing of carp MC2R and MC5R. We cloned and sequenced the carp MC2R and MC5R to be able to assess the receptor profile in the head kidney. The full-length 1,303 bp cDNA MC2R sequence, which contains an open reading frame encoding a protein of 304 amino acids, is shown in Fig. 3 (submitted to EMBL database, accession number AJ605725). We sequenced a total of 25 independent 5’- and 3’-clones, and six full coding sequence clones, but we found only a single MC2R cDNA sequence. Amino acid identity to zebrafish (83%) and Fugu (54%) sequences is higher than to chicken (47%), human (48%), and bovine (45%) MC2R. There is one potential site for N-linked glycosylation in the N-terminal extracellular domain (Asn1) and there are two potential sites for phosphorylation by protein kinase C in intracellular domains (Thr137 and Thr287). Figure 4 shows a multiple alignment of the carp MC2R amino acid sequence with that of various other vertebrate species. Predicted transmembrane regions are indicated in the figure. The third intracellular loop of the carp sequence is seven amino acids longer than that of mammalian MC2Rs.

Sequence analysis of 32 clones yielded two independent sequences of carp MC5R, arbitrarily named MC5R-I and MC5R-II (Fig. 5; submitted to EMBL database under accession numbers AJ783917 and AJ783918, respectively). Both sequences contain open reading frames that encode 328 amino acid proteins. The nucleotide sequences of MC5R-I and MC5R-II are 95% identical, while the deduced amino acid sequences differ only in a single amino acid (Val100 $\rightarrow$ Ile100), a conservative substitution. MC5R-I has highest sequence identity with that of zebrafish (MC5R-a; 98%) and goldfish (97%). Amino acid identities with other fish species (Fugu 80%, zebrafish MC5R-b 75%) are higher than with phylogenetically more distant species (chicken 75%, human 69%). Three potential sites for N-linked glycosylation (Asn2, Asn25, and Asn38) and four potential protein kinase C phosphorylation sites (Thr160, Ser18, Thr210, and Ser319) are recognized in
the sequence. Figure 6 shows an alignment of MC5R amino acid sequences from several vertebrate species.

**Phylogenetic analysis.** Figure 7 shows the relationship between carp MC2R and both MC5Rs with orthologs of other species in a consensus phylogenetic tree, which was constructed with the neighbor-joining method with 1,000 bootstrap replications. The five different members of the MCR family cluster each in a separate clade. The topology of MC2R shows clustering of the piscine sequences, with sequences from cyprinid species clustering apart from the more modern Fugu species. The chicken MC2R separates piscine from mammalian MC2R sequences. The very similar sequences of carp MC5R-I, carp MC5R-II, goldfish MC5R, and zebrafish MC5R-a sequences cluster in close proximity, while fugu and Tetraodon MC5Rs separate these from zebrafish MC5R-b, suggesting that the two zebrafish MC5Rs may be the result from an early genome duplication event.

**Tissue distribution.** To assess the tissue distribution of the carp MC2R and MC5R gene expression, we used RT-PCR (Fig. 8). The expected 131 bp MC2R product was clearly present in head kidney and to a minor extent in spleen and kidney. This relatively low abundance was consistently observed in all three replicate analyses. The head kidney-RT, which served as control to exclude genomic DNA contamination, was negative. MC5R-I and II expression was observed in brain, pituitary pars distalis, kidney, and skin, but not in head kidney. Primer design was such that both MC5R-I and II cDNAs, without distinction, were amplified. All cDNA preparations appeared proper, as judged by the amplification of a 83-bp β-actin fragment in all organs.

**Expression during stress.** Quantitatively determined MC2R mRNA levels in head kidney were significantly lower after 7 days but not after 1 day of restraint (Fig. 9). Restraint stress did not induce detectable MC5R expression in carp head kidney (not shown).

**DISCUSSION**

The present study addresses the regulation of cortisol release in common carp. We made four key observations. First, our in-vitro experiments demonstrate that α-MSH, β-endorphin, and combinations of these have no direct corticotrophic action in carp. Second, the pituitary PI of carp also, next to ACTH from the PD, synthesizes one or more factors with corticotrophic activity, of which its identity is as yet uncertain. Third, after a 1-day and 7-day restraint stress period, in which we show that plasma levels of cortisol and α-MSH are elevated, in vitro stimulation of head kidneys with a maximal dose of ACTH results in unaltered interrenal cortisol synthesis and release capacity. Fourth, molecular cloning, sequencing, identification, and quantitatively determined tissue expression of two members of the melanocortin receptor family, namely, MC2R and MC5R, show that in carp head kidney MC2R, but not MC5R, is expressed. After 7 days of restraint, MC2R expression in head kidney is downregulated, and MC5R expression is not induced (which substantiates the lack of α-MSH sensitivity). We will discuss these key findings below.

**Control of cortisol release.** Previously, the corticotropic signal from the PI in tilapia has been attributed to diacetyl α-MSH (35) or to the combined action of diacetyl α-MSH and N-Ac β-endorphin (4). Both these suggestions were postulated on the basis of studies that used HPLC fractions of PI homogenates that contained α-MSH and N-Ac β-endorphin immunoreactivity. Synthetic forms of α-MSH and N-Ac β-endorphin, alone and in combination, did not stimulate cortisol release in the present study. It seems, therefore, likely to us that the HPLC fractions also have harbored an additional factor, which alone or in combination with α-MSH or N-Ac β-endorphin, has corticotropic activity.

ACTH and α-MSH both contain the His-Phe-Arg-Trp (HFRW) core sequence, which is required for binding to MCRs (56). In mammals, MC2R binds ACTH with high affinity (0.1 nM; Ref. 56), while affinity for α-MSH is negligibly low. The mammalian MC5R is activated by nanomolar concentrations of both ACTH and α-MSH (16, 34, 57). Pignatelli et al. (50) and Liakos et al. (38) showed that MC5R was not detected under basal conditions in the mammalian adrenal gland, but became detectable after 14 days starvation stress or 18 h exposure to ACTH. This tempted us to restrain carp for 1

**Fig. 2.** Plasma concentrations of cortisol (A) and α-MSH (B) as determined by radioimmunoassay. *P < 0.05; **P < 0.01; ***P < 0.001.
and 7 days and evaluate the receptor profile in the head kidney after this stress.

One-day restraint did not alter the expression of MC2R in the head kidney, but a prolonged (7 days) restraint period considerably decreased MC2R expression. In vitro, this was reflected by an altered interrenal response to ACTH: the response differed in kinetics, characterized by an earlier return to basal release after the removal of ACTH exposure. However, absolute cortisol output, reflected by the area under the curve, was not significantly different, which indicates that interrenal synthesis and release capacity was not affected by this stressor. Apparently, although restraint is experienced as a stressor, the allostatic load, defined as the capacity of an animal to maintain stability through change of physiological limits (44), was unaffected by the stressor chosen. Our observations contrast with studies performed by Balm and Pottinger (5), in which 48-h confined fish had reduced cortisol production capacity, as indicated by lower areas under the curve. Initial cortisol release from tissue of stressed fish was higher than from controls. This is further corroborated by plasma cortisol (F) values that correlate very well with initial release (IR) in vitro (IR/F = 0.47; r² = 0.71; P < 0.001). Apparently, head kidneys of stressed fish are activated to a “cortisol-producing state” in vitro.

Observations that led to the hypothesis that α-MSH is a corticotrope in tilapia included increased MSH-cell volume and elevated plasma α-MSH following stress (35, 46, 61), CRH-controlled release of α-MSH (62) and stimulation of

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**Fig. 3.** Full-length nucleotide and deduced amino acid sequence of carp melanocortin-2 receptor (MC2R) cDNA. The deduced amino acid sequence is displayed above the nucleotide sequence. The start codon is boxed in black, the stop codon is indicated by an asterisk (*). The putative polyadenylation signal is underlined. A potential glycosylation signal is boxed in white, and two potential protein kinase C (PKC) phosphorylation sites are boxed in grey. The EMBL accession number is AJ605725.
cortisol release in vitro by an α-MSH-containing HPLC fraction (35). In our species, the common carp, we observe three parallels: 1) prolonged stress induces increased plasma cortisol and α-MSH levels (this study); 2) α-MSH release from the PI is controlled by CRH (66); and 3) the PI synthesizes a corticotrophic signal (this study). However, direct stimulation of head kidneys from both control and stressed fish with any form of α-MSH (whether combined with β-endorphins or not) did not evoke a rise in cortisol release in carp. Moreover, expression of MC5R, anticipated to mediate α-MSH-sensitivity, could not be demonstrated in head kidney nor was it induced by prolonged stress. Therefore, we now tentatively conclude that cortisol release in carp is controlled mainly by ACTH, thus not only in the acute, but also in the chronic, phase of the stress response. Interestingly, α-MSH is able to permeate the blood-brain barrier (6, 60), and thus the enhanced plasma levels seen could have effects peripherally, as well as centrally. Peripherally, α-MSH is known to induce anti-inflammatory responses (39), to interfere with reproduction (58), and to enhance lipolysis (8). Centrally, it not only suppresses food intake (10, 11, 12), but it is also very likely that it activates CRH neurons in the nucleus preopticus, since in mammals, as well as in fish, it has been established that these cells express MC4R and MC5R (9, 11, 41). Thus during stress, the enhanced α-MSH production and cortisol release could function to sustain HPI-axis activity via a short-loop positive feedback mechanism.

Taken together, we postulate that in carp, acute stress causes elevation of cortisol through an ACTH surge, which acts via the MC2R. During prolonged stress, cortisol levels remain elevated, albeit lower than during the acute phase. This slight elevation is not directly induced by α-MSH (as suggested earlier), and indeed MC5R, anticipated to mediate α-MSH-sensitivity, is not expressed in the cortisol-producing cells. CRH from the nucleus preopticus stimulates POMC expression in both the pituitary PD and PI. From the PD, ACTH is secreted, which, in turn, stimulates cortisol release; from the PI, α-MSH is released, which provides positive feedback to CRH and peripherally mobilizes extra energy via lipolysis. The
Fig. 5. Nucleotide and deduced amino acid sequences of carp melanocortin-5 receptor-I (MC5R-I) and II cDNA. The deduced amino acid sequences are displayed above and under the nucleotide sequences, respectively. The start codon is boxed in black; the stop codon is indicated by an asterisk (*). Dots indicate identities, whereas hyphens indicate gaps. Three potential glycosylation sites are boxed in white, and four potential PKC phosphorylation sites are boxed in grey. The EMBL accession numbers are AJ783917 and AJ783918, respectively.
lower plasma cortisol levels during prolonged stress compared with acute stress are in accordance with the observed down-regulation of MC2R in the head kidney.

We cannot exclude a role for the other MCR family members in the control of cortisol release, but on the basis of mammalian literature, we did not consider them in the present study: MC1R is not expressed in the adrenal gland, but primarily in skin, where it controls pigment synthesis (19). MC3R is predominantly expressed in the brain and is involved in energy homeostasis (15, 24). Moreover, MC3R is considered the receptor for γ-MSH, as among the MCR subtypes, γ-MSH has highest affinity at MC3R (23). The γ-MSH sequence is lacking in POMC of teleost fish (31), including carp (3). Accordingly, in fugu it has been reported that MC3R is absent (32). MC4R is the exclusive brain receptor (47), where it is involved in energy homeostasis. In fish however, the distribution of MC4R differs from that in mammals: MC4R is expressed not only in brain, but also peripherally (11, 51), including in the head kidney (27), but whether it is expressed in interrenal cells or other cells, is unknown. In the present study, we did not consider the MC4R.

Sequence analysis and tissue distribution. The elucidation and the relatively high homology of piscine MCRs allows us to conclude that, in analogy with other key proteins in the stress response, MCR sequences have undergone little change during evolution. Because carp is a tetraploid species (63), we anticipated to find two sequences for MC2R and MC5R. Indeed, two 95% identical genes for MC5R were found in this study, albeit that the differences found in nucleotide sequences lead to only a single, conservative amino acid substitution (in predicted transmembrane region 2). Unexpectedly, we could find only a single MC2R cDNA sequence. Although we cannot rule out the possibility that there is a second transcript in carp, it seems more likely from our repeated trials that either a second transcript is identical or, more likely, that one gene is not expressed in head kidney. A similar phenomenon has been reported for rainbow trout, in which only one of two POMC mRNAs was detected in brains of sexually immature fish.

![Multiple alignment of MC5Rs of six different vertebrate species](attachment:image.png)
whereas mature fish expressed both genes (54). In carp, one of two POMC genes is preferentially expressed in the pituitary gland during the course of a temperature shock (2). The biological significance of two gene transcripts is uncertain, yet tetraploidy is known to offer benefits over diploidy: tetraploid fish grow faster, live longer, and have greater adaptability (63), which explains the interest of aquaculture practices for such species. The frequent occurrence of tetraploidy in early vertebrates is considered to have contributed to their evolutionary success (37).

The amino acid sequence characteristics of MC2R suggests one putative glycosylation site (specific to the consensus sequence Asn-Xaa-Ser/Thr; 43), which is conserved in other piscine and mammalian MC2R sequences. Two putative protein kinase C (PKC) phosphorylation sites (70) are both conserved in the piscine sequences. In MC5R, three potential glycosylation (two of which are conserved in mammals) and four PKC phosphorylation (two of which are conserved) sites were found. Although little is known about the functions of glycosylation and phosphorylation sites in MCRs, it seems

Fig. 7. Neighbor-joining tree of melanocortin receptor amino acid sequences. Numbers at branch nodes represent the confidence level of 1,000 bootstrap replications. Clusters of different and melanocortin receptor family members are shaded in light grey, clusters of teleost fish are shaded in dark grey. The starting point for cluster formation is indicated by an open circle. Accession numbers: Tetraodon (Tetraodon nigroviridis) MC1R, AAQ55176; Fugu (Takifugu rubripes) MC1R, AAQ55176; zebrafish (Danio rerio) MC1R, NP_851301; great skua (Catharacta skua) MC1R, AA49212; chicken (Gallus gallus) MC1R, P55167; mouse (Mus musculus) MC1R, BAD16661; jaguar (Panthera onca) MC1R, Q86588; dog (Canis familiaris) MC1R, Q77616; pig (Sus scrofa) MC1R, AAS17974; sheep (Ovis aries) MC1R, O19037; orangutan (Pongo pygmaeus) MC1R, Q86410; human (Homo sapiens) MC1R, Q01726; gorilla (Gorilla gorilla) MC1R, Q86419; carp (Cyprinus carpio) MC2R, AA65725; zebrafish MC2R, NP_851302; Tetraodon MC2R, AAO55177; Fugu MC2R, AO65550; chicken MC2R, AA24002; human MC2R, AH69074; mouse MC2R, NP_032586; guinea pig (Cavia porcellus) MC2R, Q9Z159; pig MC2R, AAQ013968; cow (Bos taurus) MC2R, NP_776534; sheep MC2R, Q9TU77; spiny dogfish (Squalus acanthias) MC3R, AA866720; chicken MC3R, BAA325555; zebrafish MC3R, NP_851303; pig MC3R, CAG30767; human MC3R, AAH69599; mouse MC3R, NP_032587; rat (Rattus norvegicus) MC3R, P32244; Fugu MC4R, AA065511; spiny dogfish MC4R, NP_775385; goldfish (Carassius auratus) MC4R, CAD58853; chicken MC4R, AAT73773; cow MC4R, Q9GLJ8; rat MC4R, NP_037231; mouse MC4R, P56450; pig MC4R, O97504; human MC4R, AAH69172; macaque (Macaca fascicularis) MC4R, BAC02596; carp MC5R-I, AJ783917; carp MC5R-II, AJ783918; golden MC5R, CAE11349; zebrafish MC5R-a, NP_775386; zebrafish MC5R-b, NP_775387; Fugu MC5R, AAO65553; Tetraodon MC5R, AAQ55179; spiny dogfish MC5R, AAS67890; chicken MC5R, BAA26540; rat MC5R, NP_037314; mouse MC5R, P41149; human MC5R, AAH69545; pig MC5R, Q9MZV8; sheep MC5R, P41983; and cow MC5R, P56451.

The amino acid sequence characteristics of MC2R suggests one putative glycosylation site (specific to the consensus sequence Asn-Xaa-Ser/Thr; 43), which is conserved in other piscine and mammalian MC2R sequences. Two putative protein kinase C (PKC) phosphorylation sites (70) are both conserved in the piscine sequences. In MC5R, three potential glycosylation (two of which are conserved in mammals) and four PKC phosphorylation (two of which are conserved) sites were found. Although little is known about the functions of glycosylation and phosphorylation sites in MCRs, it seems
likely that in analogy with other mammalian seven-transmembrane receptor sequences, these sites are involved in the regulation of receptor function (20).

Carp MC2R is predominantly expressed in the head kidney. As yet, no pharmacological binding assays have been performed on any fish MC2R. Regarding the high homology, similar expression pattern and downregulated expression during prolonged stress, we suggest that, in analogy with mammals, the carp MC2R in the head kidney is the ACTH receptor that triggers cortisol production by the interrenal cells.

In mammals, MC5R is expressed in a variety of tissues, including skin, muscle, adrenal cortex (zona glomerulosa), thymus, leukocytes, lung, spleen, ovary, testis, brain, and pituitary gland (15, 19). In carp, we detected MC5R expression in brain, skin, kidney, and pituitary pars distalis. Brains were not further dissected into separate areas in this study, but a very comprehensive study in the closely related goldfish revealed MC5R expression in many regions, including ventral telencephalon, nucleus lateralis tuberis, and nucleus preopticus (9). These areas are key sites in the initiation of the stress response (28, 69) and the control of food intake (9, 29). Our future studies will focus on MCR expression profile and function in these brain areas. Rainbow trout MC5R was detected not only in several brain areas and in the ovary but also profoundly in head kidney (27). We have not succeeded in demonstrating the existence of MC5R in carp head kidney. This indicates that among different and phylogenetically distant fish species, considerable differences in MCR expression profiles may occur.

In conclusion, we demonstrate that MC2R and MC5R genes are highly conserved from fish to humans. The cloning, characterization, and expression profiles of these receptors provide an important tool for further studies to assess their roles in neuroendocrine and behavioral control. We have performed for the first time a functional expression study of MCRs in fish. We provide strong evidence that during acute and prolonged stress, plasma cortisol rises through MC2R activation by ACTH. We take the observation that MC2R expression is downregulated during prolonged stress to explain the lower chronic plasma cortisol levels seen. Increased levels of α-MSH are anticipated to activate CRH release from the NPO to continue HPI-axis activation. The pituitary PI does secrete a corticotrope factor, but this is not α-MSH, N-Ac β-endorphin, or a combination of these two. We are now seeking this unknown corticotrope.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Tom Spanings for organization of fish husbandry and Mark Huising for useful comments on the manuscript.
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This study was financially supported by the Dutch Research Council, under project number 805-46-033.

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