Multifunctional Roles of Melanocyte-Stimulating Hormone and Melanin-Concentrating Hormone in Fish: Evolution from Classical Body Color Change

Akiyoshi Takahashi, Kanta Mizusawa and Masafumi Amano

School of Marine Biosciences
Kitasato University
1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
e-mail: akiyoshi@kitasato-u.ac.jp

Abstract
The representative role of melanocyte-stimulating hormone (MSH) and melanin-concentrating hormone (MCH) in fish is regulation of pigment migration. However, our studies using barfin flounder Verasper moseri, a flatfish as a major experimental fish, have revealed that MSH and MCH are multifunctional because their receptors are widely distributed not only in the melanophores but also in the brain and systemic body. Their biological roles other than control of pigment migration would be regulation of feeding behavior, energy metabolism, cortisol release, etc. Among them, an interesting biological process on molecular level has been observed in the role of α-MSH. A fine difference in the structure—presence or absence of one acetyl group—modified the activities. Namely, desacetyl-α-MSH having no acetyl group at N-terminal stimulates pigment dispersion in melanophore and cortisol release from the interrenal gland, while α-MSH having one acetyl group has negligible effects. On the whole body level, MCH probably transfers information about photic conditions from the external environment to the body. MCH production is changeable, depending on the difference in the intensity of the light. A white background enhances production of MCH, and MCH turns body color pale by aggregating pigments in scales. It is suggested that this peptide stimulates feeding behavior. This monograph reveals molecular characteristic and biological significance of MSH and MCH systems in fish.

1. General introduction

Body color change of fish in response to background coloration is an important biological process such as environmental adaptation for camouflage. Some fish species including flounders change their surface hue to match their background. The interactions and networks of endocrine and neural systems may be responsible for the regulation of color and pattern (Fujii and Oshima 1986, 1994; Eberle 1988; Fuji 2000). In the endocrine system, two peptide hormones—melanocyte-stimulating hormone (MSH) and melanin-concentrating hormone (MCH)—produced in the hypothalamo-pituitary axis play roles as mutual physiological antagonists through opposite effects on pigment migration. α-MSH produced in the pituitary gland turns the body to a dark color by stimulating melanin dispersion, while MCH, which is produced in the hypothalamic area of the brain and released from pars nervosa (PN), turns the body to a pale color by aggregating melanin granules (Takahashi and Kawauchi 2006a).

α-MSH is synthesized as part of a large precursor protein called proopiomelanocortin (POMC) together with other peptides including β-MSH, γ-MSH, adrenocorticotropic hormone (ACTH), and β-endorphin (β-END). On the other hand, MCH is derived from a different precursor protein, proMCH. MSHs and MCH exhibit their activities by interacting with specific receptors—called melanocortin receptors (MCRs) for MSH, and MCH receptors (MCHR), respectively (Takahashi and Kawauchi 2006a). These receptors are expressed not only in skins but also in many other tissues. It is, therefore, suggested that α-MSH and MCH...
exhibit multiple effects to maintain a discreet homeostatic status in many aspects of the fishes’ endocrine system beyond the body color change.

The barfin flounder is a member of flatfish, Pleuronectiformes, inhabiting the Pacific coast of northern Japan (Andoh et al. 1999). This species is the mostly derived teleost order after Tetradontiformes containing Fugu (Nelson 2006). Using barfin flounder as experimental fish, we have obtained some interesting features of MSH, MCH, and their receptors concerning the number of each hormone and receptor genes, post-translational processing, tissue distribution, pigment synthesis/dispersion, expression profiles in relation to background color change, and receptor functions. This monograph deals with advances on the understanding of the biological significance of MSH and MCH together with their receptors in fish that has been achieved by our research group.

2. Proopiomelanocortin (POMC) of barfin flounder

POMC is the common precursor of ACTH, MSHs, and β-END (Nakanishi et al. 1979). ACTH and MSHs are collectively called melanocortin (MC). Over the past decade, considerable progress has been made on the primary structure of the POMC from a broad taxonomic group of vertebrates (Takahashi and Kawauchi 2006a, b). The results suggest that three MSHs (α-, β-, and γ-MSH) and a single END were established in early vertebrates. Thereafter, unequal crossing over may have resulted in class-specific numbers of MSH segments during the radiation of fish.

The genomic structure of the POMC gene has been reported in various species including some fish and shown to be well conserved (Gonzalez-Nunez et al. 2003; Hansen et al. 2003). Two introns are present at homologous positions, and all functional segments, ACTH, MSHs, and β-END, are encoded on exon 3. Several transcription factors synergistically participate in the initiation of transcription of the POMC gene (Therrien and Drouin 1991).

Each segment of POMC (N-POMC, ACTH, and β-lipotropic hormone: β-LPH) itself consists of small segments: N-POMC contains γ-MSH and joining peptide (JP), ACTH contains α-MSH and CLIP, and β-LPH contains β-MSH and β-END (Takahashi and Kawauchi 2006a, b). Although the pomc is expressed in both ACTH cells of the pars distalis (PD) and MSH cells in the pars intermedia (PI) of the pituitary, the final gene products are different in each cell type. In the ACTH cells, POMC is cleaved to N-POMC, ACTH, and β-LPH. A significant part of β-LPH is further cleaved to γ-LPH and β-END. In the MSH cells, N-POMC is further cleaved to form γ-MSH, ACTH is cleaved to form α-MSH and CLIP, and β-LPH is cleaved to form β-MSH and β-END. Several of the generated peptides and POMC itself undergo modifi-
cations such as glycosylation, amidation, phosphorylation, and acetylation.

Using barfin flounder as an experimental fish, we characterized the POMC on cDNA sequence, structure of each hormonal segment, genomic sequence including intron and 5' upstream region, posttranslational events such as processing, and tissue distribution.

Conventional molecular cloning methods were used to clone cDNA molecules from mRNA prepared from several kinds of fish pituitaries. Moreover, gene structure of POMC including intron and 5' upstream regions were determined using genomic DNA prepared from liver. POMC-derived peptides in an extract of one pituitary gland were analyzed by a combination of high-performance liquid chromatography (HPLC) and mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was also performed for direct profiling of the pituitary slice. POMC mRNAs were detected by reverse transcription (RT)-polymerase chain reaction (PCR) and in situ hybridization. In some case, products of RT-PCR were further analyzed by Southern hybridization. Pituitary contents of POMC mRNA were measured by quantitative real-time PCR.

2.1. Structure

Working with the barfin flounder, we identified for the first time the presence of three subtypes of pomc (Takahashi et al. 2005a). Later, the presence of three functional pomc types was also reported in rainbow trout (Leder and Silverstein 2006). Barfin flounder (bf) prePOMC-A, -B, and -C are composed of 199, 214, and 220 amino acid residues (aa), respectively. Protein sequence alignment of POMCs reveals that bfPOMC-A and bfPOMC-B are composed of an N-POMC, an ACTH segment containing α-MSH, and a β-LPH segment containing β-MSH and β-END, as in other teleost POMCs (Fig. 1). bfPOMC-C has a similar organization, whereas significant mutations are observed in the segment corresponding to the β-END essential sequence (from Tyr-Gly-Gly-Phe-Met to Ser-Gly-Arg-Phe-Met). Based on the phylogenetic tree, bfPOMC-A and bfPOMC-B retain the structural characteristics of invariant copies, because they make up a clade with the POMCs of tuna, tilapia, sea bass, sockeye salmon-A, and rainbow trout-A (Fig. 2). However, bfPOMC-C is considered to be a variant copy because it comprises a clade with POMC-B of sockeye salmon and rainbow trout.

A phylogenetic tree suggests that the lineage leading to an ancestor of bfPOMC-A and bfPOMC-B and that leading to bfPOMC-C may have diverged from a common ancestor at the first duplication event, and then the lineage of bfPOMC-A may have diverged from that of bfPOMC-B at the second duplication event (Fig. 2). Thereafter, bfPOMC-C may have accumulated a number of point mutations in the N-POMC segment and in the β-END-core sequence, causing the loss of biological activity after the first duplication. These structural characteristics suggest that bfPOMC-C has become specialized as a precursor of MC peptides.

2.2. Gene organization

Genomic structure of bfPOMC is depicted in Fig. 3. bfPOMC-A and bfPOMC-B contain α-MSH, β-MSH, and β-END as in other teleost POMCs; however, the primary structure of bfPOMC-C is different from these two precursors in a sense that it contains functional MSHs and nonfunctional β-END—a trace of β-END— at the C-terminal (Takahashi et al. 2005a). Despite the differences in the hormonal organization, bfPOMC-A, bfPOMC-B, and bfPOMC-C are each composed of three exons and two introns (Kobayashi et al. 2008b). The gene organization is the same as that of other POMCs reported in tetrapods, ray-finned fish, and agnathans (Nakanishi et al. 1981; Cochet et al. 1982; Notake et al. 1983; Drouin et al. 1985; Deen et al. 1992; Takeuchi et al. 1999; Gonzalez-Nunez et al. 2003; Hansen et al. 2003; Takahashi et al. 2005b).

Gentic information encoded in each exon of the three bfPOMCs is also similar to that of the other POMC genes; exon 1 encodes an untranslated nucleotide sequence, exon 2 encodes a signal peptide and N-terminal short region, and exon 3 encodes the remaining segment including ACTH, MSHs, and β-END—a trace of β-END in the case of bfPOMC-C. The similarity among the three bfPOMCs supports our interpretation obtained from the cDNA sequence that these genes originated from a common ancestral POMC, and the β-END in POMC-C was secondarily mutated (Takahashi et al. 2005a). We also suggested that the lineage leading to an ancestor of bfPOMC-A and bfPOMC-B may have diverged from that leading to the bfPOMC-C at the first duplication event and then the lineage of bfPOMC-A may have diverged from that of bfPOMC-B at the second duplication event. This suggestion is supported by the presence of the microsatellite consisting of CA repeat (adenylic acid–cytidylic acid dinucleotide repeat) in intron B in both bfPOMC-A and bfPOMC-B genes.

2.3. Transcription elements

While pomc-a is exclusively expressed in the pituitary, pomc-b and pomc-c are expressed in a variety of non-pituitary tissues in addition to the pituitary gland, and the expression levels of pomc-c is greater than pomc-b in non-pituitary tissues (Takahashi et al. 2005a). These differences seem to be ascribed to the tissue-specific transcription manner of each POMC.

The presence of several kinds of transcription elements suggests that the expression of the three POMC genes is regulated by synergistic interactions of a variety of regulatory factors (Fig. 4) as that in mammals (Therrien and Drouin 1991). One POMC conserved element (PPCE), which was originally found in teleost Tetraodon (Bumaschny et al. 2007), one pituitary-restricted transcription factor (Tpit), which is one of the pomc-specific transcription factors in mammals (Lamolet et al. 2001), and two TATA boxes are commonly present in the 5′-flanking regions of the three POMC genes. E boxes and cyclic AMP response element (CRE)-like elements are also commonly observed in the region flanked by the two TATA boxes, while the number and distribution of these elements are inconsistent. Besides these elements, pomc-b and pomc-c possess CCAAT boxes, but pomc-a does not (Kobayashi et al. 2008b). It is, therefore, conceivable that PPCE and Tpit may play facilitating roles for expression in the pituitary and some other elements may be associated with the stimulation and/or inhibition of this expression in a variety of tissues including the pituitary.

2.4. Tissue distribution

The results of RT-PCR (Fig. 5) and in situ hybridization (Fig. 6) demonstrated that the pomc-a, pomc-b, and pomc-c are expressed in both the PD and PI of the
Similar distributions of pomc-a and pomc-b transcripts observed in adjacent sections, and those of pomc-b and pomc-c transcripts in other adjacent sections, and those of pomc-c and pomc-a transcripts in additional adjacent sections suggest that some cells express concomitantly the three POMC genes in the barfin founder pituitary gland. The stronger signal for pomc-a than for pomc-b and pomc-c in in situ hybridization shows the presence of a greater amount of transcript from pomc-a than from pomc-b and pomc-c. The detection of pomc-c transcripts was lowest, among the three pomcs in both the PD and PI.

The POMC-A mRNA content in the neurointermediate lobe (NIL, a tissue consisting of both PI and PN) was higher than that in the PD, regardless of the background color (Table 1) (Kobayashi et al. 2008b). Given that the transcription efficiency in the corticotrophs is the same as that in the melanotrophs, barfin founder pituitary (Takahashi et al. 2006).
this may be related to the difference in the number of POMC-producing cells (Amano et al. 2005). In this context, the similar contents of POMC-B and POMC-C mRNAs between the PD and PI suggest that the transcription efficiency of these two genes in the PD is higher than that in the PI.

In barfin flounder, pomc-a transcript was detected only in the pituitary by a combination of RT-PCR and Southern hybridization (Fig. 7). The pomc-b transcript was detected in the pituitary by RT-PCR, while it was

Table 1. The pituitary contents of POMC-A, POMC-B, and POMC-C mRNAs in the PD and NIL of barfin flounder reared in black or white tanks.

<table>
<thead>
<tr>
<th>Lobe</th>
<th>Tank color</th>
<th>mRNA contents (x10^6 copies/mg total RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>POMC-A</td>
</tr>
<tr>
<td>PD</td>
<td>White</td>
<td>2.88 ± 0.71 **</td>
</tr>
<tr>
<td>NIL</td>
<td>White</td>
<td>12.2 ± 2.69 **</td>
</tr>
<tr>
<td>PD</td>
<td>Black</td>
<td>5.15 ± 1.79 **</td>
</tr>
<tr>
<td>NIL</td>
<td>Black</td>
<td>19.6 ± 3.56 **</td>
</tr>
</tbody>
</table>

Pituitaries were taken from fish on day 0. NIL means a tissue consisting of both PI and PN. Each value is expressed as means ± SEM (n = 6). Different letters indicate a significant difference (P < 0.05). **(P < 0.01) indicate the levels of statistical differences between the two lobes. Reprinted from Gen. Comp. Endocrinol., 158, Kobayashi et al., Transcription elements and functional expression of proopiomelanocortin genes in the pituitary gland of the barfin flounder, 259–267, © 2008, with permission from Elsevier.

Table 2. Identification of bfPOMC-related peptides based on molecular weight.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Peptides</th>
<th>Location on POMC</th>
<th>Mass (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Calculated</td>
</tr>
<tr>
<td>18.3</td>
<td>N-β-LPH</td>
<td>C (110–144)</td>
<td>3947.4</td>
</tr>
<tr>
<td>19.8</td>
<td>N-β-LPH</td>
<td>A (94–117)</td>
<td>2631.7</td>
</tr>
<tr>
<td>22.0</td>
<td>β-ENDOR-like</td>
<td>C (166–174)</td>
<td>1091.6</td>
</tr>
<tr>
<td>22.9</td>
<td>β-MSH</td>
<td>A (120–136)</td>
<td>2053.3</td>
</tr>
<tr>
<td>23.1</td>
<td>β-MSH</td>
<td>B (143–159)</td>
<td>1965.2</td>
</tr>
<tr>
<td>24.7</td>
<td>Des-Ac-α-MSH</td>
<td>C (67–79)</td>
<td>1623.8</td>
</tr>
<tr>
<td>25.7</td>
<td>Des-Ac-α-MSH</td>
<td>A (51–63)/B (62–74)</td>
<td>1621.8</td>
</tr>
<tr>
<td>26.4</td>
<td>α-MSH</td>
<td>C (67–79)</td>
<td>1665.8</td>
</tr>
<tr>
<td>27.7</td>
<td>α-MSH</td>
<td>A (51–63)/B (62–74)</td>
<td>1663.8</td>
</tr>
<tr>
<td>28.4</td>
<td>N-POMC</td>
<td>A (1–48)</td>
<td>5171.5</td>
</tr>
<tr>
<td>28.9</td>
<td>β-MSH</td>
<td>C (147–163)</td>
<td>2021.2</td>
</tr>
<tr>
<td>29.3</td>
<td>Di-Ac-α-MSH</td>
<td>A (51–63)/B (62–74)</td>
<td>1705.8</td>
</tr>
<tr>
<td>30.2</td>
<td>CLIP</td>
<td>C (84–107)</td>
<td>2451.8</td>
</tr>
<tr>
<td>30.5</td>
<td>CLIP</td>
<td>A (68–91)</td>
<td>2713.0</td>
</tr>
<tr>
<td>33.5</td>
<td>CLIP</td>
<td>B (79–101)</td>
<td>2628.9</td>
</tr>
<tr>
<td>34.5</td>
<td>β-ENDOR-like</td>
<td>A (139–179)</td>
<td>4652.2</td>
</tr>
<tr>
<td>34.7</td>
<td>N-POMC</td>
<td>B (1–59)</td>
<td>6479.3</td>
</tr>
<tr>
<td>36.2</td>
<td>N-Ac-β-END</td>
<td>A (139–179)</td>
<td>4694.2</td>
</tr>
<tr>
<td>37.3</td>
<td>N-Ac-β-END</td>
<td>B (162–192)</td>
<td>3727.2</td>
</tr>
<tr>
<td>38.4</td>
<td>β-END-like</td>
<td>C (177–198)</td>
<td>2507.0</td>
</tr>
<tr>
<td>40.5</td>
<td>N-POMC</td>
<td>C (17–64)</td>
<td>5326.2</td>
</tr>
<tr>
<td>42.2</td>
<td>N-β-LPH</td>
<td>B (105–140)</td>
<td>3914.3</td>
</tr>
</tbody>
</table>

See Fig. 8 for chromatogram on HPLC in which retention time of MCH was 27.7. Reprinted from Gen. Comp. Endocrinol., 141, Takahashi et al., Nucleotide sequence and expression of three subtypes of proopiomelanocortin mRNA in barfin flounder, 291–303, © 2005, with permission from Elsevier.
also detected in brain, gill, heart, spleen, liver, stomach, intestine, testis, muscle, blood, and skin by a combination of RT-PCR and Southern hybridization. The pomc-α transcript was detected in the same tissues excluding liver and blood by RT-PCR, while expression in the liver was detected by Southern hybridization of the PCR product. Note that while the pomc-a is expressed exclusively in the pituitary, the pomc-b and pomc-c are expressed in a variety of non-pituitary tissues in addition to the pituitary. These differences in pomc-expressing tissue suggest that functional ramifications may have also occurred in the promoter region, and then the pomc-b and pomc-c might have acquired a multi-tissue expression pattern after the duplication event. Thereafter, pomc-c may have accumulated a number of point mutations in the N-POMC segment and in the β-END-core sequence causing a fadeout of biological activity after the first duplication (Fig. 2). These structural characteristics suggest that POMC-C has specialized to become a precursor of MC in barfin flounder (Fig. 1). The presence of β-END in bfPOMC-B suggests that the duration since the second duplication event is not sufficient for a missense mutation to be induced in the β-END-core sequence.

The pituitary content of three POMC mRNAs seems to be inversely proportional to the systemic distribution pattern of the mRNA (Takahashi et al. 2005a). The pituitary content of the POMC-A mRNA, which is expressed exclusively in the pituitary, is the most predominant (Table 1). The pituitary content of the POMC-C mRNA is the lowest (Table 1), but its transcripts could be detected in a variety of tissues (Fig. 7). The pituitary content of POMC-B mRNA was lower than POMC-A mRNA, but greater than POMC-C mRNA; POMC-B mRNA could be detected in a variety of tissues, but the signals were lower than those from POMC-C (Takahashi et al. 2005a). It seems that the trade-off for the occurrence of multi-tissue expression ability might have reduced the transcription ability in the pituitary.

2.5. Production, processing, and tissue distribution

The mass values of the peptides extracted from the whole pituitary and separated by HPLC in Fig. 8 were compared with those calculated from the deduced amino acid sequences of hormone segments of three bfPOMCs. The bfPOMC-related peptides thus identified are summarized in Table 2. The peptides derived from bfPOMC-A were N-POMC, desacetyl (Des-Ac)-α-MSH, α-MSH, diacetyl (Di-Ac)-α-MSH, corticotropin-like intermediate lobe peptide (CLIP), N-β-LPH, β-MSH, β-END1-31, and N-α-β-END1-31. The peptides derived from bfPOMC-B were N-POMC, Des-Ac-α-MSH, Di-Ac-α-MSH, CLIP, N-β-LPH, β-MSH, and N-α-β-END1-31. The peptides derived from bfPOMC-C were N-POMC17-44, Des-Ac-α-MSH, β-MSH, CLIP, N-β-LPH, α-MSH, β-END-like peptide1-9, and β-END-like peptide21-31. The identification of N-POMCs derived from POMC-A and -B reveals that the signal sequence of pre-POMC-A and -B is composed of 18 and 21 aa, respectively. A signal sequence of prePOMC-C consisting of 22 aa was deduced by sequence comparison of fish POMCs. Thus, we identified almost all of the POMC-related peptides.
in the extract of a whole pituitary taken from a single individual barfin flounder by mass spectrometry excluding N-POMC
14 derived from bPOMC-C. The absence of ACTH and β-LPH in the present study and the dominant population of MSH cells compared
to ACTH cells (Amano et al. 2005) suggest the major source of these peptides is the PD. Consequently, a combination of two different approaches, from cDNA or peptide, demonstrated the expression of three POMC genes in a single individual of barfin flounder.

Next, we examined the production of POMC-derived peptide in the PD and PI by using MALDI-TOF MS. α-MSH/A/B (amino acid sequence of α-MSH-A is identical to that of α-MSH-B), Des-Ac-α-MSH-A/B, β-MSH-A and -B, CLIP-A, N-β-LPH-A, N-Ac-β-END-A1-41, and N-POMC-A were detected from a frozen slice of the barfin flounder NIL by (Fig. 9). Thus, the characteristics of posttranslational processing of POMC in the barfin flounder PI of pituitary are thorough cleavage of POMC at all processing signals, and N-terminal acetylation or C-terminal truncation generating α-MSH and N-Ac-β-END-A1-41 (Fig. 10). This manner of processing is comparable to that in mammals (Smith and Funder 1988; Castro and Morrison 1997).

In addition to ACTH-A, which is a typical PD peptide, Des-Ac-α-MSH-A/B, CLIP-A, and β-MSH-A, which are generally accepted as PI peptides, were detected in the PD of barfin flounder pituitary (Takahashi et al. 2006). These results indicate that ACTH-A is generated from POMC-A in the PD, however, the ACTH-A is further cleaved to Des-Ac-α-MSH-A and CLIP-A. Cleavage of ACTH into α-MSH has also been shown in the cells of the PD of the chicken pituitary gland because of co-localization of these peptides in some cells (Hayashi et al. 1991). Detection of β-MSH-
A derived from POMC-A suggests that the β-LPH-A segment is also cleaved to N-β-LPH-A and β-MSH-A (Fig. 10). This peptide profile also indicates that the PD lacks an acetylation enzyme generating α-MSH as shown by the presence of Des-Ac-α-MSH-A/B and the absence of α-MSH-A/B. Therefore, it can be concluded in general that POMC is processed differentially in the two pituitary lobes and the processed peptides undergo similar modifications to those observed in mammals, while some differences are observed.

2.6. Structure of each hormonal segment

2.6A. ACTH

Among the three ACTHs of barfin flounder, ACTH-
A and ACTH-C are composed of 41 aa and ACTH-B 40 aa. In case of barfin flounder, ACTH-B is almost identical to tuna ACTH except one amino acid residue, while ACTH-A and ACTH-C show some deviations from the ACTH-B.

2.6B. α-MSH

α-MSH refers to acetyl-ACTH1-13-amide, which is acetylated at the N-terminal residue and contains an amide at the C-terminal. Most fish α-MSH are composed of 13 aa and are identical to mammalian α-MSH. This α-MSH is referred to herein as “common α-MSH.” In barfin flounder, the C-terminal amino acid residue of α-MSH (Thr-amide) derived from POMC-
C is different from common α-MSH (Val-amide).

2.6C. β-MSHs

Barfin flounder three β-MSHs, which show different amino acid sequence each other, also comprise 17 aa.

2.6D. β-END

Three β-ENDs in barfin flounder possess independent characteristics. β-END-A is composed of 44 aa, β-END-B has identical amino acid sequence to tuna β-END, and segment of β-END-C in barfin flounder com-

Fig. 8. Capillary HPLC/Q-Tof MS of the acid-acetone extract of barfin flounder pituitary for the detection of POMC-
derived peptides and proMCH-derived peptides with selected-ion monitoring at m/z 1043 for 7+ charged ion of
t/aN-proMCH (7293.2 Da) and at m/z 705 for 3+ charged ion of
bMCH (2112.5 Da). The elution was performed using a
linear gradient of acetonitrile in 0.05% TFA from 10 to 70%
of MCH (2112.5 Da). The elution was performed using a
linear gradient of acetonitrile in 0.05% TFA from 10 to 70%
of 60 min. Reprinted from
linear gradient of acetonitrile in 0.05% TFA from 10 to 70%
of food intake in a teleost fish, barfin flounder, 1613
Possible involvement of melanin-concentrating hormone in
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prises 33 aa residues. \(\beta\)-END-C segment is separated into two fragment—\(\beta\)-END-like peptide_{1-9} and \(\beta\)-END-like peptide_{12-33}—at a dibasic amino acid sequence in the middle of the segment. As observed in sockeye salmon \(\beta\)-END-B, the \(\beta\)-END-like peptide_{1-9} has a dramatic mutation in amino terminal five amino acid residues sequence (Fig. 1). It is suggested that biological activity of \(\beta\)-END-C has disappeared during the evolution of barfin flounder.

3. Melanocortin receptor (MCR) of barfin flounder

Two PCR subclones resulting from amplification of human melanoma cDNA were determined by DNA sequencing to encode novel G protein-coupled receptors (GPCRs) that are highly related to one another (Mountjoy et al. 1992). Northern hybridization analysis demonstrated expression specifically in melanocytes and adrenal cortex. The melanocortin receptor (MCR) family, which consists of five subtypes, has been cloned from human, rat, mouse, and chicken (Mountjoy 2000; Gantz and Fong 2003; Takeuchi et al. 2003), and their pharmacological characteristics, tissue distribution, and biological significance have been characterized in mammals. MC1R shows nearly the same degree of affinity for \(\alpha\)-MSH and ACTH, although the mc1r is mainly expressed in melanocytes and melanoma where it participates in pigmentation. Leukocytes and the brain are also known
to express the gene. MC2R is a specific receptor for ACTH, and its affinity for ACTH, but not for α-, β-, or γ-MSH, distinguishes this receptor from the other MC receptor subtypes. The mc2r is preferentially expressed in adrenal cortex and in adipose tissue. MC3R, which is thought to be associated with energy metabolism, possesses roughly the same degree of affinity for ACTH and α-, β-, and γ-MSH, and this gene is expressed in the brain and in several peripheral tissues including placenta, duodenum, pancreas, and stomach. MC4R shows nearly the same degree of affinity for α-MSH and ACTH. The mc4r is expressed predominantly in the brain, especially in the hypothalamic area, and is involved in energy homeostasis. MC5R shows the highest affinity for α-MSH. The mc5r is expressed in a variety of tissues including skeletal muscle, lung, spleen, thymus, bone marrow, testis, ovary, uterus, adrenal glands, adipocytes, leukocytes, pituitary, and thyroid gland. MC5R is involved in the regulation of exocrine functions, particularly in sebaceous glands.

We have revealed some interesting features of bfPOMC concerning the number of genes, post-translational processing, tissue distribution, pigment synthesis/dispersion, expression profiles in relation to background color change, and receptor functions. The nucleotide sequence of an mc1r DNA amplified from barfin flounder genomic DNA encoded a reading frame consisting of 323 aa (Fig. 12). This amino acid sequence showed the highest sequence identity with MC1R of Japanese flounder (97%) followed by that of Fugu (82%), zebrafish (78%), chicken (61%), mouse (54%), and human (53%). The locations of the seven transmembrane domain (TM) regions are indicated by underline with TM. The DRY—a highly conserved amino acid sequence, Asp-Arg-Tyr—is present at a position homologous to that in MC1R of the other species. Three potential sites for N-linked glycosylation are present in the N-terminal extracellular domain (Asn4, Asn26, and Asn29). There are four potential sites for protein kinase C phosphorylation: one on the N-terminal extracellular domain (Ser3), one on the second intracellular loop (Thr163), and the other two on the third intracellular loop (Ser223 and Ser238). Moreover, there are four potential sites for casein kinase 2 phosphorylation: one on the N-terminal extracellular domain (Thr55), one on the first TM (Ser75), one on the

<table>
<thead>
<tr>
<th>Peak (Peptide)</th>
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<th>Calculated average mass</th>
<th>Observed average mass (M+H)</th>
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<td>1623.9</td>
</tr>
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<td>4816.4</td>
<td>4817.5</td>
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<td>NIL</td>
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<td>5172.6</td>
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</table>

Table 3. Identification of bf POMC-related peptides by MALDI-TOF MS in frozen barfin flounder pituitary slices.

seventh TM (Ser^{293}), and one on the C-terminal intracellular domain (Thr^{310}).

3.2. MC2R

The nucleotide sequence of *mc2r* amplified from barfin flounder genomic DNA and brain cDNA encoded a reading frame consisting of 300 aa (Fig. 13). This amino acid sequence showed the highest sequence identity to MC2R of Japanese flounder (91%) followed by that of *Fugu* (70%), zebrafish (57%), human (48%), chicken (47%), and mouse (45%). The locations of the seven TM regions are indicated by underline with TM. One potential site for N-linked glycosylation is present in the N-terminal extracellular domain (Asn^{2}). There are two potential sites for protein kinase C phosphorylation: one on the third intracellular loop (Ser^{214}), and the other in the C-terminal intracellular domain (Thr^{286}). There are two potential sites for casein kinase 2 phosphorylation: one on the first TM (Ser^{28}), one on the first extracellular loop (Thr^{90}). Moreover, an additional serine/threonine kinase phosphorylation site was detected in the first extracellular loop (Lys^{84}).

3.3. MC3R

Mc3r hitherto has not been identified in barfin flounder in spite of thorough studies.

3.4. MC4R

A nucleotide sequence of *mc4r* amplified from barfin flounder genomic DNA and brain cDNA encoded an open reading frame consisting of 325 aa (Fig. 14). This amino acid sequence showed the highest sequence identity to MC4R of Japanese flounder (97%) followed by that of *Fugu* (85%), zebrafish (79%), goldfish (78%), chicken (67%), human (67%) and mouse (65%). The locations of the seven TM regions are indicated by underline with TM. The DRY motif is present at a ho-
mologous position with MC4R of other species. Four potential sites for N-linked glycosylation are present: two located in the N-terminal extracellular domain (Asn2 and Asn15), one in the second TM (Asn95) and the other in the first extracellular loop (Asn109). There are two potential sites for protein kinase C phosphorylation: one located on the third intracellular loop (Thr222), and the other in the C-terminal intracellular domain (Thr311).

3.5. MC5R

The nucleotide sequence of the other mc5r amplified from barfin flounder genomic DNA encoded a reading frame consisting of 342 aa (Fig. 15). This amino acid sequence showed the highest sequence identity to the MC5R of Japanese flounder (98%) followed by that of Fugu (85%), goldfish (80%), zebrafish b (79%), zebrafish a (75%), chicken (72%), human (68%), and mouse (67%). The locations of the seven TM regions are indicated by underline with TM. The DRY motif is present at a position homologous to that in the MC5R of the other species. Three potential sites for N-linked glycosylation are present on the N-terminal extracellular domain (Asn2, Asn17, and Asn28). There are six potential sites for protein kinase C phosphorylation: one on the N-terminal extracellular domain (Thr42), one on the second intracellular domain (Thr170), one on the third intracellular domain (Ser248), and the other three on the C-terminal intracellular domain (Thr320, Ser329, and Thr339). There are three potential sites for casein kinase 2 phosphorylation: one on the first TM (Ser65), one on the seventh TM (Ser303), and one on the C-terminal intracellular domain (Thr311).

Fig. 11. Phylogenetic tree for the MC receptors of fish, chickens, and mammals with bootstrap values, including four bfMCR subtypes constructed by the neighbor-joining method. Modified from Gen. Comp. Endocrinol., 176, Kobayashi et al., Further evidence on acetylation-induced inhibition of the pigment-dispersing activity of α-melanocyte-stimulating hormone, 9–17, © 2012, with permission from Elsevier.
Moreover, an additional serine/threonine kinase phosphorylation site was detected in the N-terminal extracellular domain (Lys39).

3.6. MCR genes

In tetrapod species, the entire coding region of all MC receptors is contained in a single exon. The human and mouse mc2r are separated into two and four exons, respectively, while the coding region is located in a single exon (Naville et al. 1994; Cammas et al. 1997; Shimizu et al. 1997). Unlike tetrapod species, barfin flounder mc2r contains two introns; one is inserted into a DRY motif and the other into 3′ noncoding...
Fig. 13. The nucleic acid sequence and the deduced amino acid sequence of bMC2R cDNA (Accession No. AB541411). Positions of nucleic acid and amino acid sequences are indicated on both sides. Underlines (bold) indicate potential N-glycosylation motifs. Italicized and double-underlined bases show potential protein kinase C phosphorylation motifs. Shadows indicate potential casein kinase 2 phosphorylation motifs. Italicized residues with broken lines indicate potential serine/threonine kinase phosphorylation motifs. Stop codon. ###DRY motif. Arrow-heads indicate positions of inserted introns. Modified from Gen. Comp. Endocrinol., 170, Kobayashi et al., Melanocortin receptor subtypes in interrenal cells and corticotropic activity of α-melanocyte-stimulating hormones in barfin flounder, Verasper moseri, 558–568, © 2011, with permission from Elsevier.
Moreover the mc5r contains one intron at DRY motif (Fig. 15). In the case of Fugu mc5r, it contains three introns in the coding region (Logan et al. 2003). Fugu mc2r contains only one intron, which is in the identical position relative to the coding sequence as intron 3 of the mc5r. See Subsection 5.2 for the discussion about the evolution of GPCR regarding the insertion and deletion of introns.

### 3.7. Tissue distribution

Expression of mc5 detected by RT-PCR in various tissues of barfin flounder is shown in Fig. 16. Strong signals for mc1r transcripts were observed in the brain, eyeball, testis, eyed-side skin, and non-eyed-side skin. For mc2r, strong signals were observed in the brain, head kidney, and testis. In the case of mc4r, strong sig-
nals were observed in the brain, liver, testis and ovary, weak signals in the eyeball and eyed-side skin, and a very faint signal in the pituitary. Moreover, signals for \textit{mc5r} were observed in all the examined tissues. Among them, strong signals were observed in the brain, testis, and eyed-side skin. The wide distribution of MCRs suggests that MC peptides may exhibit a variety of functions of biological processes.

**Fig. 15**. The nucleic acid sequence and the deduced amino acid sequence of \textit{bfMC5R} cDNA (Accession No. AB540951). DNA was amplified from the genomic DNA or the brain cDNA. Positions of nucleic acid and amino acid sequences are indicated on both sides. Underlines (thin) show TMs. Italicized amino acid residues along with underlines (bold) indicate potential N-glycosylation motifs. Italicized amino acid residues along with broken lines indicate potential serine/threonine kinase phosphorylation motifs. Italicized amino acid residues along with double-underlines indicate potential protein kinase C phosphorylation motifs. Shadows indicate potential casein kinase 2 phosphorylation motifs.

--Stop codon. ###DRY motif. The arrow indicates insertion position of the intron. Modified from \textit{Gen. Comp. Endocrinol.}, 168, Kobayashi et al., Differential expressions of melanocortin receptor subtypes in melanophores and xanthophores of barfin flounder, 133–142, © 2010, with permission from Elsevier.
3.8. Repertoire of MCR in fish—discussion based on identification of MCR

The presence of mcr has been reported in a wide variety of fish classes such as Cephalaspidomorphi (lampreys), Chondrichthyes (sharks), Sarcopterygii (lobe-finned fish, including tetrapods), and Actinopterygii (ray-finned fish) (Klovins et al. 2004a, b; Schiöth et al. 2005; Haitina et al. 2007a, b). Fugu may lack mc3r, while four mcr subtypes have been identified by genomic studies. We have cloned the cDNAs for mc1r, mc2r, mc4r, and mc5r in barfin flounder (Fig. 11). These four mcr cDNAs have also been identified in Japanese flounder (Kobayashi et al. 2012). Taking the taxonomically close relationship between Pleuronectiformes including flounders and Tetradorontiformes including Fugu into consideration, flounders may lack mc3r, as is the case with Fugu.

While mammals and chickens have shown to possess five mcr subtypes (mc1r to mc5r), zebrafish was the first fish species in which the presence of a set of five subtypes was demonstrated, while its mc5r is subdivided into mc5ra and mc5rb (Logan et al. 2003). We recently demonstrated the presence of mc1r, mc2r, and mc3r in goldfish (Kobayashi et al. 2011b). These results, as well as the previous characterization of mc4r and mc5r cDNA (Cerdá-Reverter et al. 2003a, b), provide the second line of evidence for the presence of five mcr subtypes in fish. The Cypriniformes, to which both goldfish and zebrafish belong, is a group of ray-finned fish that is rather basal when compared to the Fugu of Tetradontiformes (Nelson 2006). Therefore, it is possible that the five mcr subtypes may have appeared in an early vertebrate, possibly in a common ancestor of ray-finned fish and tetrapods. Mc3r may have been deleted during the course of evolution from...
a strain from which ray-finned fishes such as flounder and Fugu were descended.

4. Melanin-concentrating hormone (MCH) of barfin flounder

The dual hormonal control of color change in response to background coloration by two antagonistic pituitary melanotropic hormones was first postulated by Hogben and Slome in 1931. MSH, known as “intermedin” at that time, is responsible for pigment dispersion in the integumentary melanophores of lower vertebrates. It was known in the early 1940s that pituitary extract from some fish induced pigment aggregation. The putative hormone was termed “melanophore-concentrating hormone,” and its hypothalamic origin was first suggested by Enami in 1955. Thereafter, no advances were made in the understanding of MCH until 1975, when Baker and Ball (1975) reinvoked the dual hormone theory for control of teleost melanophores. Rance and Bader (1979) subsequently found the highest activities in the NIL of rainbow trout pituitary using an in vitro trout scale bioassay and they called the putative hormone “melanophore-concentrating hormone.” Its hypothalamic origin was confirmed by Enami in 1955. Thereafter, no advances were made in the understanding of MCH until 1975, when Baker and Ball (1975) reinvoked the dual hormone theory for control of teleost melanophores. Rance and Bader (1979) subsequently found the highest activities in the NIL of rainbow trout pituitary using an in vitro trout scale bioassay and they called the putative hormone “melanophore-concentrating hormone.” It is noteworthy that MCH is a neurohypophysial peptide that is synthesized as a prohormone in the hypothalamus and processed to an active form, and then transported to the PN of NIL for storage and release. Consequently, the development of this bioassay facilitated the discovery of MCH in chum salmon (Kawauchi et al. 1983).

We investigated MCH system in barfin flounder for the better understanding of biological significance of MCH (Takahashi et al. 2004). Nucleotide sequence of MCH cDNA was determined by conventional molecular cloning methods. Pituitary extract was first fractionated by rpHPLC and then the molecular weight of each separated peptide was determined by mass spectrometry. Distribution of MCH was examined by immunohistochemistry.

4.1. Structure

The bfMCH cDNA consisted of 578 bp excluding the poly-A tail. The bfMCH cDNA reading frame encodes 135 aa (Fig. 17). bfMCH is located at bf-proMCH 95–111. The bf-proMCH 71–92 is named neuropeptide AL (NAL) according to the terminology used for other preproMCHs. The bfMCH 1–67 is referred to as N-terminal peptide (N-proMCH) based on the analyses of peptides identified from barfin flounder brains. Two peptides derived from bf-proMCH were detected by mass spectrometry in the extract from a single barfin flounder pituitary (Fig. 8). The molecular weight of bfMCH which consists of 17 aa and contains one disulfide bond was calculated to be 2112.5 Da. Multiple charged ions at m/z 705.03 (peak “a”) and 1057.07 observed in the HPLC were deconvoluted to 2112.1 Da. Thus, peak “a” was consistent with bfMCH. The molecular mass corresponding to bfN-proMCH was detected with a select mass analysis. Given that bfN-proMCH consists of 67 aa, the molecular weight

![Fig. 17. Comparison of bf-preproMCH amino acid sequence with that of the tilapia, chum salmon, rat and human sequences. Amino acid residues identical to those in bf-preproMCH are shaded. Double underline designates MCH. Underlines designate peptides derived from proMCH excluding MCH and N-proMCH. Numbers at right side show amino acid sequence length. Closed circle shows the N-terminal of bf-proMCH. Reprinted from Peptides, 25, Takahashi et al., Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder, 1613–1622, © 2004, with permission from Elsevier.](image-url)
is 7293.2. Selected-ion monitoring at m/z 1043 gave a single peak (peak "b") in the HPLC. Multiple charged ions at m/z 1042.82 (peak "b"), 1216.64 and 1459.68 were deconvoluted to 7293.4 Da. Thus, peak "b" is consistent with bfN-proMCH and the signal peptide of preproMCH is deduced to be a 24 aa sequence (Fig. 8).

bfMCH is located in the C-terminal region of proMCH and composed of 17 aa as in other fish such as salmonids, tilapia, bonito, and eel (Kawauchi et al. 1983; Kawauchi 1989; Minth et al. 1989; Nahon et al. 1989; Baker et al. 1995; Gröneveld et al. 1995a). There is one replacement in the amino acid sequence compared to other teleost MCHs in the N-terminal outside a ring structure formed by a disulfide bond. bfMCH is shorter than mammalian MCH which consists of 19 aa. The two additional amino acids in mammals are present in the N-terminal outside a ring structure. bfMCH possesses four replacements relative to mammalian MCH. One of them in the ring, Val7 in barfin flounder and Leu9 in mammals, is a physicochemically conservative replacement. In human MCH, the replacement of this Leu with Ala has a negligible effect on binding with the receptor (Audinot et al. 2001). The other three replacements are present outside the ring. The Arg4 residue in the N-terminal sequence and Trp15 residue in the C-terminal sequence, which are essential for binding to the receptor in humans, are conserved. The identification of bfMCH and bfN-proMCH in the pituitary extract demonstrates that the basic amino acids (Lys-Arg-Arg and Arg-Arg) act as processing signals (Takahashi et al. 2004).

In contrast to the high similarity in the MCH region...
between barfin flounder and other species, the remainder of the bf-proMCH shows a significant level of mutation in the amino acid sequence. Sequence comparison (Fig. 17) revealed that the similarity between bfNAL and corresponding regions of other species is 50% with tilapia, 23% with chum salmon, and 9% with rats and humans. Furthermore, that between bf- proMCH and corresponding regions of other species is 68% with tilapia, 32% with chum salmon, 19% with rats, and 16% with humans. In both NAL and N-proMCH regions, a deletion of consecutive amino acids is observed in addition to the replacements. Weak functional constraints might have caused the variation in these regions.

4.2. Distribution of MCH neurons in the brain

The distribution of MCH-immunoreactive (ir) neuronal somata and fibers in the adult fish is summarized in Fig. 18. MCH-ir neuronal somata were located in the nucleus lateralis tuberis (NLT) in the basal hypothalamus (Fig. 19). Projections of MCH-ir fibers from the neuronal somata of the NLT (NLT-MCH neurons) to the pituitary were observed. MCH-ir neuronal somata were also present in dorsally projecting parvocellular neurons, located more posteriorly in the area above the lateral ventricular recess (LVR) of the third ventricle. LVR-MCH neurons did not seem to project to the pituitary.

It was proposed that MCH-ir neuronal somata were located only in the NLT from which axons projected to the pituitary, and that their synthetic activity was located only in the NLT from which axons projected to the pituitary. LVR-MCH neurons did not seem to project to the pituitary.

The expression of MCH was also observed in barfin flounder as described above. Projections of MCH neurons from the NLT to the pituitary indicate that NLT-MCH neurons function as a neurophysiophyseal hormone in barfin flounder, one of which roles would be pigment aggregation. On the other hand, MCH produced in LVR may not be associated with body color change because LVR-MCH neurons do not seem to project to the pituitary.

5. Melanin-concentrating hormone receptor (MCHR) of barfin flounder

Fish reared on a white background have higher levels of MCH expression, brain concentration of MCH, and the number of MCH-ir cell body than those reared on a black background (Takahashi et al. 2004; Amiya et al. 2005). Interestingly, fish reared on a white background have a greater body length and weight (Takahashi et al. 2004; Yamanome et al. 2005). These observations suggest that the white background stimulates MCH production in the brain and this could in turn affect the food intake in barfin flounder. Thus, the characterization of MCHRs in barfin flounder is of utmost importance to elucidate the biological significance of the MCH system consisting of MCH and its receptors in this fish.

The MCHR receptor, later named MCHR1, was identified using the reverse pharmacological approach wherein a human orphan GPCR (somatstatin-like receptor-1, SLC-1) having 40% amino acid sequence identity to human somatostatin receptor specifically bound to MCH (Bächner et al. 1999; Chambers et al. 1999; Lembo et al. 1999; Saito et al. 1999; Shimomura et al. 1999). Part of the nucleotide sequence of a second MCHR receptor gene, mchr2, was initially obtained by searching the human genomic database for the MCHR1 amino acid sequence. Full-length DNA for mchr2 was determined using PCR with a primer set based on the partial sequence (Hervieu et al. 2000; An et al. 2001; Hill et al.; 2001; Mori et al. 2001; Rodriguez et al. 2001; Sailer et al. 2001). Human, rhesus monkey, dog, and ferret have been shown to express the two mchrs, while rodents, including rat, mouse, hamster, guinea pig, and rabbit, do not have a functional mchr2, or encode a nonfunctional mchr2 pseudogene (Tan et al. 2002).

The structure of the MCHR receptor in fish was first provided in zebrafish and Fugu using the whole genome shotgun datasets (Logan et al. 2003). Zebrafish has three mcr, two of which are subtypes of mchr1, mchr1-a and mchr1-b, and the third is mchr2. Fugu contains two MCHR receptors corresponding to MCHR1 or -R2. In zebrafish, the expression of mchr1-a is embryonic specific, because no transcripts are detected in adult tissues, and only a weak expression can be found in embryos younger than five days (Logan et al. 2003). Expression of mchr1-b appears stronger than mchr1-a, and can be detected in adults and young embryos. Expression of mchr2 parallels that of mchr1-b during embryonic development. Mchr2 is expressed at higher levels in the adult torso than the head.

The nucleotide sequences of two MCHRs and corresponding genes in barfin flounder were determined by conventional molecular cloning methods. The distribution of their transcripts in flounder tissues was analyzed by RT-PCR to infer the biological roles of MCH. Real-time PCR on selected tissues was performed to quantify MCHR mRNA.

5.1. Structure

We cloned two MCHR genes from the barfin flounder (Takahashi et al. 2007). The phylogenetic analysis shows that these are orthologues to the mammalian...
mchr1 and mchr2 showing 49 and 30% amino acid sequence identity to the corresponding human receptors while they have 31% amino acid sequence identity between them. Essential amino acid residues for ligand binding, signal transduction and receptor conformation, which have been shown in mammalian MCHR, are well conserved in the flounder MCHR (Fig. 20).

Mammalian MCH is a cyclic 19 aa peptide carrying a disulfide bridge between Cys7 and Cys16. In this sequence, MCHR6-16 has been shown to be sufficient for full activation of human MCHR1 and -R2 (Bednarek et al. 2001). For fish MCH consisting of 17 aa, MCHR4-14 corresponds to the minimal sequence in mammals (Takahashi et al. 2004). Fish MCHR6-14 is identical to mammalian MCHR6-16 excluding a replacement in one residue, Val at fish MCH1 or Leu at mammalian MCH. Pharmacological studies have shown that salmon MCH could activate both mammalian MCHR1 and -R2, while the activity is weaker than mammalian MCH (Chambers et al. 1999; Saito et al. 1999; Rodriguez et al. 2001; Sailer et al. 2004). The amino acid sequence of bMCH is identical to the salmon MCH, excluding the second position outside the minimal sequence (Fig. 17). As suggested by the similarity in structures and activities between fish and mammalian MCH, amino acid residues essential for MCH function are well conserved also in barfin flounder MCHR. First, Asn14 of bMCHR1 and Asp23 of bMCHR2 correspond to Asn21 of rat MCHR1, which is necessary for cell-surface expression (Saito et al. 2003). Second, Asp116 of bMCHR1 and Asp117 of bMCHR2 correspond to Asp125 of rat MCHR1, which is critical for ligand binding (MacDonald et al. 2000). Third, Arg62 of bMCHR1 and Arg63 of bMCHR2 correspond to Arg55 of rat MCHR1, which plays a critical role in receptor activation (Saito et al. 2005). Fourth, Thr137 of bMCHR1 and Thr138 of bMCHR2 correspond to Thr155 of human MCHR1, which is associated with cell-surface expression (Fan et al. 2005). Fifth, a dibasic motif (Lys206,Arg207) in bMCHR1 corresponds to Arg210–Lys212 of rat MCHR1, which is necessary for signal transduction (Tetsuka et al. 2004).

5.2. Gene structure and evolution

Barfin flounder mchr1 has one intron in the extracellular N-terminal region at a homologous position to human and rat mchr1s. Barfin flounder mchr2 has one intron in the DRY motif, which is a homologous position to one of the five introns of human mchr2. The presence of intron in the extracellular N-terminal region has also been observed in other genes of GPCRs (Probst et al. 1992; Kusakabe et al. 2003). Insertion of the intron at the DRY motif has also been observed in other GPCRs such as dopamine receptor (Probst et al. 1992; Boehmler et al. 2004) and MCSR (Logan et al. 2003; Klovins et al. 2004a). It has been speculated that this DRY intron may have been ancestral in the entire GPCR family (Bryson-Richardson et al. 2004) while other studies on the presence of introns in GPCR genes suggest that the DRY intron may have been inserted several times independently in different GPCR genes (Fridmanis et al. 2007). Our results show that this intron has been conserved within one of the mchr but not in the other subtype (Takahashi et al. 2007). It is conceivable that the ancestral mchr had at least two introns, and then, after duplication, mchr1 inherited one of the two introns and mchr2 inherited the other, so that a strain of the flounder. The intron of mchr1 orthologues has a microsatellite consisting of CA repeats in the flounder, humans, and rats, suggesting that the microsatellite occurred in early mchr1 soon after the gene duplication generated mchr1 and mchr2, and was then inherited to extant vertebrates.

5.3. Distribution of gene transcripts

In barfin flounder, mchr1 and mchr2 are expressed in the brain, indicating that both receptor subtypes are responsible for the central roles of MCH (Fig. 21). Mchr1 is exclusively expressed in the brain, whereas mchr2 is also expressed in several peripheral tissues. These distribution patterns of two subtypes of MCHR genes in the flounder differ from those of mammals. In mammals, mchr1 transcripts are distributed wider than mchr2 transcript (Boutin et al. 2002; Schlumberger et al. 2002; Eberle et al. 2004). The wide distribution of mchr2 transcripts in peripherals coincides with the role of MCH as a neurohypophyseal hormone as shown by the proteomic projections of the neuron from the hypothalamus to PN in ray-finned fish including barfin flounder (Naito et al. 1985; Batten and Baker 1988; Minth et al. 1989; Mancera and Fernández-Llibreix 1995; Baker and Bird 2002; Amano et al. 2003).

6. Background color and POMC production in barfin flounder

The PD and PI of the pituitary are differentially controlled so as to generate and secrete POMC-derived peptides in teleosts. ACTH is released from the PD in response to stress (Wendelaar Bonga 1997), and the release of MSH from the PI is modified by the change of background color (Ebelre 1988). Some hypophysiotropins participate in the release of POMC-derived peptides from these two parts with stimulatory and inhibitory functions. Although corticotropin-releasing hormone is the major hypothalamic hormone stimulating the release of ACTH from the PD, it also stimulates the release of MSH from the PI of gilthead sea bream (Rotllant et al. 2000b), tilapia (van Enckevort et al. 2000), and red porgy (Van der Salm et
Fig. 20. Amino acid sequence of bfMCHRs compared with human and rat orthologues. Common amino acid residues in MCHR1 or MCHR2 are shaded. Spaces are inserted every 20 aa. Numbers show amino acid residues essential for inter- or intramolecule interactions; 1, taken from Saito et al. 2003; 2, taken from MacDonald et al. 2000; 3, taken from Saito et al. 2005; 4, taken from Fan et al. 2005; 5, taken from Tetsuka et al. 2004; 6, taken from Vitale et al. 2004. Reprinted from Gen. Comp. Endocrinol., 151, Takahashi et al., The melanin-concentrating hormone receptor 2 (MCH-R2) mediates the effect of MCH to control body color for background adaptation in the barfin flounder, 210–219, © 2007, with permission from Elsevier.
al. 2004). Neuropeptide Y stimulates the release of MSH in goldfish (Fryer 1989), and thyrotropin-releasing hormone stimulates the release of α-MSH in fish including goldfish (Tran et al. 1989), tilapia (Lamers et al. 1991, 1994), carp (van Den Burg et al. 2003), and red porgy (Van der Salm et al. 2004). While considerable evidence regarding the release of POMC-derived peptides has been acquired, no studies have evaluated the regulation of POMC expression in the PD and PI in the pituitary of teleosts. Thus we examined the effects of background color on the expression of POMC genes in the PI of barfin flounder and on the plasma profiles of MSH.

Barfin flounder were selected from the stock tanks and transferred to a black tank. The fish were transferred to a white tank or to the other black tank 120 days later. In the other experiment, barfin flounder were selected from the stock tanks and transferred to a white tank. The fish were transferred to a black tank or to the other white tank 147 days later. In these experiments, pituitaries were collected from the fish 0, 1, and 7 days after this transfer. The content of mRNA was estimated by real-time RT-PCR. Plasma levels of MSH were measured by time-resolved fluoroimmunoassay (TR-FIA).

6.1. Gene expression

In teleost fish, MSH seems to be associated with morphological color changes rather than physiological color changes in vivo (Rodrigues and Sumpter 1984; Ebibe 1988). The same holds true for the barfin flounder because the bolus injection of MSH did not alter the body color, while chronic treatment with MSH turned the body color dark (Yamanome et al. 2007). However, the transferring experiment of barfin flounder using black and white tanks indicated that the expressions of the three POMC genes do not always show similar expression profiles (Fig. 22). We also observed no difference in the contents of the mRNAs for POMC-A, POMC-B, and POMC-C in the NIL taken from barfin flounder reared in a black tank and a white tank for 126 and 163 days, respectively (Fig. 22). It may be difficult to compare the expression levels in fish reared under black and white background because the length of time spent in these tanks is different; nevertheless, long-term rearing in black or white tanks may not make a difference in the levels of each POMC mRNA in the NIL. Moreover, changes in the mRNA contents in the NIL corresponding to the background were only observed for pomc-c in the fish transferred from a black tank to a white tank.

The non-synchronous expression of the three POMC genes may be a result of the gene-specific regulation of the transcription in which the transcription factors are differentially used in a single cell. Moreover, the expression of each pomc may be differentially regulated between the PD and PI as suggested by the non-synchronous changes in the mRNA between the two lobes in some cases (Kobayashi et al. 2008b). A set of transcription factors regulating the POMC genes in the corticotrophs may be different from that employed in melanotrophs.

Our results also suggest that the transcription of POMC genes is regulated with multiple neuroendocrine controls and that the background color may have minor effects on the transcription of POMC genes in the PI.
6.2. Plasma MSH levels

The pituitary levels of α-MSH do not always parallel the plasma levels of ir-α-MSH (Rodrigues and Sumpter 1984; Amiya et al. 2005). We demonstrated the independent profiles of pituitary POMC mRNAs and plasma ir-α-MSH (Kobayashi et al. 2008b). Despite the absence of changes in the POMC mRNAs in the NIL of barfin flounder pituitary in response to changes in the background color, the plasma ir-α-MSH

Fig. 22. Contents of mRNAs for POMC-A, POMC-B, and POMC-C in the NIL of pituitary taken from barfin flounder transferred (A) from a black tank to a white tank or to another black tank and (B) from a white tank to a black tank or to another white tank. Black bar and white bar indicate black tank and white tank, respectively. Each value is expressed as means ± SEM (n = 6). Different letters in a capital letter group or small letter group indicate a significant difference (P < 0.05). Reprinted from Gen. Comp. Endocrinol., 158, Kobayashi et al., Transcription elements and functional expression of proopiomelanocortin genes in the pituitary gland of the barfin flounder, 259–267, © 2008, with permission from Elsevier.
remarkably increased 1 day after transfer from a white tank to black tank (Fig. 23). POMC mRNAs might be constitutively expressed in the given experimental condition. Hence, the remarkable increase in plasma ir-α-MSH levels on day 1 in the black tank may indicate the release of accumulated α-MSH in response to a change in the background from white to black. The fact that no increase occurred in the ir-α-MSH levels on day 1 in the white tank supports this explanation. On the other hand, the plasma ir-α-MSH levels are not exclusively influenced by changes in the background color, as shown by the increased plasma levels of ir-α-MSH in fish 7 days after transfer from a white tank to another white tank. In this case, it seems that factors related to environmental changes other than background color changes are involved. This hypothesis is supported by the fact that no changes occurred in the plasma levels of ir-α-MSH in fish transferred from a black tank to a white tank or to another black tank.

On day 7 after transferring, the ir-α-MSH levels in the fish transferred from white to black and white to white tanks were higher than the ir-α-MSH levels in fish in a black tank on day 0 (Fig. 23). On day 0, however, the plasma levels of ir-α-MSH for fish in a black tank were similar to those for fish in a white tank, although the duration of fish being reared in each tank prior to sampling was different for each tank, as described above. It is, therefore, suggested that MSH secretion was stabilized during long-term rearing in black or white tanks. Further, in other fishes, the relationships between the plasma levels of α-MSH and background color were inconsistent. Black-adapted eel (Baker et al. 1984), rainbow trout (Baker et al. 1984; Rodrigues and Sumpter 1984; Gilham and Baker 1985), and Japanese flounder (Amiya et al. 2007) showed higher plasma α-MSH levels than white-adapted groups, while sea bream showed higher plasma α-MSH levels in white-adapted fish than those in black-adapted fish (Arends et al. 2000). Thus, the control of α-MSH secretion during background color adaptation in fish may vary from species to species.

7. Effects of MSH on pigment dispersion in barfin flounder

MSH is generated from POMC mainly in the pituitary. The barfin flounder characteristically expresses three different POMC genes (see Section 2). Among the three POMC genes, pomc-a is exclusively expressed in the pituitary, while pomc-b and pomc-c are expressed in extra-pituitary tissues in addition to the
The expression levels of *pomc-c* seem to be greater than those of *pomc-b* in extra-pituitary tissues (Takahashi et al. 2005a). *Pomc* expression in extra-pituitary tissues, including the skin, has also been shown in mammals, and POMC-derived peptides such as (ACTH, MSH, \( \beta \)-END) have been identified in cultured skin cells (Slominski and Wortsman 2000). These reports indicate the presence of autocrine and/or paracrine systems in pigment production in a wide range of vertebrates. In teleost fish, \( \alpha \)-MSH-related peptides (\( \alpha \)-MSH-rp) such as \( \alpha \)-MSH, Des-Ac-\( \alpha \)-MSH, and Di-Ac-\( \alpha \)-MSH stimulate not only melanophores but also other bright-colored chromatophores—erythrophores, xanthophores, and leucophores (Fujii and Oshima 1986; Fujii 2000). Usually, acetylation increases the melanin-dispersing activity of \( \alpha \)-MSH in teleosts (Kawamura et al. 1984) as well as amphibians (Eberle 1988). In this section, the role of \( \alpha \)-MSH-rp expressed in barfin flounder skin was evaluated. Moreover, pigment-dispersing activities of three types of \( \alpha \)-MSH with different number of acetyl group at N-terminal were examined.

RNA samples were prepared from the skin of body and fin of both dorsal and ventral surface (eyed side and non-eyed side, respectively) and the pituitary. The presence of transcripts of each *pomc* was evaluated by RT-PCR. On the other hand, absolute amounts of mRNA for POMC-A, POMC-B, and POMC-C were determined by quantitative real-time PCR. Cells were dispersed from fin skin. The cDNA was prepared from these cells and then subjected to PCR to detect POMC-C mRNA. Pigment-dispersing activities \( \alpha \)-MSH-rp were evaluated using skin pieces prepared from fin. Skin extract was fractionated by HPLC and fractions were analyzed by MALDI-TOF MS.

### 7.1. Possible paracrine function of \( \alpha \)-MSH

The POMC-C mRNA was detected in skin extract by RT-PCR (Fig. 24). The PCR using isolated cells showed the expression of *pomc-c* in isolated non-chromatophoric dermal cells from barfin flounder skin (Fig. 25). Subsequent mass spectrometry analyses of fractions of skin extract separated by HPLC revealed the presence of a peptide with a molecular mass corresponding to Des-Ac-\( \alpha \)-MSH-C derived from POMC-C (Fig. 26). These results indicate that POMC-C mRNA is translated into prePOMC-C, and subsequently cleaved into small peptides in the skin, at least Des-Ac-\( \alpha \)-MSH-C, by post-translational processing (Kobayashi et al. 2009). The presence of Des-Ac-\( \alpha \)-MSH-C in the skin suggest that, in addition to endocrine functions, MSH is associated with skin pigmentation via paracrine mechanisms. The amount of POMC transcripts in the barfin flounder skin and pituitary is summarized in Table 4. Interestingly, taking the large area of the skin into consideration, this organ seems to be another major source of POMC-derived peptides. Consequently, the control over the pigmentation of barfin flounder skin by MSHs is two-fold—endocrine control by the pituitary and paracrine control by the skin itself (Fig. 27).
The approximate total surface area (cm\(^2\)) of the dorsal and ventral surface of barfin flounder was calculated as follows: 

\[
\text{Total surface area} = \frac{\text{length} \times \text{body (dorsal) depth}}{2}. 
\]

The approximate copy number of POMC-C mRNA was calculated as follows: total surface area/area copy number in skin piece. The total length was the actual measured value. Body depth was estimated using photographs. Paired Student’s t-test indicated that, in the pituitaries of both black- and white-reared fish, the contents of POMC-A mRNA were greater than those of POMC-B mRNA\(^{\text{a}, \text{b}}\). One-way ANOVA and subsequent Fisher’s PLSD test indicated that, in the pituitaries of both black- and white-reared fish, the contents of POMC-A mRNA were greater than those of POMC-B mRNA\(^{\text{a}, \text{b}}\) and \(^{\text{c}, \text{d}}\) vs. \(^{\text{e}}\), \(P < 0.001\), and the contents of POMC-B mRNA were greater than those of POMC-C mRNA\(^{\text{a}, \text{b}}\) and \(^{\text{c}, \text{d}}\) vs. \(^{\text{e}}\), \(P < 0.05\). Student’s t-test (non-paired) indicated that the contents of POMC-C mRNA in the skin of the ventral body was significantly \(\mu \times 10^8\) higher than those of black-reared fish\(^{\text{a}, \text{b}}\). Reprinted from \textit{Gen. Comp. Endocrinol.}, 161. Kobayashi et al., Possible paracrine function of \(\alpha\)-melanocyte-stimulating hormone and inhibition of its melanin-dispersing activity by N-terminal acetylation in the skin of the barfin flounder, Verasper moseri, 419–424, © 2009, with permission from Elsevier.

### Table 4. The amount of POMC transcripts in the barfin flounder skin and pituitary. The values are expressed in units of mg and tissue (n = 6).

<table>
<thead>
<tr>
<th>POMC</th>
<th>Source</th>
<th>mRNA (copies/µg)</th>
<th>mRNA (copies/tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black-reared fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Skin (dorsal body)</td>
<td>1.25 \times 10^2 \pm 3.0 \times 10^7 ^a</td>
<td>3.42 \times 10^3 \pm 1.0 \times 10^{10}</td>
</tr>
<tr>
<td></td>
<td>Skin (ventral body)</td>
<td>1.66 \times 10^2 \pm 2.8 \times 10^6 ^b</td>
<td>5.37 \times 10^7 \pm 7.0 \times 10^{10}</td>
</tr>
<tr>
<td>A</td>
<td>Pituitary</td>
<td>2.13 \times 10^2 \pm 3.4 \times 10^{10} ^a</td>
<td>6.40 \times 10^6 \pm 9.0 \times 10^{10}</td>
</tr>
<tr>
<td>B</td>
<td>Pituitary</td>
<td>5.66 \times 10^2 \pm 7.6 \times 10^{10} ^b</td>
<td>1.73 \times 10^7 \pm 2.4 \times 10^{10}</td>
</tr>
<tr>
<td>C</td>
<td>Pituitary</td>
<td>1.55 \times 10^3 \pm 3.7 \times 10^{11} ^c</td>
<td>1.84 \times 10^9 \pm 4.6 \times 10^{10}</td>
</tr>
<tr>
<td>White-reared fish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Skin (dorsal body)</td>
<td>1.37 \times 10^2 \pm 2.8 \times 10^6</td>
<td>3.40 \times 10^3 \pm 5.6 \times 10^6</td>
</tr>
<tr>
<td></td>
<td>Skin (ventral body)</td>
<td>1.42 \times 10^2 \pm 3.8 \times 10^6</td>
<td>3.75 \times 10^7 \pm 1.1 \times 10^{10}</td>
</tr>
<tr>
<td>A</td>
<td>Pituitary</td>
<td>1.96 \times 10^2 \pm 2.7 \times 10^{10} ^c</td>
<td>7.62 \times 10^6 \pm 1.2 \times 10^{10}</td>
</tr>
<tr>
<td>B</td>
<td>Pituitary</td>
<td>7.33 \times 10^2 \pm 1.0 \times 10^{11} ^d</td>
<td>2.77 \times 10^7 \pm 3.8 \times 10^{10}</td>
</tr>
<tr>
<td>C</td>
<td>Pituitary</td>
<td>5.86 \times 10^2 \pm 1.4 \times 10^{11} ^c</td>
<td>5.89 \times 10^9 \pm 1.5 \times 10^{10}</td>
</tr>
</tbody>
</table>

7.2. Inhibition of pigment-dispersing activity by acetylation

Also, in barfin flounder, \(\alpha\)-MSH-rp such as \(\alpha\)-MSH and Des-Ac-\(\alpha\)-MSH, stimulate both melanophores and xanthophores; however, N-terminal acetylation differentially modulated the pigment-dispersing activities of these cells (Fig. 2B). Surprisingly, acetylation reduced the activity of \(\alpha\)-MSH on melanophores, while it enhanced activity on xanthophores. Reduction of the pigment-dispersing activity of \(\alpha\)-MSH by acetylation is the first evidence. Similar results were also obtained from Japanese flounder (Kobayashi et al. 2012). On the other hand, replacement of the C-terminal residue of \(\alpha\)-MSH-rp may have negligible effects on the pigment-dispersing activities, because Des-Ac-\(\alpha\)-MSH-C and Des-Ac-\(\alpha\)-MSH exhibit similar effects on the two types of chromatophores. The differences in the effects of \(\alpha\)-MSHs on pigment dispersion with modification of N-terminal residue would provide insight into the receptor type expressed in the two chromatophores as will be described below.

8. Differential expression of MCR gene in barfin flounder chromatophores

MCR is a GPCR with seven TM regions and short extracellular and intracellular domains at the N- and C-termini, respectively (Schlöth et al. 2005; Takahashi and Kawauchi 2006a, b; Metz et al. 2006). Molecular cloning studies have revealed the presence of at least five subtypes of MCR (MC1R to MC5R) in fish, as in the case of tetrapods (Cerdá-Reverter et al. 2003a, b; Logan et al. 2003; Klovins et al. 2004a; Kobayashi et al. 2011b). Four subtypes of MCRs have been identified in barfin flounder. Among these MCR genes, \textit{mc1r} and mc5r are markedly expressed in the skin of barfin flounder. This section deals with the investigation of the type of \textit{mc}r expressed in isolated skin cells of barfin flounder in order to evaluate the relationships between the molecular type of MCR present on pigment cells and the pigment-dispersing activities of \(\alpha\)-MSH and Des-Ac-\(\alpha\)-MSH. For this purpose, cells were dispersed from the skin of the fin. The cDNA was prepared from these cells and then subjected to PCR to detect MC1R.
the binding affinity of Des-Ac-α-MSH to MC5R (Schiöth et al. 1996). Therefore, it is possible that acetylation of the ligand results in the increased binding affinity of the ligand to MC5R and thus leads to increased pigment-dispersing activity.

In contrast, α-MSH did not stimulate pigment dispersion in melanophores (Fig. 28). An apparent contradiction concerning the migration of pigments in and MCR mRNA.

The MC5R mRNA was detected in single cells of xanthophores isolated from barfin flounder fins (Fig. 29). We also showed that both α-MSH and Des-Ac-α-MSH stimulated pigment dispersion in these cells, but α-MSH activity was higher than that of Des-Ac-α-MSH (Kobayashi et al. 2009). These results suggest enhancement of pigment-dispersing activity by acetylation of the N-terminus. Similar potentiating pigmentation-related functions have also been observed in frogs and rats (Kawauchi et al. 1984; Eberle 1988; Canegemi et al. 1995). Pharmacological studies on sea bass MC5R have revealed a higher efficacy of α-MSH than of Des-Ac-α-MSH to stimulate cellular activities (Sanchez et al. 2009). These results together with the high sequence identity observed between flounder proopiomelanocortin in fish with special reference to barfin flounder, 1374–1382, © 2009, with permission from Elsevier.

We cloned MCH cDNA in barfin flounder and showed that the flounder responded well to synthetic MCH by aggregating melanin granules in similar manner as for other teleosts (Takahashi et al. 2004). Fish reared on a white background have higher levels of mch expression, brain concentration of MCH, and MCH-ir cell body number than those reared on a black background (Takahashi et al. 2004; Amiya et al. 2005). These observations suggest that the white background affects MCH system consisting of MCH and its receptors in this fish. Herein, the effects of background color on the production of MCH and MCHR will be shown.

Barfin flounder were injected intraperitoneally with 1 μg/g body weight of the synthesized MCH. One hour after the injection, photographs of the eyed-side bodies were taken. On the other hand, barfin flounder were reared in white or black tanks for 155 days. Their brains and the pituitary gland were collected. The tissue contents of MCH were measured by MCH TR-FIA. Moreover, barfin flounder were reared in either a white or black background for 121 days. Their brains and skin from the eyed and non-eyed side were dissected out. The contents of MCHR mRNA were measured by quantitative real-time PCR.

9. Background color and MCH system in barfin flounder

We cloned MCH cDNA in barfin flounder and showed that the flounder responded well to synthetic MCH by aggregating melanin granules in similar manner as for other teleosts (Takahashi et al. 2004). Fish reared on a white background have higher levels of mch expression, brain concentration of MCH, and MCH-ir cell body number than those reared on a black background (Takahashi et al. 2004; Amiya et al. 2005). These observations suggest that the white background affects MCH system consisting of MCH and its receptors in this fish. Herein, the effects of background color on the production of MCH and MCHR will be shown.

Barfin flounder were injected intraperitoneally with 1 μg/g body weight of the synthesized MCH. One hour after the injection, photographs of the eyed-side bodies were taken. On the other hand, barfin flounder were reared in white or black tanks for 155 days. Blood, brain, and the pituitary gland were collected. The tissue contents of MCH were measured by MCH TR-FIA. Moreover, barfin flounder were reared in either a white or black background for 121 days. Their brains and skin from the eyed and non-eyed side were dissected out. The contents of MCHR mRNA were measured by quantitative real-time PCR.
9.1. Effects of tank color on MCH production

Administration of MCH resulted in the paling of body color in barfin flounder (Fig. 31). This result shows that MCH influences physiological color change also in this fish. In teleost hypothalami, MCH cells in the NLT have been shown to be involved in physiological color changes (Bird and Baker 1989; Gröneveld et al. 1995b; Suzuki et al. 1995). This function also exists in barfin flounder as demonstrated by the different numbers of MCH-ir cell bodies in the NLT between the fish reared in white background and black background (Takahashi et al. 2004). Moreover, the barfin flounder reared in the white tank had higher MCH levels, both in the brain and plasma, than the ones in the black tank (Fig. 32). These results indicate that MCH, released into the blood, contributes to reduced skin pigmentation in barfin flounder reared under white background.

9.2. Effects of tank color on the expression of MCHR genes

The interaction between MCH and its specific receptor in skin is the primary event initiating the aggregation of melanin granules. Indeed, barfin flounder reared in the white tank had higher MCH levels, both in the brain and plasma, than the ones in the black tank (Fig. 32). These results indicate that MCH, released into the blood, contributes to reduced skin pigmentation in barfin flounder reared under white background.

Fig. 28. Pigment-dispersing activity of synthetic Des-Ac-α-MSH-C (A and B), Des-Ac-α-MSH (C and D), and α-MSH (E and F) on melanophores (A, C, and E) and on xanthophores (B, D, and F). MI, melanophore index; XI, xanthophore index analogous to MI. Asterisk indicates significant differences compared to the control value by a posthoc multiple comparison test for the Kruskal–Wallis test at $P < 0.05$. Reprinted from Gen. Comp. Endocrinol., 161, Kobayashi et al., Possible paracrine function of α-melanocyte-stimulating hormone and inhibition of its melanin-dispersing activity by N-terminal acetylation in the skin of the barfin flounder, Verasper moseri, 419–424, © 2009, with permission from Elsevier.
responded well to exogenous MCH by a paling of body color (Fig. 31). It is certain that MCHR2 in the eyed-side skin mediates the function of MCH to control body color for background adaptation, because of the detection of mchr2 transcripts from the skin (Fig. 21).

Barfin flounder reared on a white background exhibits a higher expression of mch in the brain (Takahashi et al. 2004, 2007) indicating that mch expression is enhanced by white color. However, the expression levels of mchr2 in eyed-side skin in fish reared under white background were lower than those of black-reared fish (Fig. 33), suggesting down regulation of the receptor gene with high plasma levels of MCH (Takahashi et al. 2007). Similar reduction in receptor gene expression has been observed in corticotropin-releasing factor (CRF) receptor in the rat anterior lobe of the pituitary cultured with CRF (Sakai et al. 1996) and gonadotropin-releasing hormone

Fig. 29. Expression of mc1r, mc2r, mc4r, and mc5r in cells isolated from the dorsal fins. RT-PCR was performed using the total RNA extracted from melanophores (A), xanthophores (B), and nonchromatophoric dermal cells (C). Numbers indicate mcr subtypes. Total RNAs prepared from three single cells were combined. β-Actin was used as an internal control (D). Amplification of the β-actin cDNA fragment from melanophores “M”, xanthophores “X”, and nonchromatophoric dermal cells “D”. Genomic DNA was used as a template in “G”. The amplified DNAs for β-actin contained one intron. Reprinted from Gen. Comp. Endocrinol., 168, Kobayashi et al., Differential expressions of melanocortin receptor subtypes in melanophores and xanthophores of barfin flounder, Japanese flounder, and goldfish (A), Des-Ac-α-MSH and α-MSH stimulate pigment dispersion when one type of MCR is expressed in chromatophores, such as in xanthophores of barfin flounder, Japanese flounder, and goldfish (A). Des-Ac-α-MSH also exhibits activities in chromatophores expressing two types of MCRs, such as in the melanophores of barfin and Japanese flounders, but α-MSH does not. α-MSH may have low affinity to putative MCR heterodimers (B). Reprinted from Frontiers in Endocrinology, 168, Kobayashi et al., Melanocortin systems on pigment dispersion in fish chromatophores, doi: 10.3389/fendo.2012.00009, © 2012, with permission from Frontiers Media S.A.

Fig. 30. Schematic models of the pigment-dispersing activities of α-MSH-rp via MCRs. Both Des-Ac-α-MSH and α-MSH stimulate pigment dispersion when one type of MCR is expressed in chromatophores, such as in xanthophores of barfin flounder, Japanese flounder, and goldfish (A). Des-Ac-α-MSH also exhibits activities in chromatophores expressing two types of MCRs, such as in the melanophores of barfin and Japanese flounders, but α-MSH does not. α-MSH may have low affinity to putative MCR heterodimers (B). Reprinted from Frontiers in Endocrinology, 168, Kobayashi et al., Melanocortin systems on pigment dispersion in fish chromatophores, doi: 10.3389/fendo.2012.00009, © 2012, with permission from Frontiers Media S.A.

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(GnRH) receptor in the pituitary of cows receiving continuous infusion of GnRH (Vizcarra et al. 1997).

10. Interrelationship between α-MSH and MCH in physiological body color change

The effects of MCH are opposite from those of α-MSH. Adaptation of rainbow trout to white background activates the expression of mch in hypothalamus (Suzuki et al. 1995), elevates tissue concentrations of MCH in both hypothalamus and pituitary (Green et al. 1991), and increases plasma levels of MCH (Kishida et al. 1989). In vivo and in vitro treatment with MCH aggregates melanin granules in melanophores of various fish (Kawauchi et al. 1983; Baker 1991; Takahashi et al. 2004). Long-term administration of MCH reduces the melanin content of skin and plasma α-MSH levels in rainbow trout (Baker et al. 1986). In vitro bioassays have demonstrated the direct antagonism between α-MSH and MCH on the melanophores by titrating the two hormones against each other in rainbow trout and winter flounder, Pseudopleuronectes americanus (Baker 1993; Burton and Vokey 2000). In these experiments, melanin granules were aggregated initially with MCH and then dispersed with increasing concentrations of α-MSH added to the MCH vehicle, or inversely melanin granules were dispersed initially with α-MSH and then aggregated with increasing concentrations of MCH added to α-MSH vehicle. In this section, interrelationship between α-MSH and MCH in physiological body color change is discussed based on results revealed in previous sections.

10.1. Antagonism between α-MSH and MCH on chromatophores

Also in barfin flounder, MCH and α-MSH-rp control pigment migration with opposite actions in vitro (Table 5). Namely, MCH activity was antagonized by Des-Ac-α-MSH in both melanophores and xanthophores, whereas α-MSH antagonized MCH

Fig. 31. Eyed-side body color of barfin flounder before (A) and 1 hr after (B) intraperitoneal injection of MCH (1 µg/g body weight). Reprinted from Peptides, 25, Takahashi et al., Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder, 1613–1622, © 2004, with permission from Elsevier.

Fig. 32. (A) MCH concentrations in the brain (pg/mg brain), (B) MCH contents in the pituitary gland (ng/pituitary gland), and (C) MCH concentrations in the plasma (ng/ml) of barfin flounder reared in the white and black tanks. Each value is expressed as means ± SEM (n = 6). *p < 0.05 and ***p < 0.001 indicate the level of statistical difference between the two groups. Reprinted from Gen. Comp. Endocrinol., 143, Amiya et al., Effects of tank color on melanin-concentrating hormone levels in the brain, pituitary gland, and plasma of the barfin flounder as revealed by a newly developed time-resolved floroimmunoassay, 251–256, © 2005, with permission from Elsevier.
activity on xanthophores, but not on melanophores, where α-MSH showed negligible pigment-dispersing activity. No antagonism of α-MSH in barfin flounder melanophores may be related to the presence of two types of MCRs (Kobayashi et al. 2010, see Section 8). This type interaction between α-MSH-rp and MCH is apparent only in in vitro condition. In in vivo condition, Des-Ac-α-MSH showed no pigment dispersing activities, while MCH could aggregate pigment (Yamanome et al. 2007). These findings suggest that the activities of α-MSH-rp are overcome by MCH, probably because of cooperation of MCH and sympathetic system.

10.2. Constitutive action of α-MSH and flexible action of MCH; a model obtained from barfin flounder

In teleosts, physiological color changes are controlled by both the rapid sympathetic system, with noradrenalin as a neurotransmitter, and the slow endocrine system, with MCH and MSH as representative peptide hormones (Fujii 2000; Burton 2002). In barfin flounder, MCH exhibits melanin-aggregating activity both in vitro and in vivo (Takahashi et al. 2004; Yamanome et al. 2005, 2007), suggesting that this peptide acts more or less in concert with noradrenalin and the sympathetic nervous system in physiological body color paling. Expression of mch and biosynthesis of MCH are modulated in response to background color (Takahashi et al. 2004; Amiya et al. 2005; Yamanome et al. 2005). Indeed, the degree of pigment aggregation in response to different background colors seems to be proportional to the body color change in background color-adapted fish (Yamanome et al. 2005; Mizusawa et al. 2011). Thus, there is good agreement among background color, MCH production, pigment concentration, and body color change.

α-MSH-rp exhibit pigment-dispersing activity in vitro (Kobayashi et al. 2009), but not in vivo in barfin flounder (Yamanome et al. 2007). Expression of the pomc encoding α-MSH does not always correspond to changes in background color (Kobayashi et al. 2008b). This is also the case for the plasma profiles of α-MSH, while MCH plasma levels are higher in fish acclimated to a white tank than in those acclimated to a black tank. It is possible that α-MSH-rp possess the potential for pigment dispersing activity, although this activity is overcome by the predominant melanin-aggregating effect of MCH in the endocrine system and the sympathetic system. This is consistent with the common feature of flatfish in which the sympathetic nervous system contributes to the suppression of MSH in vivo activity (Burton 2002).

Long-term background adaptation usually affects differentiation and proliferation of pigment cells, a process known as morphological color change (Sugimoto et al. 2002). Eight months’ acclimation to a white or yellow background yielded a more pale skin color on the non-eye-side than did a black background in barfin flounder (Yamanome et al. 2005). However, the effect of long-term background adaptation on the proliferation or pigmentation of eye-side chromatophores is not known in this fish. We found that pigment dispersion of chromatophores in the dorsal skin exhibited good correlation with background color after 300 days’ acclimation in black, yellow, or white tanks, while chromatophore density remained similar, regardless of tank color (Mizusawa et al. 2011). These results suggest that, at least in the dorsal fin, long-term background adaptation might not yield differential chromatophore proliferation. In contrast, continuous MCH injection (1 µg/g body weight, once a
week for 10 weeks) suppressed pigmentation of the non-eye-side color in barfin flounder (Yamanote et al. 2005), suggesting that MCH might suppress melanophore development or melanin synthesis. Continuous MCH injections (2 μg/g body weight, once per 3 days, a total of nine doses), however, failed to change the pigment cell density (Mizusawa et al. 2011), suggesting that MCH might not affect chromatophore differentiation in the dorsal fin, at least under the experimental conditions employed in this study. Rather than a morphological color change, background color adaption of the dorsal fin might be caused by a physiological color change induced by the control of pigment migration.

Based on the properties of hormonal peptides and catecholamines described above, we propose there is a relationship between the endocrine and neural systems for pigment migration in the barfin flounder (Fig. 34). The activities of MCH, and perhaps noradrenalin, vary depending on their variable release in response to background color changes. α-MSHs may act on chromatophores, although their production and release does not always correspond to background color change. However, the concentrations of α-MSHs are inadequate to overcome the pigment-aggregating activity of MCH and catecholamines under light conditions (white background). Under dark conditions (black background), the concentrations of MCH and catecholamines decreased; hence, the plasma levels of ACTH in interrenal function has been well documented in teleost fish. Hypophysectomy results in a marked atrophy of interrenal cells and a decrease in plasma cortisol levels (Donaldson and McBride 1967; Ball and Hawkins 1976; Young 1993). These changes are prevented by treatment with ACTH. In vitro studies have also confirmed cortisol-releasing activity of ACTH by incubation of diced head kidneys from rainbow trout (Rance and Baker 1981; Takahashi et al. 1985; Young 1993) or by in vitro superfusion.

### Table 5. In vitro pigment-aggregating activities of MCH and pigment-dispersing activity of MSHs in the barfin flounder skin.

<table>
<thead>
<tr>
<th>Hormone (nM)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serially diluted MCH</td>
<td>5.0 ± 0.0</td>
<td>3.6 ± 0.1</td>
<td>2.3 ± 0.2*</td>
<td>1.7 ± 0.2*</td>
<td>1.5 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
</tr>
<tr>
<td>10 nM MCH plus serially diluted Des-Ac-α-MSH</td>
<td>3.0 ± 0.3**</td>
<td>3.6 ± 0.2**</td>
<td>3.8 ± 0.3**</td>
<td>4.3 ± 0.1**</td>
<td>4.3 ± 0.2**</td>
<td></td>
</tr>
<tr>
<td>100 nM MCH plus serially diluted Des-Ac-α-MSH</td>
<td>1.4 ± 0.1*</td>
<td>1.7 ± 0.2*</td>
<td>1.8 ± 0.2*</td>
<td>2.4 ± 0.1*</td>
<td>2.9 ± 0.2**</td>
<td></td>
</tr>
<tr>
<td>1000 nM MCH plus serially diluted Des-Ac-α-MSH</td>
<td>1.5 ± 0.1*</td>
<td>1.5 ± 0.1*</td>
<td>1.6 ± 0.2*</td>
<td>1.9 ± 0.1**</td>
<td>2.0 ± 0.2**</td>
<td></td>
</tr>
<tr>
<td>10 nM MCH plus serially diluted α-MSH</td>
<td>1.8 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>3.1 ± 0.2*</td>
<td></td>
</tr>
<tr>
<td>100 nM MCH plus serially diluted α-MSH</td>
<td>1.8 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>2.2 ± 0.3</td>
<td>2.7 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>1000 nM MCH plus serially diluted α-MSH</td>
<td>1.6 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>2.2 ± 0.2*</td>
<td>2.4 ± 0.3*</td>
<td></td>
</tr>
</tbody>
</table>

The Kruskal–Wallis test and comparisons of treated vs. untreated controls indicated that treatments with serially diluted MCH significantly concentrated pigments in melanophores (above) and xanthophores (below, P < 0.05), and treatments with serially diluted Des-Ac-α-MSH or α-MSH significantly dispersed pigments in chromatophores pretreated with MCH (10, 100, or 1000 nM; *P < 0.05). The effects of MCH pretreatment on pigment dispersal by Des-Ac-α-MSH (* vs. 0.3*, a 3.6) and α-MSH (* vs. 0.1*, b 1.8) were inhibited by MCH pretreatment in melanophores (above) and xanthophores (below), while those of α-MSH were inhibited by MCH pretreatment only in xanthophores. Reprinted from Gen. Comp. Endocrinol., 171, Mizusawa et al., Inhibiting roles of melanin-concentrating hormone for skin pigment dispersion in barfin flounder, Versasper moseri, 75–81, © 2011, with permission from Elsevier.
using diced head kidneys from tilapia (Balm et al. 1994) and gilthead sea bream (Rotllant et al. 2000a, b). In elasmobranchs, ACTH has been isolated from the pituitary of spiny dogfish (Lowry et al. 1974).

In fish, Rance and Baker (1981) demonstrated for the first time the in vitro corticotropic activity of α-MSH using diced interrenal tissue from rainbow trout. Des-Ac-α-MSH has full intrinsic activity: its molar potency is 0.9 × 10⁻²-fold less than ACTH₁⁻⁻²⁴ but nearly 100-fold greater than α-MSH. When Lamers et al. (1992) compared the corticotropic activities of Des-Ac-α-MSH, α-MSH and Di-Ac-α-MSH in a superfusion experiment with tilapia interrenal tissue, Di-Ac-α-MSH showed the highest corticotropic activity followed by Des-Ac-α-MSH and α-MSH. Although Di-Ac-α-MSH is about 120-fold less potent than ACTH₁⁻⁻³⁹, a superfusate from the PI is only 2.7-fold less active than that from the PD, in which the corticotrophic activity is caused by ACTH. Thus, α-MSH-rp released from the PI may have a significant effect on interrenal function. The difference in potency among the three types of MSH with different degrees of acetylation suggests that the N-terminal Ser residue affects binding to MCRs that are present in interrenal cells. This section deals with the role of MC systems on interrenal function of barfin flounder.

Cortisol-releasing activities of MC peptide such as ACTH₁⁻⁻₂⁴, Des-Ac-α-MSH, α-MSH, and Di-Ac-α-MSH were examined using head kidney pieces. After incubation of the tissue pieces with appropriate concentrations of MC peptides, cortisol contained in incubation medium was estimated by TR-FIA. Localization of MC2R and MC5R mRNA was examined by in situ hybridization on barfin flounder head kidney using cRNA synthesized from each recombinant DNA.

11.1. Effects of POMC-derived peptides on cortisol release and roles of MCRs on interrenal function

The effects of MC peptides on in vitro cortisol release from the head kidney of barfin flounder are shown in Fig. 36. ACTH₁⁻⁻₂⁴ stimulated cortisol secretion in a dose-dependent manner. Des-Ac-α-MSH and Di-Ac-α-MSH also stimulated cortisol release in a dose-dependent manner, while these activities were lower than ACTH. However, α-MSH showed negligible effects on cortisol release. In situ hybridization using an anti-sense cRNA probe for MC2R mRNA showed the presence of positive signals on the interrenal cells (Fig. 37C), while the sense probe yielded no signals (Fig. 37D). When an anti-sense cRNA probe for MC5R mRNA showed the presence of positive signals on the interrenal cells (Fig. 37C), while the sense probe yielded no signals (Fig. 37D). When an anti-sense cRNA probe for MC2R mRNA was used, positive signals were observed in both interrenal cells and non-interenal cells (Fig. 37E). No signals were observed when a sense cRNA probe was used (Fig. 37F).

ACTH, MC2R, and cortisol are key components of the pituitary–interrenal axis in fish (Wendelaar Bonga 1997; Metz et al. 2005). The presence of ACTH in the PD of barfin flounder pituitary was demonstrated in...
our study (Takahashi et al. 2006). We have demonstrated that MC2R, which is a specific receptor for ACTH, is present in the interrenal cells (Fig. 37), and that ACTH stimulates cortisol release from the head kidney (Fig. 36) (Kobayashi et al. 2011a). ACTH also exhibits its function via MC5R; considering the abundant mc2r transcripts in the head kidneys compared to mc5r transcripts and the generally accepted binding properties of MC2R and MC5R, MC2R is an essential receptor mediating the biological function of ACTH. In summary, these results show the presence of a classical pituitary–interrenal axis in barfin flounder.

We showed the expression of mc5r in the head kidney of barfin flounder using RT-PCR (Kobayashi et al. 2010). This observation was confirmed by detection of mc5r transcript in interrenal cells by in situ hybridization (Fig. 37), and supported the assumption that MC peptides other than ACTH are associated with cortisol release in this fish. Indeed, this was demonstrated by the fact that Des-Ac-α-MSH and Di-Ac-α-MSH stimulate cortisol release. The major source of ACTH is the PD of the pituitary, while α-MSH-rp such as α-MSH, Des-Ac-α-MSH, and Di-Ac-α-MSH are mainly produced in the PI of the pituitary (Takahashi et al. 2006). Taken together, it is conceivable that α-MSH-rp in the PI and MC5R in the interrenal cells are additional members that contribute to pituitary–interrenal axis to regulate cortisol release.

11.2. Acetylation and corticotropic activities of α-MSH-rp

Corticotropic activity of α-MSH-rp has been shown in rainbow trout (Rance and Baker 1981) and tilapia in vitro (Lamers et al. 1992). In rainbow trout, molar potency of Des-Ac-α-MSH was greater than α-MSH. In tilapia, Di-Ac-α-MSH showed the highest corticotropic activity, which was followed by Des-Ac-α-MSH and α-MSH. Our data on the cortisol-releasing activities of α-MSH-rp were comparable to the preceding two reports (Rance and Baker 1981; Lamers et al. 1992). Namely, these results suggest that the number of acetyl groups on α-MSH may influence ligand–receptor interactions, and that corticotropic activities of α-MSH...
and its related peptides are not always proportional to the degree of acetylation. Pharmacological properties of sea bass MR5R on human embryonic kidney (HEK) 293 cells (Sanchez et al. 2009) which stably express a galactosidase reporter gene may be helpful to explain the interaction between α-MSH-rp and MC5R in barfin flounder because bMC5R shares 92% amino acid sequence identity with sea bass MC5R. Sea bass MC5R activated galactosidase gene transcription in response to α-MSH. The potency of α-MSH was indistinguishable from that of Des-Ac-α-MSH and Des-Arg1-MSH to MC5R in HEK293 cells is incompatible with the corticotrophic activities of these peptides in barfin flounder—Des-Ac-α-MSH exhibits corticotrophic activity, and is more potent than α-MSH. We therefore suggest that corticotrophic activities of α-MSH-rp, which are not proportional to the degree of acetylation, may not simply depend on affinity of each peptide for MC5R.

11.3. Putative MCR heteromer and corticotrophic activities of α-MSH-rp

There are at least two similarities between corticotrophic activities and pigment-dispersing activities exhibited by α-MSH-rp (Fig. 30). One is that Des-Ac-α-MSH shows higher activity than α-MSH in both biological activities. The other is that two different mcr subtypes are expressed in both interrenal cells and melanophores (Kobayashi et al. 2010). We therefore suggested that heteromers of MC2R and MC5R may result in negligible corticotrophic activities of α-MSH in barfin flounder interrenal cells (Kobayashi et al. 2011a). This would also explain the discrepancy in the activities of α-MSH-rp observed in HEK293 and interrenal cells.

Given that the amounts of MC2R and MC5R mRNA are proportional to those of receptor molecules of MC2R and MC5R, most MC5R may be associated with MC2R to form heteromers, and most MC2R may remain in monomeric form. It is interesting in this context that mc2r and mc5r are found in tandem on the genome, a property that is remarkably well conserved through the evolution (Schiødt et al. 2005) suggesting that they could be under common transcriptional control. In addition, MC2R accessory protein may participate in the regulation of cellular functions of MC2R and MC5R in interrenal cells, because these two receptors are differentially regulated by this protein, which exerts opposite effects on the surface expression and dimerization of the two receptors (Sebag et al. 2009; Webb and Clark 2010).

12. General discussion—biological significance of melanotropic peptides beyond body color change

The processes described above concerning the activities of α-MSH-rp and MCH may be part of the complex regulation of pigment migration for delicate and subtle changes in the hues and patterns of barfin flounder, in which endocrine systems uniformly affect the entire skin, while neural systems exert local control ( Mizusawa et al. 2011). It is conceivable that MSH and MCH are involved in control processes because receptor genes of MSH and MCH are expressed in a wide variety of tissues of barfin flounder (Takahashi et al. 2007; Kobayashi et al. 2008a, 2010, 2011a). Association with food intake or energy metabolism may be a possible function.

12.1. Effects of MSH on lipolysis

POMC-derived peptides have been shown to stimulate lipolysis in mammalian adipocytes (Boston 1999). However, significant species differences have been shown with regard to the relative potency of the peptides. Both α-MSH and ACTH exhibit potent lipolytic activity against rabbit adipocytes, while only ACTH has potent lipolytic activity in rats (Ramachandran and Lee 1976; Richter and Schwandt 1987). Guinea pig adipocytes are sensitive to both α-MSH and ACTH, while adipocytes from hamsters only respond to ACTH (Ng 1990).

Among the chum salmon MSH-rp, Des-Ac-α-MSH shows the highest lipolytic activity in isolated rat adipocytes, followed by α-MSH, ACTH1–24, and β-MSH (Kawauchi et al. 1984). Yada et al. (2000) demonstrated the lipolytic activity of Des-Ac-α-MSH in rainbow trout. Intra-arterial administration of Des-Ac-α-MSH increases circulating levels of free fatty acids, whereas α-MSH has no effect. Moreover, administration of Des-Ac-α-MSH stimulates hepatic triacylglycerol lipase both in vivo and in vitro. Although the major lipotropic hormone in mammals is ACTH, the hormone shows no significant effect on lipid mobilization in rainbow trout (Takahshima et al. 1972).

The finding of lipolytic activity of MSH in rainbow trout led us to investigate the role of MSH in the "cobalt" variant of rainbow trout (Yada et al. 2002). The cobalt variant is found on rare occasions at trout experimental stations and commercial farms. It has been named after its typical “cobalt-blue” body color (Oguri 1974; Yamazaki 1974; Kaneko et al. 1993). In the cobalt variant, integumental melanophores are considerably reduced in number (Oguri 1983). This phenomenon seems to be related to a lack of most of the P1 in the pituitary, which contains MSH cells (Kaneko et al. 1993). Another characteristic of this variant is obes-
ity: enlarged liver and fat accumulation in the abdominal cavity have been reported (Yamazaki 1974; Oguri 1976; Kaneko et al. 1993). The hepatic lipase activity of the cobalt responded to Des-Ac-α-MSH as in the normal rainbow trout in vitro, whereas its activity in vivo was lower than that in the normal rainbow trout (Yada et al. 2002). No significant difference has been observed in the basal activity of triacylglycerol lipase of cultured liver slices between the cobalt and normal rainbow trout. Thus, the lower levels of lipase activity...
in the cobalt variant of rainbow trout is due to a lack of stimulatory regulation by lipolytic hormones such as Des-Ac-α-MSH. Taking into account the lipolytic activity of the peptide, the scarceness of MSH in the pituitary seems to be correlated with the abnormal fat accumulation in the cobalt variant.

12.2. Roles of MC system on food intake

Among the five different MC receptors, MC3R and MC4R are preferentially expressed in the hypothalamus, a central nervous system region that controls many physiological functions, including feeding behavior. The first evidence for an inhibitory role of MSH in the regulation of food intake was provided by studies in rats in which intracerebroventricular administration of the peptide resulted in suppression of food intake (Panson et al. 1976; Vergoni et al. 1986; Tsuji and Bray 1989). Food deprivation decreases the expression of hypothalamic POMC mRNA in rats (Brady et al. 1990; Kim et al. 1996). Several lines of evidence demonstrated the association of MC-receptors with food intake hitherto. Fan et al. (1997) showed that melanocortinergic neurons exert a tonic inhibition of feeding behavior.

The pome-c, one of the three pome subtypes in barfin flounder (Takahashi et al. 2005a) is expressed in the brain and MSH-ir cell bodies are present in the hypothalamus (Amano et al. 2005). Apparent expression of mc4r is also observed in the brain of this fish (Kobayashi et al. 2008a). These results indicate the presence of a functionally complete MC system consisting of POMCergic neurons and the corresponding receptor in the brain of the barfin flounder. One of the functions of this system is likely to be the regulation of energy homeostasis. We have shown, however, that feeding status does not seem to affect the relative amount of pome transcripts in the brain of barfin flounder (Takahashi et al. 2005a). Moreover, no difference was observed in the expression of mc4r in the brain in relation to feeding status (Fig. 38). This is contrary to the well-documented regulation of POMC and the MC4R in mammals (Adan et al. 2006; Harrold and Williams 2006). Remarkably, however, the expression of the mc4r in the liver of fish that had fasted is higher than that of fish that had been fed (Fig. 38). These results may be the first evidence of a functional role of MC4R in the periphery. It is possible that food deprivation causes an increase in the expression of the receptor because the MC system is associated with liver functions in relation to energy homeostasis. This assumption is supported by the ability of Des-Ac-α-MSH to stimulate lipid mobilization in the liver of rainbow trout (Yada et al. 2000), while this function is probably mediated by MC receptors other than MC4R in the rainbow trout, because the expression of this receptor has not been detected in rainbow trout liver (Haitina et al. 2004).

12.3. Effects of MCH on food intake

In rodents, several recent investigations have shown an association between MCH and enhanced feeding behavior: (i) intracerebroventricular injection of MCH stimulates feeding (Qu et al. 1996; Rossi et al. 1997; Gomori et al. 2003), (ii) transgenic mice overexpressing the MCH gene tend to overeat and are obese (Ludwig et al. 2001), (iii) fasting (24 or 48 hr) in rodents results in increased mch expression (Qu et al. 1996; Hervé and Fellmann 1997), (iv) increased mch expression is observed in genetically obese rodents (Qu et al. 1996; Hanada et al. 2000), (v) mice carrying a deletion of the entire coding region of the mch are hypophagic and lean (Shimada et al. 1998), (vi) MCH receptor antagonists reduce food intake in rats (Borowsky et al. 2002; Takekawa et al. 2002), and (vii) disruption of MCHR1 in mice results in lean and hyperactive animals in which hyperphagia may be a compensatory response to the increased lean mass and hypermetabolic rate (Chen et al. 2002; Marsh et al. 2002).

In fish, circumstantial evidence for the association of MCH with food intake was obtained in barfin flounder (Takahashi et al. 2004). The mch expression level in the brain was higher in fish that had fasted than controls (Fig. 39). The number of MCH-ir cell bodies in the hypothalamus was also greater in the fish that had fasted. These results are comparable to those in rodents, which showed a correlation between fasting and increased mch expression. Rearing of barfin flounder for 6 months in a tank with a white background resulted in increased mch expression in the brain compared to those reared with a black background. Furthermore, both total length and body weight were greater in the fish reared with a white as opposed to black background. These results indicate that a white background stimulated production of MCH in the brain and, in turn, MCH enhanced body growth, probably by stimulating food intake. However, MCH may not stimulate food intake throughout fish species because our recent research indicated that intracerebroventricular injection of MCH exerted an anorexigenic action in goldfish, unlike its orexigenic action in mammals (Matsuda et al. 2006).

13. Conclusions and perspective

As suggested by their names and many experimental results, the main target cells of MSH and MCH are the chromatophores in the skin, where these peptide hormones play opposing roles in regulating pigment migration. These functions are rather restricted to neopterygians including most of teleost fish. Although...
MCH is present in the hypothalamus of all vertebrates that have been examined, only neopterygians employ MCH as a circulating hormone—MCH synthesized in hypothalamus is released via PN of the pituitary (Sherbrooke and Hadley 1988; Baker 1991; Bird et al. 2001). The presence of MCRs and MCHRs in systemic body in fish indicates that these peptides exhibit a wide variety of functions. Teleost fish may harbor the other interesting MSH/MCH interrelating system than chromatophore control and feeding regulation.

The important external environmental factor for fish would be photic conditions. Synthesis and release of MCH responds well to background color and affects body color in barfin flounder, while those of MSH would be under constitutive or constant synthesis and release. Changes in photic condition could conceivably influence physiological process in many tissues and organs where MCHRs are present (Fig. 21). Indeed, MCH levels correlated well with growth, i.e. barfin flounder reared in a white tank showed faster growth speed and higher MCH production than those reared in a black tank (Takahashi et al. 2004; Yamanome et al. 2005). Given that MCH facilitated food intake also in barfin flounder, an increased amount of MCH under white background might stimulate the...

Fig. 38. Amounts of MC4R mRNA in the brain (A) and liver (B) of barfin flounder after 21 days rearing with feeding (black bar) or without feeding (white bar). Unpaired t test was used to compare two groups. Values are given as means ± SEM. Asterisks show statistical significance; *P < 0.05, n = 7. Reprinted from Gen. Comp. Endocrinol., 155, Kobayashi et al., Food deprivation increases the expression of melanocortin-4 receptor in the liver of barfin flounder, 280–287, © 2008, with permission from Elsevier.

Fig. 39. Total length (A), body weight (B), and relative amount of MCH cDNA compared with β-actin cDNA in the brain (C) of barfin flounder after 14 days fasting. Black bar and white bar show control group and fasted group, respectively. The values are given as means ± SEM. Asterisks show statistical significance; *P < 0.05, ***P < 0.001. The number of fish is given in parentheses. Modified from Peptides, 25, Takahashi et al., Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder, 1613–1622, © 2004, with permission from Elsevier.
feeding center in the hypothalamus, or modified di- 
gestive functions or metabolism in intestine or in
clinator muscle.

Fish adapt to various photic environments through 
changes in both spectral sensitivity and the number of 
visual pigments (Lythgoe 1979). A complete set of 
visual opsins genes such as red-sensitive, green-
sensitive, blue-sensitive, and ultraviolet-sensitive 
opsins along with rod opsins have been identified in 
 freshwater fish living in shallow water, such as 
zearfish (Chinen et al. 2003; Takechi and Kawamura 
2005) and medaka (Matsumoto et al. 2006), whereas 
only rod opsins have been identified in various marine 
deep-water species of fish (Hope et al. 1997; Hasegawa 
et al. 2008). In flatfish members, Atlantic halibut, 
Hippoglossus hippoglossus, has been shown to pos-
sess the five opsins observed in shallow freshwater 
species (Helvik et al. 2001). Recently, we cloned a set of 
opsin cDNAs from barfin flounder eyeballs (unpub-
lished data), suggesting that color vision in this fish is 
well developed. Interestingly, during the experiments 
evaluating the effects of particular wave length of 
light on physiological status, we found that green light 
stimulates growth of barfin flounder (Yamanome et al. 
2009). It is conceivable that particular wave length of 
light would affect neuroendocrine systems associating 
with food intake and metabolism via MCH and MSH 
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