Pigment-dispersing activities and cortisol-releasing activities of melanocortins
and their receptors in xanthophores and head kidneys of the goldfish *Carassius*
auratus

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Abstract

The five subtypes of melanocortin receptors (MCRs) mediate the functions of α -melanocyte-stimulating hormone (α -MSH) and adrenocorticotrophic hormone (ACTH). In fish, these hormones are involved in pigment dispersion and cortisol release, respectively. α -MSH-related peptides exhibit ACTH-like activity in certain fishes. We recently found that multiple *Mcr* transcripts are expressed in some cell types in the barfin flounder, which is related to regulation of α -MSH activities. Similar results were also observed for the cortisol-releasing activity of α -MSH-related peptides in the head kidney. The present study was undertaken to assess relationship between the expression of multiply expressed *Mcrs* and α -MSH activities using goldfish. We also determined if α -MSH-related peptides exhibit ACTH-like activity in goldfish. The transcripts of *Mc1r*, but not those of other subtypes, were observed in xanthophores. α -MSH, which has an acetyl group at the N-terminus, was found to disperse pigment in a dose-dependent manner in xanthophores. This potency was found to be slightly greater than that of desacetyl- α -MSH. These results support our findings that MCR has a higher affinity for α -MSH when single *Mcr* subtype is expressed. On the other hand, transcripts of *Mc2r*, but not those of other subtypes, were observed in the head kidney. ACTH₁₋₂₄-stimulated cortisol release was observed in a dose-dependent manner, while α -MSH-related peptides showed no activity. It therefore appears that MC2R also acts as an ACTH-specific receptor in goldfish and that association of α -MSH-related peptides upon release of cortisol is uncommon in fishes.

Keywords: adrenocorticotrophic hormone (ACTH); cortisol-release; goldfish; melanocortin; melanocortin receptor; melanocyte-stimulating hormone (MSH); pigment-dispersion.

1. Introduction

α -Melanocyte-stimulating hormone (α -MSH) and adrenocorticotrophic hormone (ACTH) are peptide hormones liberated from a common precursor known as proopiomelanocortin (POMC) [54–56]. While POMC is biosynthesized in both the pars distalis (PD) and the neurointermediate lobe (NIL) of the pituitary in teleost fish, ACTH is mainly produced in the PD, and α -MSH in the NIL in a manner that depends upon tissue-specific proteolytic cleavage [6, 21, 35, 39, 51]. While ACTH is generally composed of 39 amino acid residues, α -MSH is identical to *N*-acetyl-ACTH₁₋₁₃-amide. ACTH, α -MSH, and their related peptides are collectively classified as melanocortins (MCs) on the basis of the presence of the common amino acid sequence—His-Phe-Arg-Trp [12].

MC receptors (MCRs) are members of the G protein-coupled receptor (GPCR) family. The members of this family have seven transmembrane domains [16, 33, 34]. MC system consisting of POMC and MCRs in fish have been shown to be similar to that of mammals. For example, the ACTH signal is mainly mediated by the MC2 receptor (MC2R), which is one of the five subtypes of MCRs [23, 48]. While MC2R selectively binds ACTH, ACTH can bind to other MCRs in addition to MC2R [1, 23, 48]. Although α -MSH does not bind to MC2R, the signal of α -MSH, is mediated by other MCRs. The representative biological activities of ACTH and α -MSH (including cortisol release from interrenal cells [57–60] and pigment dispersion [12, 14, 15], respectively) are related to tissue-specific expression of different MCR subtypes [33]. However, the studies on the biological activities of the MC system using barfin flounder, *Verasper moseri*, a teleost, have shown interesting relationships between different molecular forms of α -MSH-related peptides and MCRs [25–27, 56].

The barfin flounder is a large commercially important flatfish that inhabits the Pacific coast of northern Japan. We demonstrated the existence of pigment-dispersing activities of α -MSH-related peptides in skin parts [26]. Interestingly, while α -MSH modified with a monoacetyl group at N-terminus was found to mediate dispersion of pigments in xanthophores, this peptide exhibited negligible pigment-dispersing activities in melanophores. On the contrary, desacetyl (Des-Ac)- α -MSH, which lacks the acetyl group, has pigment-dispersing activities in

both xanthophores and melanophores, while the activities in xanthophores were found to be lower than the activities caused by α -MSH. Subsequently, we identified expression of only *Mc5r* in xanthophores and concomitant expression of *Mc1r* and *Mc5r* in melanophores [27]. Similar relationships between the degree of acetylation and biological activities were also observed for *in vitro* cortisol-releasing activities in the barfin flounder. While Des-Ac- α -MSH-stimulated cortisol release from interrenal cells was observed, α -MSH showed negligible effects. Moreover, both *Mc2r* and *Mc5r* were expressed in the interrenal cells [25].

There is a growing body of evidence indicating that many GPCRs form heterodimers that may affect ligand affinity [2, 5, 13, 28, 32, 36, 38, 43, 47]. Therefore, the concomitant expression of the different *Mcr* subtypes in melanophores and interrenal cells led to the assumption that a heterodimer consisting of MC1R and MC5R in melanophores, or MC2R and MC5R in interrenal cells, may have low binding affinity for α -MSH [25, 27]. This assumption conversely suggests that expression of only one *Mcr* subtype may lead to enhancement of biological activity of α -MSH relative to that of Des-Ac- α -MSH. In fact, acetylation enhances the activities of α -MSH-related peptides via MCRs expressed in human embryonic kidney-293 cells [44–46]. The present studies were undertaken to examine these possibilities using pigment cells and head kidney tissues from goldfish, *Carassius auratus*. We also determined if the cortisol-releasing activities of α -MSH-related peptides are common to goldfish by determining whether other *Mcrs*, in addition to *Mc2r*, are expressed in goldfish head kidney.

2. Materials and Methods

2.1. Fish

Immature goldfish, *C. auratus*, were obtained from a commercial dealer in Shizuoka, Japan, and all experiments were conducted according to the Guidelines for the Care and Use of Animals of Kitasato University. The fish were reared in indoor tanks with circulating freshwater under a natural

photoperiod. The average body sizes of the fish used for molecular cloning and gene expression tests and pigment-dispersing activities were 4.9 cm, standard length (SL), and 3.9 g body weight (BW). For these experiments, tissue samples were collected from fish anesthetized with 0.2% 2-phenoxyethanol, and subsequently frozen in dry ice/ethanol bath. Skin samples used for measurements of pigment-dispersing activities and for cell dispersion were collected from fish (4.9 cm SL, 4.1 g BW on average) anesthetized with ice-cold water. Head kidneys used in experiments for cortisol-releasing activities were collected from fish (13.5 cm SL, 87 g BW on average) anesthetized with 0.2% 2-phenoxyethanol.

2.2. Peptides

α -MSH was purchased from the Peptide Institute (Osaka, Japan). Diacetyl (Di-Ac)- α -MSH was purchased from Sigma Chemical (St. Louis, MO, USA). Des-Ac- α -MSH and ACTH₁₋₂₄ were synthesized and purified according to the previously described methods [53]. The amino acid sequence of ACTH₁₋₂₄, which is identical to that of barfin flounder ACTH-A, salmon ACTH-A, and tuna ACTH [52], differs by one residue with respect to the sequence of goldfish ACTH at position 20 (Ile in goldfish *vs* Val in others) [11].

2.3. Molecular cloning

2.3.1. Nucleic acid preparation for sequence determination

Total RNA was extracted from brain and head kidney using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNAs were synthesized from total brain RNA for amplification of *Mc1r* and *Mc3r* cDNA, and from head kidney RNA for *Mc2r* cDNA with the SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA). Custom oligonucleotides were synthesized at Nihon Gene Research Labs Inc. (Sendai, Japan).

2.3.2. Amplification of DNA fragments for sequence determination

Polymerase chain reaction (PCR) using a thermal cycler (MJ Mini, BIO-RAD, Hercules, CA, USA) under conventional conditions was performed to

amplify the DNA fragments with HotStar Taq Master Mix (Qiagen, Hilden, Germany), or Takara LA Taq (Takara, Otsu, Japan). Amplification procedures were common for all three *Mcr* cDNAs. First, the middle segment of the cDNA of each *Mcr* was amplified from first-strand cDNA by PCR using HotStar Taq DNA polymerase with primers designed from fish *Mcr* nucleotide sequences. Then, the 3' region of each *Mcr* cDNA was amplified from first-strand cDNA by 3' rapid amplification of cDNA ends (3'RACE) using HotStar Taq DNA polymerase with a gene-specific primers and Universal Primer A Mix (UPM) provided in the SMART RACE cDNA Amplification Kit. The 5' region of each *Mcr* cDNA was amplified from first-strand cDNA by 5'RACE using HotStar Taq DNA polymerase with UPM and gene-specific primers. Finally, each *Mcr* cDNA containing the full-length reading frame was amplified from first-strand cDNA by PCR using HotStar Taq DNA polymerase with gene-specific primers. Primer sequences are listed in Table 1.

2.3.3. *Sequence determination and data processing*

PCR-amplified DNA was purified by agarose gel electrophoresis (NuSieve GTG Agarose; Cambrex Bio Science, Rockland, ME, USA). DNA was extracted from the agarose gel using a QIAEX II Gel Extraction Kit (Qiagen), ligated into plasmid pT7 Blue T-Vector (Novagen, Madison, WI, USA) or pSTBlue-1 AccepTor Vector (Novagen) and transfected into JM109-competent cells. Recombinant plasmid DNA was prepared using the alkaline-SDS method and both strands were sequenced using a capillary DNA sequencer (3130-Avant Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing Ready Kit ver. 3.1. DNASIS-Pro (Hitachi Software Engineering, Yokohama, Japan) was used to process nucleotide and amino acid sequences, to calculate amino acid sequence identity, to align amino acid sequences, and to construct a phylogenetic tree by the neighbor-joining (NJ) method [37]. Transmembrane domains were predicted using a program for the prediction of transmembrane helices in proteins "TMHMM Server v2.0" (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

2.4. Reverse transcription (RT)-PCR for tissue distribution

Dorsal skin, caudal fin, and head kidney tissue were taken from three goldfish. A sample of RNA was prepared using Isogen (Nippon Gene). RNA was then treated with TURBO DNase (Ambion, Austin, TX, USA) for 4 h at 37°C. RNA yield was estimated by spectrophotometry. For each tissue, an equal amount of total RNA (100 ng) from three individuals was combined and subjected to amplification using a One-Step RT-PCR kit (Qiagen) with primer sets shown in Table 2. *β -actin* cDNA was used as a positive control. PCR products were electrophoresed on 3% agarose gel (Agarose S, Nippon Gene) and visualized with 0.025% ethidium bromide. Photographs were taken using a Densitograph (Atto, Tokyo, Japan).

2.5. Skin cell dispersion and RT-PCR

Skin cell dispersion was performed as described previously [26, 27]. Small parts of caudal fin or dorsal skin were rinsed in Hanks' balanced salt solution (HBSS). The samples were allowed to stand for 20 min at room temperature in a dissociation medium [DM: 1 mg/mL collagenase type III (Worthington, Freehold, NJ, USA), 1×10^{-4} M epinephrine (Sigma-Aldrich, St. Louis, MO, USA), 2 mg/mL bovine serum albumin (Sigma-Aldrich), 0.1 mg/mL soybean trypsin inhibitor (Roche, Indianapolis, IN, USA), and 5 U/mL DNase I (Takara)] and then gently agitated for 10 min in the same solution. The DM was removed, and the samples were rinsed three times with HBSS. Finally, during the gentle agitation in fresh DM, dispersed single xanthophores and other nonchromatophoric dermal cells were isolated using glass capillaries under a microscope. cDNA from an isolated cell was synthesized using the Super-Script III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA obtained from three cells was dissolved in 90 μ L H₂O, and a 5- μ L aliquot was analyzed using PCR to detect *Mcr* mRNAs. As a control, 2 μ L was analyzed to detect *β -actin* mRNA. The primer sets for *Mcrs* and *β -actin* mRNA were the same as those used in section 2.4. The PCR conditions programmed into the thermal cycler (MJ Mini; BIO-RAD) included reverse transcription at 50°C for 50 min followed by

amplification of the appropriate cDNA fragment with activation of the enzyme at 95°C for 15 min followed by 40 cycles of (i) denaturation for 15 s at 94°C, (ii) annealing for 30 s at 60°C, and (iii) extension for 40 s at 72°C. The PCR products were electrophoresed and visualized as described above. The series of experiments from cell dispersion to PCR was repeated three times.

2.6. Incubation of fish scales with MC peptides for measurements of pigment-dispersing activities

Scales removed from the dorsal skin were incubated in HBSS for 1 h at 20°C. After changing HBSS, the scales were incubated under the same conditions. Subsequently, the scales were incubated in HBSS containing serially diluted MSH at final concentration of 1 nM to 1 µM for 1 h. Photographs were taken using a light microscope equipped with a digital still camera (PDMCII, Olympus, Japan), and subsequently ten randomly selected xanthophores from five skin parts were observed for each peptide concentration. The xanthophore index (XI) was calculated to evaluate the pigment-dispersing activity of each peptide by analogy with melanophore index [11]. An average XI obtained from 10 xanthophores represented XI of each skin part (n = 5).

2.7. Incubation of head kidney parts with MC peptides for measuring cortisol-releasing activities

L-15 medium was used for incubation. The washing and incubation temperature was 20°C. Head kidney tissues dissected from several goldfish were diced to approximately 1 mm³ and combined. A flask containing the diced tissue (1 g/100 mL) was incubated for 30 min with occasional shaking. This incubation for washing was repeated three times and the medium was exchanged at the end of each incubation period. Following the transfer of approximately 20 mg of the diced tissues to each well of a 48-well plate, the tissue was preincubated for 90 min in 0.5 mL medium. After changing the medium, the tissue was incubated for an additional 60 min in 0.5 mL medium containing MC peptides (ACTH₁₋₂₄, Des-Ac-α-MSH, α-MSH, or Di-Ac-α-MSH). Final concentrations ranged from 10 nM to 100 nM. Control experiments were carried

out for the same time periods without including hormone in the medium. Each assay was performed in duplicate ($n = 5$). Preincubation and incubation media were used for the cortisol assay. Cortisol levels after incubation were expressed as percentage of the levels present in the preincubation media. Cortisol was extracted from the medium as described previously [63]. The cortisol levels were measured using a time-resolved fluoroimmunoassay for cortisol [62].

2.8. Statistics

All data are expressed as the mean $\pm$ standard error values. The variances of the xanthophore index were tested by the Kruskal–Wallis test [50], a nonparametric test analog to one-way analysis of variance (ANOVA). When significant differences were detected, comparisons of treatments versus the control were performed with a post hoc multiple comparison test for the Kruskal–Wallis test. The amounts of cortisol in the head kidney were compared by one-way ANOVA. Significance was determined at 5% level.

3. Results

3.1. Nucleic acid sequence determination

Because *Mc4r* and *Mc5r* cDNAs have been cloned in goldfish [8, 10], the nucleotide sequences of *Mc1r*, *Mc2r*, and *Mc3r* cDNAs were determined in the present study. Fig. 1 shows the assembly of cDNA fragments amplified by RT-PCR for each *Mcr* cDNA. Nucleotide sequences were determined using these fragments.

3.1.1. Nucleic acid sequence of goldfish *Mc1r* cDNA

The nucleotide sequence of a cDNA amplified from goldfish brain total RNA was found to encode a reading frame consisting of 321 amino acid residues (Fig. 2). Phylogenetic analysis revealed that the new sequence is localized in a clade of MC1R of various species in a phylogenetic tree for the five different MCRs (Fig. 3). This indicates that the new receptor sequence encodes MC1R of goldfish. The locations of the seven transmembrane domains were predicted as

shown in Fig. 2. The DRY motif is present at a position homologous to that of MC1R of the other species [33]. There are three potential sites for *N*-linked glycosylation [4] and eight potential sites for phosphorylation by protein kinase C [61] (Fig. 2).

3.1.2. *Nucleic acid sequence of goldfish Mc2r cDNA*

The nucleotide sequence of a cDNA amplified from goldfish head kidney total RNA was found to encode a reading frame consisting of 302 amino acid residues (Fig. 2). Phylogenetic analysis revealed that the new sequence is grouped into a clade together with the remaining MC2Rs (Fig. 3). This indicates that the new receptor sequence encodes for the goldfish MC2R. The locations of the seven transmembrane domains are shown in Fig. 2. The DRY motif is present at a position homologous to that of MC2R of the other species. There is one potential site for *N*-linked glycosylation as well as three potential sites for phosphorylation by protein kinase C (Fig. 2).

3.1.3. *Nucleic acid sequence of goldfish Mc3r cDNA*

The nucleotide sequence of a cDNA amplified from total RNA of goldfish brain was found to encode a reading frame consisting of 327 amino acid residues (Fig. 2). Phylogenetic analysis revealed that the new sequence is related to the MC3Rs in other species (Fig. 3). This indicates that the new receptor sequence encodes a goldfish MC3R. The locations of the seven transmembrane domains were predicted as shown in Fig. 2. The DRY motif is present at a position homologous to that of MC3R of the other species. There are three potential sites for *N*-linked glycosylation and two potential sites for phosphorylation by protein kinase C (Fig. 2).

3.2. *Expression of Mcrs in goldfish skin, fin and head kidney*

Fig. 4 shows the detection by RT-PCR of all cloned goldfish *Mc*rs in the fin, skin and head kidney. The negative control for the expression of each *Mc*r and amplification of positive control *β -actin* are also shown. Expression of *Mc1r* was observed in skin and fin. Expression of *Mc2r* was observed in head kidney.

When RT-PCR was performed for the all five goldfish *Mcrs* by using total RNA extracted from single cells of the fin and skin, a cDNA fragment of *Mc1r* was amplified from the total RNA derived from xanthophores (Fig. 5). None of the cDNA fragments of the five subtypes of *Mcr* were amplified from the total RNA derived from nonchromatophoric dermal cells. Because of the limitations of the kit used in this experiment, the absence of genomic DNA in the total RNA samples was indirectly evaluated using β -actin amplification. The β -actin fragment containing the intron was never amplified (Fig. 5).

3.3. Biological activities of α -MSH-related peptides

3.3.1. Pigment-dispersing activities

Fig. 6 shows the effects of α -MSH-related peptides on *in vitro* pigment dispersion in goldfish scales. Des-Ac- α -MSH was found to stimulate the dispersion of pigments in xanthophores in a dose-dependent manner (Fig. 6A). α -MSH and Di-Ac- α -MSH also showed similar effects on pigment dispersion (Figs. 6B and C). At a concentration of 1 nM, the effect of α -MSH was greater than that of Des-Ac- α -MSH. At a concentration of 100 nM, the effect of Di-Ac- α -MSH was greater than that of Des-Ac- α -MSH.

3.3.2. Cortisol-releasing activities

The effects of MC peptides on *in vitro* cortisol release from the head kidney of goldfish are shown in Fig. 7. ACTH₁₋₂₄ was found to stimulate cortisol release in a dose-dependent manner. However, Des-Ac- α -MSH, α -MSH, and Di-Ac- α -MSH were found to have negligible effects on cortisol release.

4. Discussion

4.1. Phylogenetic distribution of MCR and the MC system in goldfish

The presence of MCR has been demonstrated across a wide spectrum of vertebrate classes, including Cephalaspidomorphi (lampreys), Chondrichthyes (sharks), Sarcopterygii (lobe-finned fish including tetrapods), and Actinopterygii (ray-finned fish) [17, 18, 24, 48]. While mammals and chickens possess five

MCR subtypes (MC1R to MC5R), zebrafish is the only fish species in which the five subtypes were demonstrated with MC5R subdivided into MC5Ra and MC5Rb [30]. *Fugu* lacks *Mc3r*, while four *Mcr* subtypes have been identified by genomic studies [23, 30]. In goldfish, the presence of *Mc4r* and *Mc5r* has been shown in our previous studies [8, 10]. Therefore, the identification of *Mc1r*, *Mc2r*, and *Mc3r* cDNA provides a second line of evidence for the presence of five MCR subtypes in fish. The cypriniformes including goldfish and zebrafish are a rather primitive group of ray-finned fish when compared to tetradontiformes [37]. It is therefore possible that the five subtypes of MCR may have appeared in a common ancestor of ray-finned fish and tetrapods. Subsequently, in the branch leading to derived group of ray-finned fish such as tetradontiformes, *Mc3r* may have been deleted during the course of evolution.

In goldfish, the primary structure of POMC has been reported previously [11]. MC peptides such as Des-Ac- α -MSH, α -MSH, Di-Ac- α -MSH, and some ACTH variants released from pituitary cells have been identified [58]. Moreover, primary structures of agouti-signaling protein and agouti-related protein as endogenous antagonists for α -MSH have been reported in goldfish [7, 9]. Here, we demonstrated the presence of five MCR subtypes thus showing that the goldfish MC system possesses a molecular repertoire comparable to that of tetrapods.

4.2. Effects of α -MSH-related peptides on pigment dispersion

The pigment-dispersing activity of Des-Ac- α -MSH, α -MSH, and Di-Ac- α -MSH are almost equal in goldfish, but the activity of α -MSH is somewhat greater than that of Des-Ac- α -MSH. These results are similar to those observed in xanthophores of barfin flounder [26]. In both species, the xanthophores express only one *Mcr* subtype, i.e. *Mc1r* and *Mc5r* in goldfish and barfin flounder, respectively. On the other hand, the response of goldfish xanthophores to α -MSH is quite different from that of melanophores of barfin flounder in which α -MSH has negligible effects on melanin dispersion in a range from 1 nM to 1 μ M. Goldfish xanthophores are also different from barfin flounder melanophores because two different subtypes of *Mcr*—*Mc1r* and *Mc5r*—are expressed in the

flounder melanophores [27]. We recently observed that *Mc1r* and *Mc5r* are expressed in melanophores of Japanese flounder where α -MSH showed no effects on pigment dispersion, while Des-Ac- α -MSH is effective. Moreover, both α -MSH and Des-Ac- α -MSH are effective in xanthophores where only *Mc5r* is expressed (manuscript in preparation). Taken together, these results indicate that there is a good correlation between pigment-dispersing activities, the degree of acetylation, and the specific *Mcr* subtype(s) expressed in chromatophores. α -MSH-related peptides exhibit pigment-dispersing activities irrespective of the degree of acetylation when one type of *Mcr* is expressed and monoacetylation, which leads to generation of α -MSH, cancels the activities when two types of *Mcrs* are concomitantly expressed.

Monoacetylation of the N-terminus may contribute to an increase in pigment-dispersing activity of α -MSH-related peptides in goldfish because the activity of α -MSH was found to be slightly but significantly higher than Des-Ac- α -MSH when their effects at the concentration of 1 nM were compared. Pharmacological studies on sea bass MC1R have revealed that α -MSH is more effective than Des-Ac- α -MSH in stimulation of cellular activities [44]. Acetylation-mediated augmentation of the binding affinity of α -MSH-related peptides was also observed during pharmacological studies with human and mouse MC1R [3, 49]. Taken together, these results suggest that α -MSH may have higher activity than Des-Ac- α -MSH as a result of increased affinity for an MCR when only one MC subtype (at least MC1R or MC5R) is present. Similar enhancing effects of pigment-dispersion caused by monoacetylation have also been observed in grass carp, tilapia, and frogs [12, 22, 59]. According to our data, it is reasonable to propose that only one *Mcr* subtype may be expressed in melanophores of these species.

The pigment-dispersing activity of α -MSH was found to be indistinguishable from that of Di-Ac- α -MSH in the goldfish xanthophores. In pharmacological studies of sea bass, α -MSH was found to have efficacy similar to that of Di-Ac- α -MSH for stimulation of cellular activities via MC1R [45]. Given that goldfish MC1R may have similar properties to sea bass, it is likely that α -

MSH and Di-Ac- α -MSH interact with goldfish MC1R with similar binding affinities.

4.3. *Effects of ACTH and α -MSH-related peptides on cortisol-release*

Recently, we reported on interesting relationships among molecular forms of α -MSH-related peptides with respect to N-terminal acetylation, cortisol-releasing activity, and *Mcr* subtypes expressed in interrenal cells of barfin flounder [25]. First, Di-Ac- α -MSH and Des-Ac- α -MSH were found to stimulate cortisol release, and the former showed greater activity than the latter, while the activity of α -MSH was negligible. Second, transcripts of *Mc2r* and *Mc5r* were detected in the interrenal cell. These relationships are similar to those observed for pigment-dispersing activities of α -MSH-related peptides in melanophores of this specie [26, 27]. Hence, we assumed that a heterodimer consisting of MC2R and MC5R is associated with the differences in the activity of these peptides in the interrenal cells. The present study was undertaken to characterize the relationships between all the three players in the goldfish. The *Mc2r* was found to be the only *Mcr* expressed in head kidney containing interrenal cells and, supporting our hypothesis, the three α -MSH-related peptides were found to not have cortisol-releasing activities.

Among the five MCR subtypes, MC2R has been shown to be an ACTH-specific receptor because MC2R exclusively binds ACTH but not α -MSH-related peptides [1, 23, 48]. A classical study has shown that a central region of the ACTH molecule, which consists of basic amino acid residues, is important for binding to the receptor [19, 40]. These properties have been confirmed using native and expressed ACTH-receptors (namely MC2R) [19, 20, 41]. In the present study, it was found that ACTH₁₋₂₄, which consists of an N-terminal region corresponding to α -MSH, a central basic region corresponding to ACTH₁₅₋₁₈, and a short C-terminal region corresponding to ACTH₁₉₋₂₄, exhibits dose-dependent cortisol releasing activity. These results confirm that ACTH is a major secretagogue of cortisol in goldfish and MC2R the main link of the pituitary-interrenal axis.

Because MC2R is a specific receptor for ACTH, the other MC peptides never interact with this receptor [23, 33, 48]. Therefore, the finding that there are no cDNAs for *Mcr* subtypes other than *Mc2r* cDNA is in agreement with the lack of cortisol-releasing activities of α -MSH-related peptides in goldfish head kidney tissues. Also in carp, ACTH stimulates cortisol release, but α -MSH does not [31]. The presence and absence of the MC2R and MC5R, respectively, have been shown for this species, while investigations of the other MCR subtypes have not been reported. Even though cypriniformes such as goldfish and carp generally have only the MC2R subtype in interrenal cells, two MCR subtypes may heterodimerize in tilapia, rainbow trout and barfin flounder because cortisol-releasing activities of α -MSH-related peptides have been observed in these species [25, 29, 42]. Interestingly, tilapia has some similarities to barfin flounder in relation to potency of cortisol-releasing activities and degrees of acetylation. Di-Ac- α -MSH has greater activity than Des-Ac- α -MSH, and α -MSH exhibits the lowest activity among the three α -MSH-related peptides. Similar results have also been observed in investigations of rainbow trout, while data are not available for Di-Ac- α -MSH.

5. Conclusions

We recently hypothesized that coexpression of different types of *Mcr* leads to the formation of heterodimers of MCRs, which decrease the pigment-dispersing activities of α -MSH. The activity of this peptide is enhanced only when one subtype of MCR is present [26, 27]. In goldfish xanthophores, where only *Mc1r* was expressed, α -MSH displayed similar activity to that of Des-Ac- α -MSH and Di-Ac- α -MSH; moreover, the activity of α -MSH was greater than that of Des-Ac- α -MSH. These results support our assumption. On the other hand, ACTH₁₋₂₄, but not any α -MSH-related peptides, was found to stimulate cortisol release from goldfish head kidney where only the *Mc2r* subtype is expressed. These results indicate that MC2R is specific for ACTH and the link member in the pituitary-interrenal axis of the goldfish.

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795 Figure legends

796 Figure 1. Schematic representation of the relative positions of the
797 DNA fragments of *Mc1r* (A), *Mc2r* (B), and *Mc3r* (C) in goldfish. DNA fragments
798 were amplified from brain or head kidney cDNA. Boxes show reading frames.
799 Horizontal arrow shows relative positions and direction of primers. The
800 numbers show positions on each cDNA. For details regarding the nucleotide
801 sequences, see Accession No. AB618067 for *Mc1r*, AB618068 for *Mc2r*, and
802 AB618069 for *Mc3r*.

803

804 Figure 2. Amino acid sequences of five subtypes of goldfish MCRs. Common
805 amino acids with MC1R are shaded. Transmembrane domains (TM) were
806 deduced for each MCR subtype. Italicized amino acids show potential *N*-
807 glycosylation motifs. Lowercase letters show potential protein kinase C
808 phosphorylation motifs. Numbers in parentheses show sequence identity with
809 MC1R sequence. ###DRY motif.

810

811 Figure 3. Phylogenetic tree for MC receptors of fish, chickens, and mammals
812 including 5 MCR subtypes of goldfish constructed by the NJ method. Accession
813 numbers of MC1R for *Fugu*: AAO65548, flounder: AB287974, goldfish: AB618067,
814 zebrafish: NP_851301, chicken: P55167, mouse: BAD16661, and human:
815 Q01726; MC2R for *Fugu*: AO65550, flounder: AB541411, goldfish: AB618068,
816 zebrafish: NP_851302, chicken: BAA24002, mouse: NP_032586, and human:
817 AAH69074; MC3R for goldfish: AB618069, zebrafish: NP_851303, chicken:
818 BAA32555, mouse: NP_032587, and human: AAH69599; MC4R for *Fugu*:
819 AAO65551, flounder: AB287975, goldfish: CAD58853, zebrafish: NP_775385,
820 chicken: AAT73773, mouse: P56450, and human: AAH69172; MC5R for *Fugu*:
821 AAO65553, flounder: AB540951, goldfish: CAE11349, zebrafish-a: NP_775386,
822 zebrafish-b: NP_775387, chicken: BAA25640, mouse: P41149, and human:
823 AAH69545.

824

825 Figure 4. Expression of *Mcrs* in caudal fin (A), dorsal skin (B), and head kidney
826 (C). Total RNA samples prepared from these tissues were subjected to RT-PCR

(RT+) using specific primers for *Mcrs* (see Table 2 for primer sequences). Numbers indicate *Mcr* subtypes. “RT” indicates negative control (PCR for *Mcrs* in total RNA). “Positive control” shows amplification of *β-actin* fragment in each tissue.

Figure 5. Expression of *Mcrs* in cells isolated from the caudal fin (A) and dorsal skin (B). RT-PCR was performed using the total RNA extracted from xanthophores and nonchromatophoric dermal cells (see Table 2 for primer sequences). Numbers indicate *Mcr* subtypes. Total RNAs prepared from three single cells were combined. *β-Actin* was used as an internal control. Amplification of the *β-actin* fragment from xanthophores “X,” and nonchromatophoric dermal cells “D” was not observed. Genomic DNA was used as a template in “G.” The amplified DNA for *β-actin* contained one intron.

Figure 6. Pigment-dispersing activity of synthetic Des-Ac- α -MSH, α -MSH, and Di-Ac- α -MSH on xanthophores. XI, xanthophore index analogous to the melanophore index. Asterisks show significant differences compared to the control value determined by a post hoc comparison test for the Kruskal–Wallis test at $P < 0.05$. Alphabetical letters indicate the differences in potency among the three peptides at one dose according to the results of the same test at $P < 0.05$ ($n = 5$). For example, the pigment-dispersing activity of 1 nM α -MSH was greater than that of 1 nM Des-Ac- α -MSH.

Figure 7. Effects of MC peptides, including ACTH₁₋₂₄, Des-Ac- α -MSH, α -MSH, and Di-Ac- α -MSH on *in vitro* cortisol release from the head kidney of goldfish. Head kidney tissue parts were preincubated for 1 h and then followed by incubation with each MC peptide for 1 h. The concentration of cortisol in the incubation medium was estimated relative to that of the preincubation medium. Asterisks show significant differences compared to the control value by ANOVA at $P < 0.05$ ($n = 5$).

A

B

C

MC1R	MNDssrHYFsmkHMDYIYNIDNWI7LN77LGEMNATGIAQIMIPQELFLMLGLISLVENILVVAIIKN	69
MC2R	MWSS-----TEALSTHPTDCAEVQVPSQVFMIAIVASLSENILVILAVIKN	46
MC3R	MNDSYLQFLKGQKPAWS7SLPPNGSTVDPPAG---ALCEVQVQIAEVFLTLGIVSLENILVILAVVKN	66
MC4R	MW7SHHHGPHHsyrWH5QGALPVGKPDQGERGSTSGCYEQLLISTEVFLTLGLVSLLENILVIAAIIKN	69
MC5R	MMW7SEATLSLWASISANSSPVLDLLN77ETPSHAKPKACEQLNIATEVFLILGLISLLENILVICAIVKN	70
TM1		
MC1R	RNLHSPMYFICCLAVSDMLVSVSNVVETLFMLLKEHGLLLVtakMLQHLDNVIDIMICSSVVSSLSFLC	139
MC2R	RNLHSPMYCFICNLAVFNTISSLCKSLETILLLFKEAGHLN--GRFELNIDDIMDSLLCMCFLGSIFSIL	114
MC3R	KNLHSPMYFFLCSLAAADMLVSVSNSLETIVIAVLNSRLLVASDHFVRLMDNVFDSMICISLVASICNLL	136
MC4R	KNLHSPMYFICSLAVADLLVSVSNASETVVMALITGGWtyrESIKNMDNIFDSMICSSLLASIWSLL	139
MC5R	KNLHSPMYFVCSLAVADMLVSVSNAWETIVIYLLtnrQLVVEDHFIRQMDNVFDSMICISVVASMCSSL	140
TM2		
TM3		
MC1R	TIAADRYITIFYALRYHSIMTtqrAVAIIVVWLTSTITSSSLFIVYHTDNAVIACLVTFFGLTLVFTAVL	209
MC2R	TIAVDRYISIFHALRYHTLmtmrRVVVTLSLIWVFCGTSGVLMIGFSNAAtvkIFFVVLFFTALLLILL	184
MC3R	AIAVDRYVTIFYALRYHSIVtvrRALVAIAGIWLVCVVCVGVFIVYSESKTVIVCLITMFFAMLVLMATL	206
MC4R	AIAVDRYITIFYALRYHNIMtqrRAGTIITCIWTLCTVSGVLFIVYSESTTVLICLISMFFIMLALMASL	209
MC5R	AIAVDRYVTIFYALRYHNIMtvrRAAFIIGGIWTFCTSCGVFIIYSdN7SVIVCLVSMFFIMLALMASL	210
###		
TM4		
TM5		
MC1R	YLHMFILAHVHsrriMALH-----KsrrQATsmkGAITLTILLGVFVICWGPFFLHLILILICPTNP	271
MC2R	YVHMFLLARHHANRIASMP-----GLHARQRQSGLRGALTTLILIGVFVACWAPFSLHLILISMICPENP	248
MC3R	YVHMFLLARLHVQRIAAALPPAAAAAGNPAPRQRSCMEGAVTISILIGVFVCCWAPFFLHLILLVSCPHHP	276
MC4R	YVHMFLLARLHMKRIAALP-----GNGPIWQAANMKGAITITILLGVFVVCWAPFFLHLILMISCPRNP	273
MC5R	YSHMFMLARSHVKRIAALP-----GYNSIHQRASmkAAVTLTILLGIFIVCWAPFFLHLILMISCPRNL	274
TM6		
MC1R	YCKCYFSHFNLFLILITICNSLIDPLIYAYRSQELRKtLkEMIFCSWLFAM	- 321
MC2R	YCECYRSLFQLHVLLLVSHAVIEPAIYAFRSTELRNtykKVFLCSASRIFKECV	(40%) 302
MC3R	LCLCYMSHFTTYLVLMCNSVIDPLIYACRSLEMRKtfkeILCCFGCQPPL	(49%) 327
MC4R	YCICFMSHFNMYLILIMCNSVIDPLIYAFRSQEMRKtfkeICCCWYGLASLCV	(53%) 326
MC5R	YCMCFMSHFNMYLILIMCNSVIDPLIYAFRSQEMRKtLkEITCCYsLrNVFGMSR	(51%) 329
TM7

Table 1

Custom oligonucleotide primers used for PCR to amplify cDNA fragments of goldfish *Mc1r*, *2r*, and *3r*.

Primer	Target	Nucleotide sequence
MC1-fw1	MC1R	5'-ATG (TC)TA CTG A(CA)G GA(GC) CAT GG-3'
MC1-rv1	MC1R	5'-(GT)GC TGA A(AG)T AGC ACT TGC AG-3'
GSP-MC1-fw1	MC1R	5'-CGT CAC GTT TTT TGG CTT GA-3'
GSP-MC1-rv1	MC1R	5'-GGA AAC GAC GGA ACT GCA TA-3'
GSP-MC1-fw2	MC1R	5'-GTC AAA GGT GTG CTG AAG GA-3'
GSP-MC1-rv2	MC1R	5'-CAA CGC AGA TGC TCC TTA AG-3'
MC2-fw1	MC2R	5'-AC(TA) GAC TGC GCT GAG GTC CA-3'
MC2-rv1	MC2R	5'-CAC ATG CAG AGT AGA GAG TC-3'
MC2-fw2	MC2R	5'-GTT GTT TAA AGA CGC CGG AC-3'
MC2-rv2	MC2R	5'-GAG TGA (AG)CG GTA GCA (TC)TC AC-3'
GSP-MC2-fw1	MC2R	5'-TTG ATT GGG GTG TTT GTG GC-3'
GSP-MC2-rv1	MC2R	5'-CTT GAT GTC GGC TAG GAT CA-3'
GSP-MC2-fw2	MC2R	5'-CCA GAC TCA TGT CTC TGA GA-3'
GSP-MC2-rv2	MC2R	5'-GTG AAG CAT GTA TTG CTG GG-3'
MC3-fw1	MC3R	5'-TAT GTG ACG AGG TCC (AC)(AG)A T(CT)C A-3'
MC3-rv1	MC3R	5'-AG(AG) A(CT)C AGG TAT GTG (GT)TG AA(AG)-3'
GSP-MC3-fw1	MC3R	5'-CCA CCT CAT TCT GCT GGT GT-3'
GSP-MC3-rv-1	MC3R	5'-CCA AGA TGA CGA GGA TGT TC-3'
GSP-MC3-fw2	MC3R	5'-CAG TCC ACC ATC TGA ATC AG-3'
GSP-MC3-rv2	MC3R	5'-ACC ACC ATG CTT TGG CAT CT-3'
UPM	MC1R, 2R, 3R	5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT -3'

Synthesis of primers was performed by Nihon Gene Research Lab. (Sendai, Japan).

Table 2

Custom oligonucleotide primers used for tissue distribution of goldfish *Mcrcs*.

Primer	Target	Nucleotide sequence
MC1-TD-fw1	MC1R	5'-GCT TGT CAC GGC AAA GAT GT-3'
MC1-TD-rv1	MC1R	5'-TGG CTT GTC GGC GAC TCT TA-3'
MC2-TD-fw1	MC2R	5'-ACA CCT GAA CGG TCG TTT CG-3'
MC2-TD-rv1	MC2R	5'-CTC AAG CCA CTT TGT CTC TG-3'
MC3-TD-fw1	MC3R	5'-TGT CTG TTC TTC CCC ATC TC-3'
MC3-TD-rv1	MC3R	5'-GGC GAT TGT TTA GTA CAG CA-3'
MC4-TD-fw1	MC4R	5'-TGC CTC CGA AAC GGT AGT GA-3'
MC4-TD-rv1	MC4R	5'-GCT GAT AAG GCA GAT GAG AA-3'
MC5-TD-fw1	MC5R	5'-CTG TCA CTT TGG GCC ATC AG-3'
MC5-TD-rv1	MC5R	5'-TCT GAT GAA ATG GTC CTC CA-3'

Synthesis of primers was performed by Nihon Gene Research Lab. (Sendai, Japan).