Characterization of melanocortin receptors from stingray *Dasyatis akajei*, a
cartilaginous fish

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Abstract

Melanocortin (MC) systems are composed of MC peptides such as adrenocorticotrophic hormone (ACTH), several molecular forms of melanocyte-stimulating hormones (MSHs) and MC receptors (MCRs). Here we demonstrated that the cartilaginous fish, *Dasyatis akajei* (stingray) expresses five subtypes of MCRs genes—*mc1r* to *mc5r*—as in the case of teleost and tetrapod species. This is the first evidence showing the presence of the full repertoire of melanocortin receptors in a single of cartilaginous fish. Expression of respective stingray *mcr* cDNAs in Chinese hamster ovary cells revealed that Des-acetyl- α -MSH exhibited cAMP-producing activity indistinguishable to ACTH(1-24) on MC1R and MC2R, while the activity of Des-acetyl- α -MSH on MC3R, MC4R, and MC5R were similar to or slightly greater than that of ACTH(1-24). Notably, in contrast to the other vertebrates, MC2R did not require coexpression with a melanocortin receptor-2 accessory protein 1 (*mrp1*) cDNA for functional expression. One of the roles of MC system resides in regulation of the pituitary-interrenal (PI) axis—a homologue of tetrapod pituitary-adrenal axis. In stingray, interrenal tissues were shown to express *mc2r* and *mc5r* as major MCR genes. These results established the presence of functional PI axis in stingray at the level of receptor molecule. While MC2R participates in adrenal functions together with MRAP1 in tetrapod species, the fact that sensitivity of MC5R to Des-acetyl- α -MSH and ACTH(1-24) were two order of magnitude higher than MC2R without coexpression with MRAP1 suggested that MC5R could play a more important role than MC2R to transmit signals conveyed by ACTH and MSHs if MRAP1 is really absent in the stingray.

1. Introduction

Melanocortin (MC) is a collective term for peptide hormones such as adrenocorticotropin (ACTH) and melanocyte-stimulating hormones (MSHs) derived from the common precursor proopiomelanocortin (POMC), from which an opiate peptide β -endorphin is also derived (Dores and Baron, 2011; Sundstrom et al., 2010; Takahashi and Kawauchi, 2006; Takahashi et al. 2014). Melanocortin peptides exhibit their biological functions by binding with melanocortin receptors (MCRs), which are members of G protein-coupled receptors with seven transmembrane domains (Cone, 2006). Five subtypes of MCRs (MC1R, MC2R, MC3R, MC4R, and MC5R) have been identified so far in bony fishes and tetrapod species (Cerdá-Reverter et al., 2011; Gantz and Fong, 2003; Metz et al., 2005). Moreover, another protein called melanocortin-2 receptor accessory protein (MRAP) is required for the specific activation of MC2R among the five MCRs (Chan et al., 2009; Dores and Garcia, 2015; Hinkle and Sebag, 2009; Metherell et al., 2005; Webb and Clark, 2010). The melanocortin systems thus consisting of melanocortin peptides, MCRs, and MRAP is suggested to have emerged in early vertebrates because these repertoires have been identified in lampreys (Superclass Agnatha) the oldest group of vertebrates, but not found in any of extant protochordates such as the cephalochordates or the urochordates (Dores and Garcia, 2015; Haitina et al., 2007; Takahashi et al., 1995; Vastermark and Schiöth, 2011).

Among the several melanocortin peptides, ACTH is the largest peptide consisting of 39 amino acid residues in many vertebrate species including cartilaginous fish, whereas the length of MSH peptides are shorter than ACTH; for example α -MSH is typically composed of 13 amino acid residues (Dores and Baron, 2011; Takahashi et al., 2001). In addition to α -MSH, teleost POMC

contains β -MSH, and mammalian POMC contains β -MSH and γ -MSH (Takahashi and Kawauchi, 2006). These melanocortin peptides possess specific binding properties to the five MCR subtypes. For example, MC2R specifically binds ACTH but not shorter MSH-sized ligands, in the presence of MRAP, while the other MCRs bind all melanocortin peptides in an MRAP independent manner (Dores and Garcia, 2015).

The cartilaginous fishes (Class Chondrichthyes) are divided into two subclasses: Holocephali including ratfishes and elephant shark, and the Elasmobranchii including sharks and rays (Nelson, 1994). Compared with teleost and mammals, melanocortin systems of cartilaginous fish possess unique properties which have not yet been observed in teleosts and mammals. Regarding ligand structure, cartilaginous fish POMC characterized from sharks, rays, and ratfish contains δ -MSH in addition to α -MSH, β -MSH, and γ -MSH (Amemiya et al., 1999; Amemiya et al., 2000; Dores et al., 2003; Takahashi et al., 2004). Concerning ligand activation properties, elephant shark MC2R was shown to be MRAP-independent for the functional activation (Reinick et al., 2012b). These characters may have emerged after the divergence of the ancestral cartilaginous fish and the ancestral bony fishes more than 400 million years ago (Reinick et al., 2012b).

Given the significance of the cartilaginous fishes for understanding the evolution of melanocortin systems, there is limited information on the number of MCRs and the ligand selectivity of these receptors from current studies on dogfish MC3R, MC4R, and MC5R, and elephant shark MC2R and MC3R (Klovins et al., 2004; Liang et al., 2013; Reinick et al., 2012a; Ringholm et al., 2003), because a complete set of MCR paralogs has not been characterized currently in these fish (Dores and Garcia, 2015). In the stingray *Dasyatis akajei*, no information about

MCRs are reported so far. However, for the stingray *Dasyatis akajei*, although no information is currently available about the MCRs, a *pomc* cDNA has been cloned for this species (Amemiya et al., 2000); hence, the melanocortin peptides for pharmacology are available on the basis of deduced amino acid sequence. Using the stingray as a model system, the present study was undertaken to demonstrate the presence of all repertoires of MCRs in a single cartilaginous fish, and to investigate the pharmacological properties of these MCR paralogs. In addition, we sought to determine whether the activation of the MC2R ortholog of the stingray was MRAP independent and could be activated by either ACTH or a form of α -MSH, in a manner similar to the MC2R ortholog of the elephant shark (Reinick et al., 2012b).

2. Materials and Methods

2.1. Fish samples

The stingray, *D. akajei*, were captured at the northern side of the eastern region of Inland Sea of Japan (Setonaikai) and kept at Ushimado Marine Institute, Okayama University. Fish (3 females of 1.6–8.7 kg bodyweight and 3 males of 1.9–3.1 kg) were treated according to Guidelines for the Care and Use of Animals of Kitasato University. Prior to tissue collection, all individuals were anesthetized by 0.05% 2-phenoxyethanol (Wako, Osaka, Japan). The hypothalamus, interrenal tissue, and kidney were immediately dissected and frozen on dry ice; the samples were stored at -80°C until analysis.

2.2. Nucleic acid preparation and amplification

Total RNA was isolated from the hypothalamus and interrenal tissue using ISOGEN2 reagent (Wako) and MixerMill MM300 (Reche, Haan, Germany).

Total RNA was treated with RNase-free DNase (TaKaRa, Otsu, Japan) to eliminate genomic DNA contamination and then purified by phenol-chloroform extraction followed by isopropanol precipitation. First-strand cDNA was prepared using the PrimeScript II 1st Strand Synthesis kit (TaKaRa), in 20 µl of cDNA synthesis mixture containing 5 µg of total RNA. Genomic DNA was isolated from the kidney according to a previously described method (Sambrook and Russell, 2001). Genomic DNA was treated with RNase A (Macherey-Nagel GmbH & Co. KG, Düren, Germany) to eliminated RNA contamination and then purified by phenol-chloroform extraction followed by isopropanol precipitation. Polymerase chain reaction (PCR) to amplify cDNA and genomic DNA was performed using TaKaRa Ex Taq Hot Start Version kit (TaKaRa) under conventional conditions. For rapid amplification of cDNA ends (RACE), the 5' and 3' end of synthesized cDNA were prepared using the SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). Amplified DNA was purified using the Nucleospin gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany) after agarose gel electrophoresis. Custom oligonucleotides for PCR that are shown in Table 1 were synthesized at Life Technologies (Carlsbad, CA, USA) or Nihon Gene Research Labs, Inc. (Sendai, Japan). The full-length cDNA fragments were subcloned into pSTBlue-1 Acceptor™ Vector (Merck Millipore, Billerica, MA, USA) followed by standard alkaline-SDS plasmid preparation (Sambrook and Russell, 2001).

2.3. cDNA cloning and sequence determination

First, the middle segment of a MCR cDNA was amplified from the first-strand cDNA that was synthesized from the hypothalamus. Primers were designed based on the nucleotide sequences of *mcr* genes of dogfish *Squalus acanthias* (GenBank accession numbers, *mc3r*: AY560605, *mc4r*: AY169401, and

mc5r: AY562212), elephant shark *Callorhinchus milii* (GenBank accession
 numbers, *mc1r*: BR000855, *mc2r*: BR000856, *mc3r*: BR000857), goldfish
Carassius auratus (GenBank accession numbers, *mc1r*: AB618067, *mc2r*:
 AB618068, *mc3r*: AB618069, *mc4r*: AJ534337, and *mc5r*: AJ576322), and mouse
Mus musculus (GenBank accession numbers, *mc1r*: BC119296, *mc2r*: NM_008560,
mc3r: NM_008561, *mc4r*: BC116959, and *mc5r*: BC100720). For all *mcr* genes
 except *mc1r*, second, the 5' and 3' regions of each MCR cDNA were amplified from
 first-strand cDNA by RACE systems using gene-specific primers that were
 designed based on the sequences of the middle segment. In the case of *mc1r*,
 upstream and downstream DNA fragments were amplified from genomic DNA by
 inverse PCR (see [Sambrook and Russell, 2001] for the principles). Finally, a MCR
 cDNA containing the entire coding region was amplified from first-strand cDNA
 by PCR using gene specific primers based on the sequences from 5'- and 3'-RACE
 systems or inverse PCR. The full-length cDNAs were cloned into the pSTBlue-1.
 DNA sequencing analyses were carried out for the purified PCR products and the
 cloned DNA using a Big Dye Terminator v3.1 cycle sequencing kit (Thermo Fisher
 Scientific, Waltham, MA, USA) and ABI 3130xl genetic analyzer (Thermo Fisher
 Scientific).

2.4. Sequence data analyses

Transmembrane domains were predicted using "MEMSAT3"
 (<http://bioinf.cs.ucl.ac.uk/psipred/>). Alignments and phylogenetic relationships
 among the amino acid sequences were inferred for the stingray and other
 vertebrates (dogfish, *S. acanthias*; elephant shark, *Callorhinchus milii*; goldfish,
Carassius auratus; and mouse, *M. musculus*) MCRs by using MEGA 6.0,
 together with bovine *Bos taurus* rhodopsin (RHO: NM_001014890) as an outgroup.

The evolutionary distance of amino acid substitutions were estimated using the Jones-Taylor-Thornton model (Jones et al., 1992), and a phylogenetic tree was constructed by applying the neighbor-joining method (Saitou and Nei, 1987). The reliability of tree topology was evaluated by bootstrap analysis with 500 replications.

2.5. Reverse transcription polymerase chain reaction

Reverse transcription was done as described in section 2.2. PCR was carried out using TaKaRa Ex Taq HS (TaKaRa). The PCR mixture (25 μ l) contained 10 pM of each forward and reverse primer, together with 0.5 μ l of hypothalamus cDNA or 125 ng (equal to the amount of template RNA for 0.5 μ l of cDNA) of total RNA, or 25 ng of genomic DNA. The primers for the amplification of MCR cDNA and β -actin are shown in Table 1. The amplification protocols was as follows: 95°C for 5 min (activation of TaKaRa Ex Taq HS DNA polymerase), 30 cycles at 94°C for 20 s (denaturation), 60°C (for MCR) or 66°C (for β -actin) for 15 s, and 72°C for 60 s (extension). PCR products were electrophoresed on 2% agarose gel (Agarose S, Wako) and visualized with ethidium bromide. Photographs were taken using a Densitograph (Atto, Tokyo, Japan).

2.6. Quantification of *mcr* mRNA expression

A series of mRNA concentrations was prepared by *in vitro* transcription of the pST-Blue-1 plasmid clones with the *mcr* cDNAs that contained the entire coding region (see section 2.3), which were used as standard references to quantify *mcr* mRNA concentrations. The plasmid clones were digested with *Sal*I or *Bam*HI at the 3' terminal end of the inserts. The sense-strand RNA was synthesized according to the manufacturer's instructions using a MAXIscript SP6/T7 kit

(Ambion, Austin, TX, USA) and quantified for serial dilution.

Quantitative reverse-transcription PCR (qRT-PCR) was carried out using Thermal Cycler Dice® Real Time System II MRQ (TaKaRa) and OneStep RT-PCR kit (QIAGEN, Hilden, Germany). The TaqMan probes and primers that are listed in Table 2 were designed using Primer Express (Thermo Fisher Scientific). The PCR mixture (10 µl) contained QIAGEN OneStep RT-PCR buffer, QIAGEN OneStep RT-PCR Enzyme Mix, 400 µM of the dNTP mix, 2.5 pM of each forward and reverse primer, and 2.5 pM of TaqMan probe. The reaction was started with reverse transcription at 50°C for 30 min, followed by amplification protocols: 95°C for 15 min (activation of HotStarTaq DNA polymerase), 50 cycles at 95°C for 20 s (denaturation), 55°C for 20 s (annealing), and 72°C for 15 s (extension). The levels of mRNA of each *mcr* gene in the total RNA was quantified based on the amplitude scaled to the dilution series of the reference RNA (1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 RNA copies) for each *mcr* gene.

2.7. Functional expression assays

In order to analyze the ligand selectivity of the stingray (sr) MC1R, srMC2R, srMC3R, srMC4R, and srMC5R paralogs in a heterologous mammalian cell system, the nucleotide sequences for these melanocortin receptors were synthesized by GenScript (Piscataway, NJ, USA) with a V-5 epitope tag at the N-terminal of the receptor. These cDNAs were separately inserted into a pcDNA3.1 expression vector, and each stingray *mcr* cDNA construct was separately transiently transfected into Chinese Hamster Ovary (CHO) cells. The CHO cells were grown at 37° in a humidified 5% CO₂ incubator in DMEM/F12 with 5% fetal calf serum. For these experiments, 2 µg of the stingray *mcr* cDNA was co-transfected with 2 µg of a CRE/Luciferase reporter plasmid (Chepurny and

Holz, 2007) using the Solution T Cell Line Nucleofector Kit (Amaya Inc., Gaithersburg, MD, USA) and program U-23 as describe in Reinick et al (2012a). The transiently transfected cells were seeded on a 96-well plate at a density of 10^5 cells/well. After an incubation period of 48 hours, the transfected cells were stimulated with synthetic ACTH(1-24), Des-acetyl- α -MSH, β -MSH, γ -MSH, δ -MSH or β -endorphin based on stingray *pomc* cDNA sequence (Fig. 1, Amemiya et al., 2000), at concentrations ranging from 10^{-6} M to 10^{-12} M, in serum-free CHO media for four hours at 37°C. At the end of the incubation period, 100 μ l of Bright-Glo luciferase assay reagent (Promega Inc., Madison, WI, USA) was added to each well and incubated for 5 minutes at room temperature. Luminescence was measured with a Bio-Tek Synergy HT plate reader (Bio Tek, Winooski, VT, USA), and the dose response curves were analyzed by using Kaleidagraph software (Synergy Software, Reading, PA, USA). All experimental treatments were performed in triplicate.

2.8. Statistical analyses

For qRT-PCR, statistical differences were determined using one-way analysis of variance (ANOVA) followed by Games Howell test by using StatView 5.0 for Windows (SAS Institute Inc, Cary, NC, USA). For pharmacology, statistical differences were determined using one-way ANOVA followed by Tukey's multi-comparison test by using GraphPad Prism 2 (GraphPad Software Inc, La Jolla, CA, USA). Significance was set at $P < 0.05$.

3. Results

3.1. Determination of nucleic acid sequence

It has been generally accepted that vertebrates possess five subtypes of

mcr genes (Cone, 2006). Both previous cloning studies in dogfish and recent analysis of the genome in elephant shark uncovered only three subtypes of MCR (Klovins et al., 2004; Ringholm et al., 2003; Vastermark and Schiöth, 2011). By using conventional PCR and RT-PCR methods we have obtained the sequence of five stingray *mcr* paralogs—*mc1r* from genomic DNA, and *mc2r*, *mc3r*, *mc4r*, and *mc5r* from total RNA of stingray brains (Fig. 2A). Amino acid sequence comparison of srMCRs with those from other species are shown in Figs. 1 to 5 in Takahashi et al., submitted. srMCR sequences show the highest amino acid sequence identity to MCR sequences from other cartilaginous fishes (i.e., dogfish and elephant shark) (Fig. 3).

3.2. Expression of *mcr* genes in hypothalami and interrenal tissues

In RT-PCR analyses amplifying whole reading frames, signals for the all five *mcr* mRNA corresponded to the lengths calculated based on nucleotide sequences (Fig. 4A). The size of each amplified *mcr* cDNA was similar to that amplified from genomic DNA (Fig. 4B). Figs. 4B also show successful removal of genomic DNA from RNA samples for reverse transcription. These results indicated that these five stingray *mcr* genes did not contain introns in the reading frames. Table 3 shows the amounts of *mcr* mRNAs in hypothalami and interrenal tissues. In the hypothalami, *mc3r* mRNA was the most predominant, based on average, and followed by *mc5r* mRNA, *mc4r* mRNA, *mc2r* RNA, and *mc1r* mRNA in descending order. However, no detectable level of *mc3r* mRNA was observed in stingray #5. In interrenal tissues, *mc2r* mRNA was most predominant in average, and both *mc3r* and *mc5r* were secondary predominant. The expression of *mc1r* and *mc4r* mRNA were also detected, but these were lower than the other *mcr* mRNAs. The expression of *mc3r* mRNA was also not detected in stingray #5. In

the case of *mc4r* mRNA, the expression was not detected in four individuals #2, 4, 5, and 6.

3.3. Ligand selectivity of stingray MCRs

Previous studies on the ligand selectivity of dogfish MC3R, MC4R and MC5R, and elephant shark MC3R showed that these cartilaginous fish MCRs had a higher sensitivity to ACTH(1-24) than to any of the tested α -MSH analogs (Ringholm et al., 2003; Klovins et al., 2004; Reinick et al. 2012a). In addition, the MC2R ortholog of elephant shark could be activated by either ACTH or α -MSH (Reinick et al., 2012b). Based on these observations, the srMCRs were separately expressed in CHO cells, and stimulated with each of the stingray melanocortin ligands (i.e., ACTH(1-24), Des-acetyl- α -MSH, β -MSH, γ -MSH, and δ -MSH). Des-acetyl- α -MSH was used in this study due to the fact that this ligand is the predominant form of α -MSH in the pituitaries of cartilaginous fishes, whereas, the N-acetylated form (i.e., α -MSH) has not been detected in the pituitaries of cartilaginous fishes (Takahashi et al., 1999, 2008). The efficacy of stingray β -endorphin(1-20) was also tested to confirm strict ligand selectivity of MCRs.

The ligand selectivity profile of srMC1R is presented in Tables 4 and 5, and this is the first cartilaginous fish MC1R ortholog to be analyzed in an activation assay. As shown in Table 4, the EC₅₀ values for the melanocortin ligands were not statistically different. Hence, this stingray receptor can be activated by any stingray melanocortin ligand with equal efficacy. Note that there was no stimulation with stingray β -endorphin(1-20) (Fig. 6 in Takahashi et al., submitted).

srMC2R could be stimulated with stingray ACTH(1-24) and Des-acetyl- α -MSH (Table 4 and Fig. 7A in Takahashi et al., submitted). In

addition, as seen for the elephant shark MC2R ortholog (Reinick et al., 2012b), srMC2R could be stimulated by hACTH(1-24) or NDP-MSH (Table 4 and Fig. 7B in Takahashi et al., submitted). Among the five srMCRs, srMC2R had the lowest sensitive for melanocortin ligands.

In contrast to srMC1R and srMC2R, there was a hierarchy of activation for srMC3R (Tables 4 and 5). The receptor had the highest sensitivity for Des-acetyl- α -MSH and ACTH(1-24), and this ligand was statistically more potent than γ -MSH (Table 4). srMC3R was much less sensitive for β -MSH and δ -MSH, and the receptor could not be activated by β -endorphin(1-20) (Fig. 8 in Takahashi et al., submitted).

The ligand selectivity profile of srMC4R was slightly different from that of srMC3R (Tables 4 and 5). The most potent ligand was Des-acetyl- α -MSH, and this ligand was statistically more potent than β -MSH and δ -MSH (Table 4). Once again, srMC4R could not be stimulated by β -endorphin (Fig. 9 in Takahashi et al., submitted).

The ligand selectivity for srMC5R had features distinct from srMC3R and srMC4R (Tables 4 and 5). First, from a statistical view, the efficacy of Des-acetyl- α -MSH and ACTH(1-24) were equipotent, yet more potent than β -MSH and γ -MSH. In addition, srMC5R could not be stimulated by δ -MSH and β -endorphin (Fig. 10 in Takahashi et al., submitted).

4. Discussion

4.1. Establishment of pituitary-interrenal axis in stingray

To elucidate how the POMC-derived peptides are selected by the receptors in stingray, we isolated all five *mcr* cDNAs as the first step. Determination of MCR sequences, together with previous determination of POMC sequence

containing several melanocortin peptide sequences (Amemiya et al., 2000), have enabled homologous assay for ligand binding to clarify properties of MCRs. The presence of pituitary-interrenal (PI) axis in cartilaginous fish has been shown for a long time only by the presence of POMC containing ACTH in the pituitary (Lowry et al., 1974; Nozaki et al., 1999; Takahashi et al., 1999, 2008), and by activity of ACTH to stimulate the corticosteroid (1α -hydroxycorticosteron) secretion from the interrenal tissue, a homologous tissue to mammalian adrenal cortex (Anderson, 2012; Hazon and Henderson, 1985; Nunez and Trant, 1999). The present study has finally established the (functional) PI axis at the level of MCRs by demonstrating the expression of the responsible genes in stingray interrenal tissues.

4.2. Phylogeny and ligand selectivity of MC receptors

MCRs may have multiplied by 1R genome duplication event, subsequent 2R genome duplication event, and additional local duplication events (Dores and Garcia, 2015; Västermark and Schiöth, 2011). The ancestral MC1R and MC2R might have split from ancestral lamprey MC(a)R. On the other hand, the ancestral MC3R, MC4R, and MC5R might have split from another ancestral lamprey MC(b)R. These scenarios for diversification of MCRs is in good agreement with their pharmacological properties as follows.

Five subtypes of srMCR can be grouped into three categories on the basis of ligand selectivity; the first category contains srMC1R, the second category contains srMC2R, and the third category contains srMC3R, srMC4R, and srMC5R. srMC1R of the first category was equally activated by ACTH(1-24), Des-acetyl- α -MSH, β -MSH, γ -MSH, and δ -MSH. srMC2R of the second category was equally activated by ACTH(1-24) and Des-acetyl- α -MSH, but the potencies

evoked by these peptides were lower than those of srMC1R. srMC3R, srMC4R, and srMC5R of the third category showed the highest sensitivity to Des-acetyl- α -MSH and followed by other melanocortin peptide, while ACTH(1-24) and γ -MSH exhibited similar potencies to that of Des-acetyl- α -MSH depending of subtypes of receptors. These suggest that srMCRs in each category acquired specific ligand selectively after their emergence, and the properties might have been inherited to the present MCRs in stingray.

Additionally, ligand selectivity of srMCRs is highly restricted to melanocortin peptide as shown by almost no activity of β -endorphin. Even though β -endorphin is concomitantly released with melanocortin peptides from pituitary, these POMC-derived peptides exhibit their activities on corresponding receptors—MCRs for melanocortin peptides and opioid receptors for β -endorphin.

4.3. Roles of MC2R and MC5R on interrenal

In many teleosts and tetrapods, srMC2R has shown to be a major MCR transmitting ACTH signal with high ligand-selective functions, as shown by exclusive binding of MC2R with ACTH (Buckley and Ramachandran, 1981; Gants and Fong, 2003). Moreover, trafficking of teleost and tetrapod MC2R to the plasma membrane and the activation of the receptor by ACTH require association with MRAP1 (Agulleiro et al., 2010, 2013; Davis et al., 2013; Liang et al., 2011; Metherell et al., 2005), suggesting that functional activation of ACTH is MRAP1 dependent in teleost and tetrapod. However, MRAP1 dependency and strict ligand selectivity observed in teleost and tetrapod MC2R are not seen in the MC2R of the elephant shark (Reinick et al., 2012b).

With respect to srMC2R, this receptor was activated by ACTH(1-24) and Des-acetyl- α -MSH in CHO cells without co-expression of MRAP1. These

properties are similar to elephant shark MC2R, but different from MC2R orthologs in teleosts and tetrapods. The present results support the hypothesis that the relatively low ligand-selectivity, and independency to MRAP1 appears to be common among cartilaginous fish MC2R. However, it should be noted that for srMC2R the potency of ACTH(1-24) and Des-acetyl- α -MSH are two orders of magnitude lower than the other srMCRs. In addition, although srMC2R could be activated by either hACTH(1-24) or NDP-MSH, the degree of ligand selectivity was three orders of magnitude lower for hACTH(1-24) and one order of magnitude less than for NDP-MSH as compared to the elephant shark MC2R ortholog (Fig. 11 in Takahashi et al., submitted). The differences in ligand sensitivity may be due to amino acid sequence differences between elephant shark MC2R and stingray MC2R in the N-terminal domain, extracellular loop 2, or TM5 of the two orthologs (Fig. 2B). Finally, at present it is not clear if stingray MC2R requires interaction with an MRAP to enhance the ligand sensitivity of the receptor. While we tried to isolate an MRAP1 sequence by PCR using cDNA and genomic DNA sample during the present study, we could not find them. Nevertheless, we cannot exclude the possibility of the presence of MRAP1 in stingray.

It would appear that srMC5R could play important roles in PI axis, because it was activated by ACTH(1-24) and Des-acetyl- α -MSH with similar potencies, and was more sensitive to these peptides than srMC2R. Contribution of other MCRs are thought to be low, because the amounts of *mc1r* and *mc4r* mRNA were lower than those of *mc2r* and *mc5r* mRNA. Moreover, *mc3r* and *mc4r* does not always express detectable levels of mRNA, suggesting minor effects of these MCRs on the regulation of interrenal tissue. Based on these observations, two possibilities emerge to explain the regulation of the PI axis. Assuming that MRAP1 is really absent in the stingray, srMC5R may act as the major MCR

transmitting signals conveyed by either ACTH or Des-acetyl- α -MSH.

Alternatively, if MRAP1 is present in interrenal tissue, this accessory protein may facilitate the activation of srMC2R and/or srMC5R to initiate steroidogenesis in the stingray interrenal tissue.

4.4. Possible dual pituitary regulation on MCR functions

ACTH is the major secretagogue of corticosteroid and MSH peptides exhibit partial roles in teleosts (Takahashi and Kawauchi, 2006). However, Des-acetyl- α -MSH stimulated both srMC2R and srMC5R with similar potency to that of ACTH(1-24) (Fig. 5B). Also in cartilaginous fish, ACTH and MSH peptides are produced in the pars distalis and pars intermedia of the pituitary, respectively, as in other vertebrates (Takahashi et al., 2008). These findings have lead us to suggest that the two pituitary regions are associated with interrenal functions of stingray with dual regulation using ACTH and Des-acetyl- α -MSH (Liang et al., 2013). In teleosts, release of ACTH and MSHs are controlled with different stimuli; for example, ACTH is released in response to stress stimuli, and MSHs are releases in response to background color change (Takahashi and Kawauchi, 2006). Also in cartilaginous fishes, differential factors might be associated with release of the ACTH and MSH peptides from the two pituitary parts, suggesting that the PI axis of cartilaginous fish, at least in stingray, is more complex than those of other vertebrates.

Differential potencies of stingray melanocortin peptides on srMC1R, srMC3R, srMC4R, and srMC5R suggest the roles of the pars distalis and pars intermedia in the regulation of the tissues expressing one or more MCRs involved in group 1 and 3. For srMC1R, the four MSHs and ACTH(1-24) are associated with equipotent activities (Fig. 5A). This suggests that activity of the pars

intermedia with combined effects of four MSH peptides are more potent than pars distalis with a single ACTH to stimulate srMC1R. In contrast, among the MSH peptides in the pars intermedia, Des-acetyl- α -MSH is a major peptide associating with srMC3R, srMC4R, and srMC5R functions, while γ -MSH may contribute to activate MC3R (Fig. 5C). Thus, Des-acetyl- α -MSH is a representative peptide of the pars intermedia on the activation of group 3 MCRs to which the effects of pars intermedia is similar to that of the pars distalis. Based on these interpretation, it is conceivable that in stingray biological processes srMC1R is under relatively potent regulation of the pars intermedia, while srMC3R, srMC4R, and srMC5R receive rather equipotent effects of either the pars distalis and pars intermedia. Thus, stingray melanocortin systems provide insight into the functional differences of melanocortin peptides from the pars distalis and pars intermedia of the pituitary on tissues where multiple MCR subtypes are present.

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Figure legends

Fig. 1. Amino acid sequence of melanocotin peptides and β -endorphin(1-20) used for the experiments of ligand selectivity. Peptides were synthesized at GL Biochem (Shanghai), China.

Fig. 2. Amino acid sequences of stingray MCRs deduced from their nucleotide sequence (A) and sequence comparison between stingray and elephant shark MC2R (B). Spaces were inserted every ten amino acid residues. Asterisks indicate DRY motif, and underlines show transmembrane domains. In (A) dots show identical amino acid residues to MC1R, and dashes gaps. Accession numbers: LC108746 for MC1R, LC108747 for MC2R, LC108748 for MC3R, LC108749 for MC4R, and LC108750 for MC5R. In (B) positions that are shaded in gray are identical in both sequences. Abbreviations: es (elephant shark), sr (stingray); TM (transmembrane domain), IC (intracellular loop), and EC (extracellular loop). Accession number of esMC2R is FAA704.1.

Fig. 3. Phylogenetic tree for cartilaginous fish MCRs (stingray, *Dasyatis akajei*; dogfish, *Squalus acanthias*; and elephant shark, *Callorhynchus milii*) with those of goldfish (*Carasius auratus*) and mouse (*Mus musculus*) constructed by the neighbor-joining method. Bootstrap values (%) are represented at nodes. The scale bar indicates the genetic distance. Bovine rhodopsin (RHO) was used as an outgroup.

Fig.4. Amplification of *mcr* genes and cDNA prepared from hypothalami. (A), sets for primers amplifying reading frames were used. Expected nucleotide lengths amplified from genomic DNA by PCR were 1,037 bp for *mc1r*, 1,163 bp for *mc2r*, 1,124 bp for *mc3r*, 1,097 bp for *mc4r*, and 986 bp for *mc5r*. (B), PCR amplifications

677 were done with (RT+) or without (RT-) reverse transcription. Amplicons originated
678 from hypothalamus RNA were compared with those originated from genomic DNA.
679 *β-actin* was used as a positive control. The sizes of amplicons were different from
680 each other to distinguish mutual contamination of each *mcr* cDNA. Expected
681 nucleotide lengths by RT-PCR were 604 bp for *mc1r*, 620 bp for *mc2r*, 502 bp for
682 *mc3r*, 450 bp for *mc4r*, and 407 bp for *mc5r*.

683

684 Fig. 5. Schematic diagrams for possible dual pituitary regulation on MCR
685 functions. The size difference in arrow shows relative potency of melanocortin
686 peptides on each MCR.

687

Table 1. List of oligonucleotide primers used for cDNA cloning and PCR.

Gene	Usage	Amplicon size (bp)	Sequence (5'→3')
<i>mc1r</i>	Internal forward	509	TAYTTYATHGTGYTGYTTIGC
	Internal reverse		ATDGTCA RIGT DATIGCICCYTT
	Inverse-PCR forward	604	CAGCTGCGATGGCAGCCAAGAAAGAC
	Inverse-PCR reverse		CGATACCATTTGAGGTACAGATCATCAG
	External forward	1,037	CATTGTCTATTTCGGAGAGTAGTGCTG
	External reverse		GATGCTGGTGATCATGGGCGGCTTG
<i>mc2r</i>	Internal forward	410	GACCAGCTACGTTCAAAGTG
	Internal reverse		CAGAAGCTGCAGATTGAGAC
	5'-RACE	604	CAGCTGGTGGCGAGGAATG
	3'-RACE		CTTGGTATGCATTTGAGCAAGC
	External forward	1,163	ATYTTYCAYGCHCTIMGITAYCA
	External reverse		ACCTGRAAIARISWCATR TARCA
<i>mc3r</i>	Internal forward	620	GCATCTGTGCCAACAGGAACATGTAG
	Internal reverse		GAAGCTGATGATGCCTTGGCTGTTGTG
	5'-RACE	555	GGCACAGATGCATGCCAGGAAGATTAG
	3'-RACE		AGCCTTCACAGTAACTGTCCTCCTCG
	External forward	1,124	CACACTCATTGCTGAAGAGTAATTCTCC
	External reverse		CACCTGGCAATCACAGCATGGTAG
<i>mc4r</i>	Internal forward	266	CATTACATCCCACCATCTCC
	Internal reverse		GATGCCTTGGCTGTTGTGAAAG
	5'-RACE	502	GGITTYTGYGARCARGTICCIAT
	3'-RACE		GCICCYTTCATRC AIGTICKYTG
	External forward	1,097	GCATTGGATACACTTACCAGCATATCAG
	External reverse		GGTTACATGGGAGAATGGAGGTTCTTG
<i>mc5r</i>	Internal forward	266	CGTCATGCTTTTCCTCATGACTACTC
	Internal reverse		GTCCACATGTT CATGCTTGCTCGACTAC
	5'-RACE	450	GCCAACCTCAAGCCTCTGCACTATG
	3'-RACE		GACAGGTACGAAATACTGTGAGGAAC
	External forward	1,097	ATTGCCATCGACAGGTACATCAC
	External reverse		GCATCTCTTGACTCCGGAAGG
<i>mc6r</i>	Internal forward	266	AAYMGRAAYCTICAYWSICCIATG
	Internal reverse		TGRTAICKIAGIGYRTARAADAT
	5'-RACE	450	GCTCTTGATCAGCTTGTCTTGAGCCAG
	3'-RACE		GTCAGCTACTGCTAAGCTGCAGATG
	External forward	1,097	GCTCAAGACAAGCTGATCAAGAGCATAG
	External reverse		GACTCGGTGATCTGTAGTTCCCTCCTTG
<i>mc7r</i>	Internal forward	545	GCCAGGCAATCCTCAGTCATAG
	Internal reverse		CTTTCCTGCATCCAGGGAGAATTG
	5'-RACE	407	CGACGGGCTCTGACTGTTATC
	3'-RACE		GAAGGTCTTCCGCATTTCTCTG
	External forward	986	TGYWSHCTGGCIGTIGCIGAYATG
	External reverse		TGCADRAARA AIGGIGCCCARCA
<i>mc8r</i>	Internal forward	545	CTCTTCATGCTCATGATGTGGTGGTAG
	Internal reverse		CGACAGCAATGGCCAAGAGACTGCAC
	5'-RACE	407	CTCATCATGGCATCCCTCTATAGCCAC
	3'-RACE		CCATGCCAAGCGGATTGCTGCTATGTC
	External forward	986	CGGCTTTCTCTACAAGCACCTCAC
	External reverse		GCCAACAACCTTCGCTCACTATAGTC
<i>β-actin</i>	RT-PCR forward	782	GCAGGCATTTGGTTGTCAAAG
	RT-PCR reverse		ACCTTTCATGCTTGCTTGCTG

I: (inosine), M: (A or C), R: (A or G), S: (C or G), W: (A or T), Y: (C or T), and H: (A or C or T).

Table 2. List of oligonucleotide PCR primers and TaqMan probes used for real-time RT-PCR.

Gene	Usage	Amplicon size (bp)	Sequence (5'–3')
<i>mc1r</i>	Forward Reverse TaqMan probe	77	TCCACATGTTTATGCTTGCTC AGCCTGGTGAGTTGGTCTCT TGCATACCAAGAAGATTATGGCCCAA
<i>mc2r</i>	Forward Reverse TaqMan probe	60	CCTGACATGATGATCCCAGGAT CATGGGCAGGATGCCATT TGGCACATTGCTGGA
<i>mc3r</i>	Forward Reverse TaqMan probe	84	GTGCCAATTAAAGCCGAACT CTTAGCAACAGCCAGGATGA TCTGTCTGGGCATCATCAGCTTCC
<i>mc4r</i>	Forward Reverse TaqMan probe	61	GGCAGATACCACCCAGAACAG CAAGATGTTGGCACCAGTGAA CCAGAGCGTGTCGGG
<i>mc5r</i>	Forward Reverse TaqMan probe	62	CCAAGCTTTCCTGATGAGTGT AGAGCTCCTTGTCCTCGTTCA ACTCTGGCTGAGGCAA

All TaqMan probes were labeled with 6-FAM at 5' end, and labeled TAMRA at 3' end.

Table 3. Copy number of *mcr* mRNA detected in hypothalami and adrenal tissues of stingray individually (RNA copies/ μ g).

Tissue	Fish Number	<i>mc1r</i>	<i>mc2r</i>	<i>mc3r</i>	<i>mc4r</i>	<i>mc5r</i>	sex	body weight (kg)
Brain	1	1.96×10^3	8.96×10^4	4.89×10^6	1.69×10^5	1.70×10^6	F	1.6
	2	5.80×10^3	5.03×10^4	1.06×10^7	1.75×10^5	1.96×10^6	M	3.1
	3	1.01×10^4	2.75×10^4	1.59×10^7	2.49×10^5	2.70×10^6	M	2.5
	4	5.63×10^3	5.38×10^4	4.32×10^5	1.80×10^5	3.43×10^6	F	8.6
	5	1.03×10^4	3.48×10^4	ND ^x	3.01×10^5	2.90×10^6	F	8.7
	6	2.00×10^4	4.23×10^4	1.10×10^6	3.14×10^5	2.94×10^6	M	1.9
	(mean $\pm$ SEM)	8.98×10^3 $\pm 2.80 \times 10^3$, a	4.97×10^4 $\pm 9.75 \times 10^3$, b	5.50×10^6 $\pm 2.90 \times 10^6$, a,b,c,d	2.31×10^5 $\pm 2.95 \times 10^4$, c	2.60×10^6 $\pm 2.92 \times 10^5$, d		
Adrenal tissue	1	5.64×10^2	9.66×10^6	9.66×10^6	1.16×10^4	4.80×10^6		
	2	7.33×10^2	7.97×10^6	4.67×10^5	ND ^y	1.14×10^6		
	3	1.68×10^3	3.63×10^6	1.06×10^6	8.21×10^5	1.12×10^6		
	4	5.20×10^2	6.93×10^6	5.29×10^5	ND ^y	1.41×10^6		
	5	2.40×10^3	4.77×10^6	ND ^x	ND ^y	9.13×10^5		
	6	1.53×10^3	6.94×10^6	1.14×10^6	ND ^y	2.28×10^6		
	(mean $\pm$ SEM)	1.24×10^3 $\pm 3.38 \times 10^3$, a	6.65×10^6 $\pm 9.71 \times 10^5$, b	2.14×10^6 $\pm 1.66 \times 10^6$, a,b	1.39×10^5 $\pm 1.49 \times 10^5$, a	1.94×10^6 $\pm 6.62 \times 10^5$, a		

ND (not detected) means that the number of mRNA copies was under minimum detectable level 1×10^3 for *mc3r* (x) and 1×10^2 *mc4r* (y).

In “interrenal tissue” sex and body weight were omitted. The same alphabets after average values indicate no significant difference.

Table 4. EC₅₀ values for each melanocortin ligand on stingray MCRs, and the order of ligand selectivity

MCRs	Ligands	EC ₅₀ (n = 3)
MC1R ¹	Des-Ac-α-MSH	$9.2 \times 10^{-9} \pm 2.5 \times 10^{-9}$ a
	ACTH(1-24)	$1.1 \times 10^{-8} \pm 2.7 \times 10^{-9}$ a
	β-MSH	$5.2 \times 10^{-9} \pm 1.1 \times 10^{-9}$ a
	γ-MSH	$9.2 \times 10^{-9} \pm 2.5 \times 10^{-9}$ a
	δ-MSH	$1.8 \times 10^{-8} \pm 7.7 \times 10^{-9}$ a
	β-endorphin	ND
MC2R ²	Des-Ac-α-MSH	$5.1 \times 10^{-7} \pm 2.8 \times 10^{-7}$ a
	ACTH(1-24)	$2.3 \times 10^{-7} \pm 1.3 \times 10^{-7}$ a
	NDP-MSH	$1.6 \times 10^{-7} \pm 2.5 \times 10^{-8}$ a
	Human ACTH(1-24)	$3.7 \times 10^{-7} \pm 1.2 \times 10^{-7}$ a
MC3R ³	Des-Ac α-MSH	$4.6 \times 10^{-11} \pm 2.5 \times 10^{-11}$ a
	ACTH(1-24)	$2.6 \times 10^{-10} \pm 6.8 \times 10^{-11}$ a
	β-MSH	$1.4 \times 10^{-9} \pm 4.5 \times 10^{-10}$ ab
	γ-MSH	$2.7 \times 10^{-10} \pm 5.7 \times 10^{-11}$ a
	δ-MSH	$8.0 \times 10^{-9} \pm 3.3 \times 10^{-9}$ b
	β-endorphin	ND
MC4R ⁴	Des-Ac α-MSH	$9.3 \times 10^{-10} \pm 3.1 \times 10^{-10}$ a
	ACTH(1-24)	$2.6 \times 10^{-9} \pm 6.1 \times 10^{-10}$ ab
	β-MSH	$2.4 \times 10^{-8} \pm 4.3 \times 10^{-9}$ bc
	γ-MSH	$1.7 \times 10^{-8} \pm 7.4 \times 10^{-9}$ ab
	δ-MSH	$4.3 \times 10^{-8} \pm 8.4 \times 10^{-9}$ c
	β-endorphin	ND
MC5R ⁵	Des-Ac α-MSH	$9.0 \times 10^{-9} \pm 1.1 \times 10^{-9}$ a
	ACTH(1-24)	$9.6 \times 10^{-9} \pm 1.4 \times 10^{-9}$ a
	β-MSH	$1.4 \times 10^{-7} \pm 1.3 \times 10^{-8}$ b
	γ-MSH	$1.3 \times 10^{-7} \pm 1.0 \times 10^{-8}$ b
	δ-MSH	ND
	β-endorphin	ND

The same alphabets after mean ± SEM indicate no significant difference.⁴

¹ to ⁵, see Figure 6 to 10 in Takahashi et al. submitted.

Table 5. The order of ligand selectivity of stingray MCRs

MCRs	The order of ligand selectivity
MC1R	Des-Ac- α -MSH = ACTH(1-24) = β -MSH = γ -MSH = δ -MSH
MC2R	Des-Ac- α -MSH = ACTH(1-24) = NDP-MSH = human ACTH(1-24)
MC3R	Des-Ac- α -MSH = ACTH(1-24) = γ -MSH $\geq$ β -MSH $\geq$ δ -MSH
MC4R	Des-Ac- α -MSH $\geq$ ACTH(1-24) = γ -MSH $\geq$ β -MSH $\geq$ δ -MSH
MC5R	Des-Ac- α -MSH = ACTH(1-24) > β -MSH = γ -MSH >> δ -MSH

ACTH(1-24)	: SYSMEHFRWGPKGRKRRPIKVSP
Des-acetyl- α -MSH	: SYSMEHFRWGPK-NH ₂
β -MSH	: DAKYKFGHFRWSSPL
γ -MSH	: NYVMGHFRWNKF-NH ₂
δ -MSH	: DEKNYKMT H FRW-NH ₂
β -Endorphin(1-20)	: YGGFMKSSDERGQKPLLTLF

Fig. 1

A

MC1R		MMNITTL--	-APRGNEQKD	ISFHWLPRNV	NNSYNASSMQ	36
MC2R	MPDMMIPGYG	TLLDSNGILP	MPPDA.ISPH	SH.TISPWLP	YGTEVVIDTI	QTNMNATEE 60
MC3R			.S.PS--	-ISFHPAMR	NGTED.NESS	ILNNRNGTGF 35
MC4R			.DLSYTRG	P.DTTQNRNQ	SVSGFTGA.I	LH.NGS.GC 38
MC5R			.L-----	----TKLSLM	SVT--.AEAM	V.GTRS.TAL 27
		TM1		TM2		
MC1R	CKHINIPPEV	FLTLGILSFV	ENILVIIAII	KNQNLHSPMY	YLICCLAMAD	TLVMSMNTIE 96
MC2R	.SQ.E.T..	Y.I..LV.LL	.L..V..VL	.KK..F...	FF..S..VS.	I.LCL.KAW. 120
MC3R	.EQVP.KA.L	.FC...I..L	.V...L.VA	.K.....	LFL.S..V..	M...V..AL. 95
MC4R	NEQLW.ST..	.VM...V.LL	A...VA..V	.K.....	FF..S..V..	M.I.V..AW. 98
MC5R	.EQSVAV...	.I...I.LL	A...	.R.....	FFV.S..V..	M...V..AW. 87
		TM3		***		
MC1R	TIVLILMEKE	VLTQVNHILK	QIDNIIDLMI	CTSMVSSLSE	LAAIAADRYI	TIFYALRYHV 156
MC2R	AFTIS.VNNH	EDLFIQTF.L	SL..VF.TL.	.I.FLA.IFN	TT....	S..H.....N 180
MC3R	..MAFLKNG	F.IANDQLIQ	.M..VF.S..	.I.L.A.ICN	.LV..I....	S 155
MC4R	..TIAMLKSR	H.LA.DKLI.	S...VF.SV.	.S.LLA.ICN	.L.V.V....	N 158
MC5R	..IT.LHSR	H.V.KDSFV.	HV..VF.S..	...V.A.MCS	.L...V....	H 147
		TM4		TM5		
MC1R	IMTTRKAVII	IVGIWIVSCT	SSIMFIVYSE	SSAVIICLIS	FFFMMLVIMG	GLYFHMFMFLA 216
MC2R	..GKRVAFA	.A...VFCTA	TG.I.M.NFHN	.QGI.SFY.I	..LLSV.LIV	S..IY..L.. 240
MC3R	..VKR.I.L	.V...FCIF	CG.I..I...	.QT.....	T M..V..FL	T T..V.... 215
MC4R	..V.R.LTV	.A...A.CIG	.G.L..I...	.TTAV....	A M..A..A..A	S..V.... 218
MC5R	..SMKR.AF.	.A...ALCIG	CG.I..I...	.PT.....	VT M..I..L..A	S..S...L.. 207
		TM6				
MC1R	QMHTKKIMAO	RKKRPTHQAA	NMKGAITLTI	LLGLFLICWS	PFFLHLLII	SCPKNPYCLC 276
MC2R	..AR..RIL	PG-HTA..GI	.F...F.V.V	...V.IF..A	.LS..FI.FL	L..SD...A. 299
MC3R	RL.I.R.ATL	PVPGVV..RT	C.....I..	...I.I..A	IM..	I. 275
MC4R	RL.L.R.A.L	PSSGAIC...	V.VV..A	I.M.	...R...I.	278
MC5R	RS.A.R.A.M	SSNSI..Q.	S.....I.IV..A	.L....I.M.	...R.L..T.	267
		TM7				
MC1R	FNSHFNMFLI	LIICNSVFDP	IIYAFRSOEL	RKTLKEFIPC	SW	318
MC2R	.M.L.QID..	F.M.H.II..	L....DP..	SN.F.KMMF.	HKKQWYFHAS	PSFLNI 355
MC3R	YT...TY..	.M...I..	M	..F..ILC.	YCMNFNFRCK	325
MC4R	.M...Y..	.L...II..	L.....M	..F..I.C.	YSLRGSCDLL	SNINTH 334
MC5R	.M...LY..	.M...II..	L..S....M	I.C.	HSLRAVCRLT	VK 319

B

N-terminal extracellular domain						
es	M-----	-----	-----	SGADTSASPW	LANVTTAVMN	TSGFMNGSGG 31
sr	MPDMMIPGYG	TLLDSNGILP	MPPDATISPH	SHPTISPWLP	YGTEVVIDTI	NQTNMNATEE 60
		TM1	IC1	TM2		
es	ICRQLEIPLE	VYLILGGVGM	LENLLVIIAV	VNNRNLHSPM	YLFICSLAMA	DMLVSVGKAS 91
sr	-CSQIEIPTE	VYLILGLVSL	LENLLVVIIV	LKNKKLHFPM	YFFICSLAVS	DILLCLSKAW 119
		EC1	TM3	***	IC2	
es	EAVIIFLDQN	-SHLLTETLI	DHLDYLFDSL	ICISLIASIL	SLGAIATDRY	LTIFHALRYH 150
sr	EAFITISLVNN	HEDLFTQTFI	LSLDNVFDL	ICISFLASIF	NLAAITDRY	ISIFHCLRYH 179
		TM4	EC2	TM5		
es	QIMTVKRAAL	IISALWTFCT	FSGSFIIKEN	RRKNAFPGSL	ITMYFTTLFV	IVSLYVYMFL 210
sr	NIMTGKRVAF	AIAGIWWFCT	ATGILMINF-	HNSQGIISFY	IIFLLSVVL	IVSLYIYMFL 238
		IC3	TM6	EC3		
es	LARRHAQCIR	SLPGQVRHQG	TSLKGAITLT	ILLGIFII-W	APFFLHLILV	LACPSNPYCT 269
sr	LAQMHARKIR	ILPGHTAHQG	INFKGAFVT	VLLGVFIFCW	APLSLHFILF	LLCPSPDYCA 298
		TM7	C-terminal intracellular domain			
es	CYMSLFQVDL	ILIMCNSIID	PLIFAFRSPE	LRNTFKKCI	CFNKQLY	316
sr	CFMSLFQIDL	IFIMCHSIID	PLIYAFRDPE	LSNTFKKMMF	CHKKQWYFHA	SPSFLNI 355

Fig. 2

Fig. 3

A

B

Fig. 4

Fig. 5