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Analyzing the Interactions Between Xenopus tropicalis MC2R, MC5R, and the MRAPs: Modeling the Regulation of Frog Interrenal Cells

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Advisor: Dr. Robert M. Dores
Abstract

The role of Melanocortin 2 Receptor (MC2R) in adrenal/interrenal glucocorticoid secretion has been well documented in many organisms. Studies in mammals have shown that in the adrenal gland two melanocortin receptors and two melanocortin receptor accessory proteins are expressed: MC2R, MC5R, MRAP, and MRAP2. The MRAPs have an opposite effect on the cell surface expression of MC2R and MC5R. In mammals, MRAP aids MC2R but inhibits MC5R cell surface expression. This thesis aims to explore the functional relationship between MC2R, MC5R, MRAP, and MRAP2 in *Xenopus tropicalis* to determine if the MRAPs have a similar effect on amphibian MC2R and MC5R when the receptors are stimulated with ACTH, or α-MSH. Expression of these four genes in the frog interrenal was verified through RT-PCR. Then CHO-K1 cells were transfected with plasmid constructs containing *X. tropicalis* MC2R, MC5R, MRAP2, and a mouse MRAP, and stimulated with ACTH or α-MSH. Levels of receptor activation were measured using a CRE-luciferase reporter gene. The findings presented here indicate that in the frog the MRAPs do not have a negative effect on MC5R, since activation of MC5R was not inhibited in the presence of MRAPs. The sensitivity of MC5R for ACTH increased from an EC$_{50}$ of $1.2 \times 10^{-9}$ M to an EC$_{50}$ of $8.7 \times 10^{-11}$ M in the presence of MRAP ($p<0.05$) and to an EC$_{50}$ of $1.3 \times 10^{-10}$ M in the presence of MRAP2 ($p<0.01$).
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Introduction

The mammalian melanocortin system is composed of five distinct melanocortin receptors: MC1R-MC5R, two accessory proteins: MRAP and MRAP2, the melanocortin ligands: ACTH, α-β- and γ-MSH, and two endogenous antagonists: agouti and AGRP.

Melanocortin Receptors (MCRs)

Melanocortin receptors (MCRs) belong to the Class A family of G-protein coupled receptors (GPCRs), which fall under the same family as the rhodopsin/β2-adrenergic-like receptors (Rodrigues et al., 2013). A common characteristic of all GPCRs is that they consist of seven α-helical transmembrane-spanning domains that are connected by alternating intracellular and extracellular loops (Rosenbaum et al., 2006). MCRs couple to an intracellular heterotrimeric G-protein, with a stimulatory Gα subunit that when activated will stimulate the production of cyclic adenosine monophosphate (cAMP) via adenylyl cyclase (Figure 1). This intracellular buildup of cAMP, will generate downstream signaling through activation of protein kinase A (PKA) (Gantz and Fong, 2003; Rosenbaum et al., 2006). MCRs are considered to be the smallest GPCRs that have been identified since they have short domains in the extracellular N-terminal and intracellular C-terminal, small fourth and fifth transmembrane-spanning domains, and a very small second extracellular loop (Mountjoy et al., 1992). Currently, five melanocortin receptors have been cloned, and they are numbered consecutively in the
Figure 1: GPCR/adenylyl cyclase signaling transduction pathway. Signaling begins when the ligand/hormone binds to the G-protein coupled receptor (GPCR) and this causes a conformational change in the intracellular heterotrimeric G-protein. This results in the activation of the Gα-subunit that will stimulate adenylyl cyclase to produce cAMP. cAMP will build up in the cytoplasm and act as a second messenger to activate protein kinase A (PKA).
order of their discovery: MC1R, MC2R, MC3R, MC4R, and MC5R (Rodrigues et al., 2013). These receptors are involved in a wide range of physiological functions such as skin and hair pigmentation, inflammation, glucocorticoid secretion, energy homeostasis, appetite regulation, sexual function, and sebaceous gland secretion (Yang, 2011).

**Proopiomelanocortin (POMC): The precursor for the MCR ligands.**

Proopiomelanocortin (POMC) is placed under the opioid/orphanin gene family and it is a precursor for β-endorphin, lipotropin, and the melanocortin ligands (Dores et al., 2002). POMC is predominantly expressed in the pituitary and the hypothalamus (Benjannet et al., 1991) as well as keratinocytes, melanocytes and some peripheral tissues such as GI-tract, adrenal, spleen, lung, thyroid and some cells of the immune system (Gantz and Fong, 2003). In the anterior pituitary, POMC is expressed by the corticotropic cells and processed into ACTH and β-lipotropin; whereas in the intermediate pituitary the melanotropic cells will process POMC into α-β- and γ-MSH, β-endorphin, and corticotropic-like intermediate lobe peptide (CLIP) (Dores et al., 2002). POMC is a biologically inactive molecule until it undergoes proteolytic cleavage at specific pairs of amino acids by enzymes known as prohormone convertases PC1/3 and PC2 (Benjannet et al., 1991; Catania et al., 2004). As shown in Figure 2, these enzymes will process POMC to yield the melanocortin ligands, which are the melanocyte stimulating hormones α-MSH, β-MSH, and γ-MSH, and adrenocorticotropic hormone (ACTH). The processing of POMC is tissue specific and different cell types will produce different melanocortin ligands depending on which enzymes, PC1/3, PC2, and other processing enzymes are present (Gantz and Fong 2003). A common feature of the melanocortin ligands is that
Figure 2: Processing of Proopiomelanocortin (POMC). Enzymes involved in the posttranslational processing of POMC in the pituitary to produce the melanocortin ligands ACTH, α-MSH, β-MSH, and γ-MSH (Figure from Patel et al., 2011).
they all share the His-Phe-Arg-Trp (HFRW) motif (Catania et al., 2004), which is required for proper activation of MCRs as well as the Lys-Lys-Arg-Arg-Pro (KKRRP) motif in ACTH (Costa et al., 2004; Liang et al., 2013). MCRs have a varying degree of affinity for the ligands that are derived from the precursor peptide hormone POMC, as well as for two known antagonists: agouti and agouti-related peptide (AGRP) (Table 1) (Gantz and Fong, 2003).

**Activation of Mammalian MC1R, MC3R, MC4R and MC5R by the POMC derived ligands**

Out of all the melanocortin ligands, MC1R seems to have the highest affinity for α-MSH (Gantz and Fong, 2003). MC1R is mainly expressed in melanocytes (Mountjoy et al., 1992) where it mediates skin and hair pigmentation. MC1R has also been found in other cell types such as keratinocytes, fibroblasts, endothelial cells, and other immune cells (Gantz and Fong, 2003). Some studies have also investigated the involvement of MC1R in the anti-inflammatory process (Catania et al., 1996; Lipton and Catania, 1997).

MC3R has been linked to energy homeostasis due to its expression in the regions of the central nervous system (CNS) known to control feeding behavior. It is largely expressed in the cortex, thalamus, hippocampus, hypothalamus, GI-tract, and placenta. It can bind ACTH and the MSH-ligands roughly with equal affinity (Gantz et al., 1993; Gantz and Fong 2003).

Similar to MC3R, MC4R is expressed in the CNS and is also involved in energy homeostasis, in addition to the regulation of sexual function (Gantz and Fong, 2003).
**Table 1:** Function of the melanocortin receptors, ligands, and antagonists. Ligand selectivity of the receptors for the proopiomelanocortin (POMC) derived ligands: melanocyte stimulating hormones (α-β- and γ-MSH), and adrenocorticotropic hormone (ACTH). Agouti has the ability to bind and antagonize all the receptors, with the exception of MC5R. Agouti-related peptide (AGRP) can potentially regulate the activity of MC3R and MC4R. There is no known antagonist for MC5R.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand selectivity</th>
<th>Antagonists</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1R</td>
<td>α-MSH=ACTH &gt; β-MSH &gt; γ-MSH</td>
<td>Agouti</td>
<td>Pigmentation, inflammation</td>
</tr>
<tr>
<td>MC2R</td>
<td>ACTH</td>
<td>Agouti</td>
<td>Steroidogenesis</td>
</tr>
<tr>
<td>MC3R</td>
<td>α-MSH=β-MSH = γ-MSH=ACTH</td>
<td>Agouti, AGRP</td>
<td>Energy homeostasis</td>
</tr>
<tr>
<td>MC4R</td>
<td>α-MSH=ACTH &gt; β-MSH &gt; γ-MSH</td>
<td>Agouti, AGRP</td>
<td>Energy homeostasis, erectile function</td>
</tr>
<tr>
<td>MC5R</td>
<td>α-MSH &gt; ACTH &gt; β-MSH &gt; γ-MSH</td>
<td></td>
<td>Sebaceous gland secretion</td>
</tr>
</tbody>
</table>

*Adapted from Gantz and Fong, 2003.
Deleting this receptor in mice, results in an obese phenotype consistent with hyperphagia, hyperinsulinemia, and hyperglycemia, which suggests that MC4R plays a role in regulating feeding behavior (Huszar et al., 1997).

The last melanocortin receptor to be cloned was MC5R. This receptor has a wide range of tissue distribution with detectable expression levels in skeletal muscle, brain, RT2-2 retinal neuronal cells, lung, spleen, kidney, testis, heart, liver (Fathi et al., 1995) and adrenal gland (Sebag and Hinkle, 2009a). Also, it has been detected in numerous exocrine tissues like Harderian gland, lacrimal, preputial, and sebaceous glands (Chen et al., 1997). There is not much is currently known about the function of this receptor in all of these tissues. However, its role in exocrine gland secretion was determined by targeted deletion of MC5R in mice, and the observation that mutant mice exhibited defects in thermoregulation due to decreased surface lipids, along with decreased secretion of proteins from the lacrimal gland, and a deficiency of porphyrin from the Harderian glands compared to wild type littermates (Chen et al., 1997). In mammals, this receptor seems to have a preference for α-MSH (Gantz and Fong 2003).

**Melanocortin 2 Receptor (MC2R): The ACTH-Receptor**

MC2R was the second melanocortin receptor to be characterized (Mountjoy et al., 1992). MC2R is expressed in murine adipocytes (Catania et al., 2004), but it is primarily involved in stress regulation via the Hypothalamic-Pituitary-Adrenal (HPA) Axis. MC2R is also known as the ACTH-receptor because it exclusively binds ACTH, and unlike the other MCRs, it does not bind any of the MSH-ligands (Gantz and Fong, 2003). MC2R is expressed in the adrenal cortex zona fasciculata and part of the zona glomerulosa, which are sites involved in the synthesis and release of glucocorticoids (Mountjoy et al., 1992;
Xia and Wikberg, 1996). The finding that roughly 25% of individuals with a rare disease, known as Familial Glucocorticoid Deficiency (FGD) Type 1, have mutations in MC2R supports the involvement of this receptor in regulating glucocorticoid secretion (Chung et al., 2008). Some of the phenotypes associated with individuals suffering from FGD are low levels of cortisol (a glucocorticoid), insensitivity to ACTH, hypoglycemia, susceptibility to infections, and intense skin pigmentation. This disease can be fatal in early childhood if left untreated (Metherell et al., 2005; Chung et al., 2008).

Another unique aspect of MC2R is that since its discovery in 1992, many researchers failed to functionally express MC2R in heterologous mammalian cell lines unless the receptor was expressed in an adrenal-derived cell line. Noon et al. (2002) were the first to suggest that MC2R must require an adrenal-specific accessory factor, such as a chaperone, that enabled proper trafficking to the plasma membrane and activation by ACTH. This observation was based on the fact that the same epitope-tagged version of the receptor would function properly if expressed in an adrenal cell line, but not in other cell types. Thus, MC2R must be interacting with another “cofactor” that is specifically expressed in adrenal cells. A few years later that interacting partner was identified and named Melanocortin 2 Receptor Accessory Protein (MRAP).

**Melanocortin 2 Receptor Accessory Protein (MRAP)**

The eventual discovery of MRAP was done by comparing individuals with FGD Type 2 and their unaffected family members through microarray analysis of SNPs, and this revealed a region on a chromosome that codes for a small protein that is now known as Melanocortin 2 Receptor Accessory Protein (MRAP) (Metherell et al., 2005). Individuals are diagnosed with FGD Type 2 because they lack mutations in MC2R, but
regardless of having an intact receptor they still display phenotypes associated with FGD, such as resistance to ACTH and a deficiency in cortisol production. Metherell et al. (2005) also proved that this small protein consists of a single transmembrane-spanning domain. When MRAP is coexpressed with MC2R in a non-adrenal cell line, it allows for the proper trafficking of MC2R to the plasma membrane as well as activation by ACTH. Further studies on the structure of this protein have shown that MRAP forms homodimers (Cooray et al., 2008) and that these two proteins are oriented in an anti-parallel manner with one MRAP being oriented with its N-terminal facing the exterior environment and the other MRAP with its N-terminal facing the cytoplasm. These dual orientations of MRAP homodimers form a stable interaction with MC2R (Sebag and Hinkle, 2007).

A homologue of MRAP was later discovered, called MRAP2, which is 39% identical to MRAP in amino acid sequence at the N-terminal and transmembrane domain (Chan et al., 2009). MRAP2 can interact with MC2R in vitro and enables receptor trafficking to the cell surface, but it is not nearly as efficient as MRAP when the cells are stimulated with ACTH (Chan et al., 2009; Sebag and Hinkle 2010). MRAP2 is similar to MRAP in that it also consists of a single transmembrane-spanning domain, forms homodimers, and can form a heterodimer with MRAP (Chan et al., 2009); but differs in that it lacks an important LDYI motif, which is necessary for MC2R activation by ACTH (Sebag and Hinkle, 2009b). These two accessory proteins interact with all five MCRs and have the potential to regulate their function by increasing/decreasing cell surface expression, in addition to the ligand affinity of the receptor (Chan et al., 2009; Cerdá-Reverter et al., 2013).
Studies on the evolutionary origin of MCRs propose that MCRs are the result of two genome duplication events and one local gene duplication that occurred during the evolution of the chordates. The evolution of MCRs is also closely linked to the co-evolution of POMC, MRAPs, and the AGRP/ASIP gene families (Dores, 2013). The discovery of an MRAP-independent MC2R in the cartilaginous fish *Callorhinchus milii*, which can be functionally activated by either ACTH or any of the MSH-ligands (Reinick et al., 2012), suggests that the ancestral MC2R must have been MRAP-independent. As mutations accumulated in both MC2R and MRAP, the receptor and the accessory protein developed a functional relationship where MRAP is required to facilitate MC2R trafficking to the plasma membrane and activation by ACTH (Dores, 2013). This interaction of MC2R with MRAP, and the ligand selectivity for ACTH has been well established not only for mammals, but also for birds (Barlock et al., 2014), fish (Agulleiro et al., 2010), and amphibians (Liang et al., 2011).

**The Hypothalamic-Pituitary-Adrenal (HPA) Axis**

In mammals, the Hypothalamic-Pituitary-Adrenal (HPA) Axis is a regulated system of signaling events that lead to the synthesis and release of glucocorticoids from the adrenal cortex in a circadian pattern and in response to organismal stress. Signaling begins after the parvocellular neurons in the hypothalamus release corticotropin-releasing hormone (CRH) directly into the median eminence of the anterior pituitary via the Pituitary Portal Vein. CRH will bind to receptors on corticotropic cells where ACTH will be synthesized and released into the vascular system. Then ACTH will bind to and activate MC2R in the glucocorticoid producing cells in the adrenal cortex leading to the synthesis and release of cortisol (Figure 3). Glucocorticoids will induce several
Figure 3: The Hypothalamic-Pituitary-Adrenal (HPA) Axis. The cerebral cortex will signal the hypothalamus to release corticotropin-releasing hormone (CRH) directly to the anterior pituitary, where the precursor hormone proopiomelanocortin (POMC) will be processed into ACTH and released into the bloodstream. ACTH will reach the adrenal gland and will induce the synthesis and release of cortisol. Cortisol will reach its target tissues and will also negatively feedback (-) to stop the release of CRH in the hypothalamus and to stop the synthesis and release of ACTH in the pituitary.

*Illustration adapted with artist permission (Samson, 2012).
physiological changes in the organism such as improvement in motor reflexes, cognitive function, alertness, pain tolerance, and decreased appetite and sexual arousal.

Glucocorticoids will also regulate the HPA-axis, through a negative feedback loop, in order to stop the release of CRH in the hypothalamus and to stop the synthesis and release of ACTH in the pituitary (For review see Papadimitriou and Priftis, 2009).

The HPA-axis is an evolutionary conserved response to stress and a similar system exists in fish and amphibians. The interrenal tissue in the head kidney of fish and the interrenal tissue in amphibians is the equivalent to the adrenals in mammals; therefore, in these species the HPA-axis is referred to as the Hypothalamic-Pituitary-Interrenal (HPI) Axis. The interrenal tissue contains the glucocorticoid producing cells and stress activation is also in response to ACTH via an MRAP-dependent MC2R.

**A closer look at the Adrenal/Interrenal**

MC2R and MC5R are coexpressed in the mammalian adrenal gland (Sebag and Hinkle, 2009a) and in the interrenal tissue of fish (Kobayashi et al., 2011), which is relevant to this study because despite the presence of MC5R, α-MSH does not induce glucocorticoid secretion *in vivo* (Kobayashi et al., 2011; Aluru and Vijayan 2008). In the mammalian adrenal gland, the two accessory proteins MRAP and MRAP2 are also expressed (Sebag and Hinkle, 2009a) and studies dating back to the 1970’s have shown that α-MSH does not stimulate the mammalian adrenal cortex to release glucocorticoids (Schwyzer, 1977).

In trying to characterize the functional interaction of these proteins, Sebag and Hinkle (2009a) found that MC2R requires MRAP for trafficking to the plasma membrane, but when MC5R was coexpressed with either of the accessory proteins,
MRAP or MRAP2, the receptor failed to reach the cell surface and was trapped in the endoplasmic reticulum (ER) (See Figure 4). Based on their observations it seems that in the mammalian system MC2R will preferentially bind MRAP to reach the cell surface and to be activated by ACTH, and the interaction of MC5R with either MRAP or MRAP2 will have a negative effect on the receptor by interfering with the ability of the receptor to dimerize (Figure 4). These observations raise some interesting questions about how the stress response has evolved and how these proteins might interact in other systems such as in fish and amphibians.

**Objectives and Hypotheses**

This study will use *Xenopus tropicalis* as a model system to test the hypothesis that the interaction of MC2R, MC5R, MRAP, and MRAP2 in the interrenal tissue of amphibians is similar to that previously reported for the mammalian adrenal gland. It is hypothesized that all of these proteins will be found in the interrenal tissue of the frog. Therefore, it is expected that MRAP will aid MC2R translocation to the plasma membrane and activation by ACTH, but MRAP or MRAP2 will prevent MC5R from reaching the cell surface and this will inhibit activation by either ACTH or α-MSH. It is also hypothesized that MRAP does exist in the *Xenopus tropicalis* genome. In the genome of *Xenopus tropicalis* all five receptors and MRAP2 have been sequenced, but the sequence for MRAP has not yet been identified. This will be accomplished through RNA extraction, cDNA synthesis, and PCR on the interrenal tissue of *Xenopus tropicalis* using gene specific primers for MC2R, MC5R, and MRAP2, and degenerate primers from conserved regions of MRAP.
This study will also make use of an *in vitro* approach doing transient transfections with CHO cells and using different combinations of cDNA constructs of *Xenopus tropicalis* MC2R, MC5R, MRAP2 and *Mus musculus* MRAP to test the interaction of these proteins. To measure cAMP production upon stimulation with ACTH or α-MSH, the CHO cells will also be transfected with a CRE-Luciferase reporter gene.
Figure 4: Observations from Sebag and Hinkle (2009) on the opposite effect that MRAP/MRAP2 have on MC2R and MC5R. A. MRAP and MRAP2 will promote trafficking of MC2R to the plasma membrane. However, MRAP is much more effective and required for activation of MC2R by ACTH. B. MRAP and MRAP2 will prevent dimerization of MC5R and will prevent the receptor from reaching the cell surface. C. In the adrenal cell all four: MC2R, MC5R, MRAP, and MRAP2 are expressed, but it is still not well understood how these four proteins interact.
Materials and Methods

Total RNA extraction

Mature female *Xenopus tropicalis*, six months or older, were purchased from Nasco (Modesto, CA) for tissue dissection. The frogs were first anesthetized with MS-222 (Sigma-Aldrich) followed by decapitation. Tissues were extracted and frozen immediately on dry ice and stored at -80°C for subsequent use in RNA extraction. Tissue dissections were done by Dr. Robert Dores and approved by the University of Denver Institutional Animal Care and Use Committee (IACUC Approval #472436-1).

A section of interrenal/kidney tissue (between 50-100mg) was used for total RNA extraction using 1mL of TRIzol® Reagent in a glass-Teflon homogenizer. After the tissue was homogenized on ice, it was transferred into a 1.75mL microcentrifuge tube and was centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean microcentrifuge tube and incubated at room temperature for 5 minutes. 200µL of phenol-chloroform-isoamyl alcohol was added and the tube was shaken vigorously by hand. After 3 minutes at room temperature, the sample was centrifuged again at 12,000 x g for 15 minutes at 4°C and the upper aqueous phase, which contains the RNA, was transferred into to a clean microcentrifuge tube. The RNA was precipitated by using 500µL isopropyl alcohol and incubated at room temperature for 10 minutes, followed by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the
RNA pellet was washed with 1mL RNase-free 75% EtOH, vortexed briefly, and then centrifuged at 7,500 x g for 5 minutes at 4°C. The RNA pellet was air dried for 10 minutes, and dissolved in 30μL RNase-free water. Afterwards, DNA-free™ DNase Treatment & Removal (Ambion, Life Technologies) was used to remove any genomic DNA contamination in the sample. The total RNA was treated with 1μL 10x DNase I Buffer and 1μL rDNase I and incubated at 37°C for 30 minutes. Then 2μL of DNase Inactivation was added, incubated at room temperature for 2 minutes and centrifuged at 10,000 x g for 1.5 minutes. The supernatant was transferred to a clean microfuge tube, leaving the pellet with any genomic DNA contamination behind. RNA concentration was measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific), and the tube was stored at -80°C.

The same protocol was repeated using 50-100mg of *Xenopus tropicalis* heart, brain, liver, muscle, ovaries, and intestinal tissue for expression patterns of MC2R, MC5R, MRAP, and MRAP2 through cDNA synthesis and PCR.

**cDNA Synthesis**

cDNA synthesis was performed using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) following manufacturer’s instructions. The reaction was carried out using Oligo(dT)20 primer and 0.5μg of total RNA was used per reaction. For the negative control, reverse transcriptase was omitted from the reaction components.

**PCR**

Gene specific primers were designed for *X. tropicalis* MC2R, MC5R, and MRAP2 from sequences obtained from [www.xenbase.org](http://www.xenbase.org) and [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).
Degenerate primers were designed for MRAP based on regions of sequence conservation for the MRAP sequences of human, mouse, chicken, rat, rhesus macaque, rainbow trout, and Chinese soft-shelled turtle. The primers were ordered from Integrated DNA Technologies (www.idtdna.org). Primer sequences, annealing temperature (Ta), and expected size PCR products are listed in Table 2. A standard 20μL PCR reaction was set up using 2μL 10X PCR buffer, 0.6μL 50mM MgCl$_2$, 2μL 10mM dNTPs, 2μL 10μM Forward Primer, 2μL 10μM Reverse Primer, 0.2μL Taq Polymerase 5U/μL (Invitrogen), 9.2μL sterile water, and 2-3μL interrenal/kidney cDNA or negative control. A positive control was also set up with plasmid cDNA constructs containing the full-length xtMC2R, xtMC5R, and xtMRAP2. Five 20μl PCR reactions were done for each gene. The cycling parameters for the thermal cycler were: initial denaturation at 94°C for 3 minutes, a second denaturation at 94°C for 45 seconds, primer annealing for 30 seconds, primer extension at 72°C for 1 minute and 30 seconds, cycle 32 times to second denaturation step, a final extension at 72°C for 10 minutes, and reaction was held at 4°C. The PCR reactions for each respective gene were combined and purified using QIAquick PCR purification kit (Qiagen). 5μL 6X loading dye was added to each PCR reaction and the PCR products were ran at 60 Volts for 1 hour on a 2% TAE gel stained with Ethidium Bromide for visualization using Quantity One Gel Doc Imager (BioRad). exACTGene Low Range Plus DNA Ladder (Fisher Scientific) was used to verify the size of products. The bands were excised from the gel based on the expected product length and purified using Wizard SV Gel and PCR Clean-Up System (Promega) and stored at -20°C. If PCR
Table 2: Sequences of *X. tropicalis* gene specific PCR primers for MC2R, MC5R, MRAP2, and degenerate PCR primers for MRAP. Annealing temperature (Ta) for each primer pair and expected size of PCR product in base pairs (bp).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Ta</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>xtMC2R</td>
<td>Forward: 5’ GGTGCTCCTAGCTGTGATAAA 3’ Reverse: 5’ GGAGACCAACAGCAATGAATA 3’</td>
<td>59.8°C</td>
<td>594 bp</td>
</tr>
<tr>
<td>xtMC5R</td>
<td>Forward: 5’ AATACAGTGCCAACTGGAAG 3’ Reverse: 5’ GTCATGACTTTGCTCTGGTG 3’</td>
<td>61.6°C</td>
<td>645 bp</td>
</tr>
<tr>
<td>xtMRAP</td>
<td>Degenerate Forward: 5’ ATGGCCAACRGRACMRACKCMCTCTG 3’ Degenerate Reverse: 5’ CRGGACATGAGCARRATARRAA 3’</td>
<td>61.6°C</td>
<td>185 bp</td>
</tr>
<tr>
<td>xtMRAP2</td>
<td>Forward: 5’ AAGCAGTTGTCAAATTCTGATTAC 3’ Reverse: 5’ GAGAGACGTGATTCTTCTGT 3’</td>
<td>57°C</td>
<td>312 bp</td>
</tr>
</tbody>
</table>
yielded a faint band, the excised band that was purified was submitted through another
round of PCR to increase product yield.

**Ligation and Bacterial Transformation**

The purified gel products: MC2R, MC5R, MRAP, and MRAP2 were ligated into
pGEM-T Easy Vector System I (Promega) using 3μL of PCR product and left overnight
at 4°C. Mix & Go Competent DH5α E. coli cells (Zymo Research) were used for
transforming 3-5μL of each of the ligations according to manufacturer’s instructions. The
cells were plated on LB-Agar+Carbenicillin/X-gal/IPTG and incubated overnight at
37°C. Colonies were selected based on blue/white screening and insert was verified using
PCR with SP6 and T7 primers. White colonies were picked with a sterile toothpick and
resuspended in microfuge tubes with 20μL sterile water. They were heated for 2 minutes
at 96°C and 2μL was added to a tube containing PCR mix (as previously described, but
with an extra 5μL sterile water). The same PCR cycling parameters mentioned above
were used, but with a primer annealing temperature of 55°C. A 20μL aliquot was taken
from each PCR reaction and stored at 4°C and the remaining 5μL were run on a 2% TAE
gel to verify the correct size of insert in the plasmid. The 20μL PCR reactions for the
colonies that were positive for the insert were treated with 0.5μL Exonuclease I (USB)
and 0.5μL Shrimp Alkaline Phosphatase (USB) and incubated at 37°C for 45 minutes and
at 80°C for 45 minutes to degrade any remaining primers and dNTPs. These PCR
samples were sent for sequencing with SP6 or T7 primer at the University of Colorado
DNA Sequencing & Analysis Service.
Cell Line

Adherent Chinese Hamster Ovary cells (CHO-K1) from ATCC, were grown in a 25cm² cell culture flask with a vented cap with CHO Media prepared by using Kaighn’s Modification of Ham’s F-12 Medium (F-12K) supplemented with 10% Fetal Bovine Serum, 1% penicillin/streptomycin, and 1 mg/ml Normocin™(InvivoGen). The cells were maintained in a 37°C incubator with a relative humidity of 80-90%, and 5% CO₂. CHO cells were sub-cultured when they reached about 90% confluence. The media was removed and the cells were rinsed with 3 mL 1X phosphate buffered saline (PBS). 2mL of Trypsin/0.53mM EDTA was added and incubated at 37°C for 3 minutes to aid cell detachment. Then 3 mL fresh CHO media was added to inactivate the trypsin. The cells were either counted with a hemocytometer for use in transfection experiments, or a small aliquot was subcultured in a new flask with fresh media being renewed between subcultures.

Plasmid Constructs

cDNA plasmid constructs were synthesized by GenScript (Piscataway, NJ) in a pcDNA3.1(+) vector: *Xenopus tropicalis* MC2R with a FLAG epitope tag at the N-terminal, *Xenopus tropicalis* MC5R, *Xenopus tropicalis* MRAP2 with a FLAG epitope tag at the N-terminal, *Mus musculus* MRAP, *Xenopus tropicalis* MC5R mutant with alanine substitutions at positions 92, 114, and 119, *Xenopus tropicalis* MC2R mutant with an alanine substitution at isoleucine 175 (xtMC2R I-175), and a *Gallus gallus* MRAP with a FLAG epitope tag at the N-terminal.
cAMP Assay using a CRE-Luciferase Reporter Gene

CHO cells were transiently transfected using the Amaxa Nucleofector™ II Device and the Cell Line Nucleofector® Kit T (Lonza). For each transfection 2.5x10⁶ cells were used with 100µL of Solution T and 2µg of different combinations of cDNA plasmid constructs. All transfections contained 2µg of the CRE-Luciferase Reporter cDNA plasmid construct, kindly donated by Dr. Patricia M. Hinkle. The cells were transfected using Program U-023. After transfection, the cells were allowed to recover for 10 minutes in 500µL of CHO media in the 37°C incubator. Then the cells were diluted in 7.5mL of CHO media and plated in a 96-well plate at a density of approximately 1x10⁵ cells per well. Each transfection was plated, three columns per transfection, so each plate could hold a total of four transfections. The plate was placed in the 37°C incubator with fresh media being replaced 24 hours after transfection. 48 hours post-transfection the cells were stimulated with varying peptide concentrations of frog ACTH 1-24 or frog α-MSH prepared in Serum-Free CHO Medium (F-12K Medium, 1 mg/ml BSA, and 1% penicillin/streptomycin). The stimulating solutions ranged in concentration from the control, which was Serum-Free CHO Medium only, and 10⁻¹² M to 10⁻⁶ M. Each concentration was done in triplicates. After a period of four hours in the 37°C incubator, the plate was allowed to sit at room temperature for 10 minutes. The stimulating solutions were removed and 100µL of a 1:1 ratio of Serum-Free CHO Medium and luciferase substrate (Bright-Glo Luciferase Assay System, Promega) was added to each well. After five minutes at room temperature, luminescence was read using the Bio-Tek Synergy HT Plate Reader. For each transfection the average control value was subtracted from each
experimental value, and the mean for each data point was graphed using KaleidaGraph Software. A Michaelis-Menten curve was fit through the data points to obtain an EC\textsubscript{50} value as well as standard error. Comparison of the EC\textsubscript{50} values were done using a Student’s \textit{t}-test with equal variance and significance set at p<0.05.
Results

Cloning MC2R, MC5R, MRAP, and MRAP2

PCR on interrenal/kidney tissue

PCR was performed using cDNA synthesized from *X. tropicalis* interrenal/kidney tissue in order to verify the presence of MC2R, MC5R, MRAP, and MRAP2 (Figure 5). The interrenal tissue was difficult to extract without taking a small portion of the kidney, so it is labeled as interrenal/kidney tissue. A band consistent with the expected product size was observed for each gene: MC2R (594bp), MC5R (645bp), MRAP (185bp), and MRAP2 (312bp). No visible band was detected in any of the negative controls (Lane 1: 5A, 5B, 5C, 5D), which had all the RT-PCR components with the exception of reverse transcriptase. The band for each gene that was amplified from the cDNA was excised from the gel, ligated into a vector, and sequenced to confirm that the primers had amplified the gene of interest. Figure 6 shows the full-length nucleotide and amino acid sequence of MC2R with the primer regions in bold and the underlined 594 base pair region that was verified through sequencing. The same was done for MC5R (Figure 7) and underlined is the 645 base pair region that was sequenced, as well as the 312 base pair region that was sequenced for MRAP2 (Figure 8). For MRAP, a 185 base pair region was amplified with the degenerate primers and when sequenced matched the chicken MRAP. An alignment of the nucleotide and amino acid sequence was done to show sequence homology between the chicken MRAP and the sequence that was obtained from
Figure 5: Gel electrophoresis of PCR fragments of MC2R, MC5R, MRAP, and MRAP2 (A-D) using cDNA synthesized from X. tropicalis interrenal/kidney tissue. Ladder (L) sizes in base pairs (bp) listed on the right panel. A. MC2R (594 bp): Lane 1-Negative control (no reverse transcriptase (RT)), Lane 2-Positive control-plasmid containing full-length xtMC2R insert, Lane 3- cDNA. B. MC5R (645 bp): Lane 1-Negative control (no RT), Lane 2-Positive control-plasmid containing full-length xtMC5R insert, Lane 3- cDNA. C. MRAP (185 bp): Lane 1-Negative control (no RT), Lane 2- cDNA. D. MRAP2 (312 bp): Lane 1-Negative control (no RT), Lane 2-Positive control-plasmid containing full-length xtMRAP2 insert, Lane 3- cDNA.
**Figure 6:** *X. tropicalis* MC2R nucleotide and amino acid sequence. Underlined sequence shows the 594 base pair region sequenced from the interrenal/kidney tissue. Sequences in bold indicate the forward and reverse primer target sequences.
**Figure 7:** *X. tropicalis* MC5R nucleotide and amino acid sequence. Underlined sequence shows the 645 base pair region sequenced from the interrenal/kidney tissue. Sequences in bold indicate the forward and reverse primer target sequences.
Figure 8: X. tropicalis MRAP2 nucleotide and amino acid sequence. Underlined sequence shows the 312 base pair region sequenced from the interrenal/kidney tissue. Sequences in bold indicate the forward and reverse primer target sequences.
the MRAP degenerate PCR primers (Figure 9). The regions targeted by the primers are in bold and sequence identity is highlighted in gray. The region that was sequenced from the frog interrenal/kidney tissue contains the region of MRAP that is responsible for trafficking to the plasma membrane and the transmembrane spanning domain (Liang et al., 2011).

Expression of MC2R, MC5R, MRAP, MRAP2 in other tissues

The same PCR conditions that were used for the interrenal/kidney tissue were used to look at the expression patterns of MC2R, MC5R, MRAP, and MRAP2 in the frog heart, brain, liver, muscle, ovaries, and intestine. The interrenal/kidney was used as a positive control in order to verify PCR efficiency and to compare the size of the expected products in the other tissues that were analyzed. For MC2R the interrenal/kidney (positive control) yielded the expected band of 594 base pairs (Figure 10: Lane 2). Faint bands were also detected in the heart (Figure 10: Lane 4) and ovaries (Figure 10: Lane 12). In addition, an extremely faint band that was visible in muscle (Figure 10: Lane 10). No visible bands were detected in any of the negative controls (Figure 10: Lanes 1, 3, 5, 7, 9, 11, 13). MC5R in interrenal/kidney (positive control) yielded the expected band of 645 base pairs (Figure 11: Lane 2) as well as a band of a similar size in the brain, muscle, ovaries, and intestine (Figure 11: Lanes 6, 10, 12, 14 respectively). No visible bands were detected in any of the negative controls (Figure 10: Lanes 1, 3, 5, 7, 9, 11, 13). For MRAP, the degenerate primers yielded the expected band at about 185 base pairs for interrenal/kidney (Figure 12: Lane 1), but no band of a similar size was detected in any of the other tissues, or the negative controls. The expected product size of MRAP2 was 312
Figure 9: MRAP nucleotide and amino acid sequence alignments of chicken (Gg) MRAP and *X.tropicalis* cDNA sequence. xt cDNA sequence was obtained from sequencing PCR products using degenerate primers for MRAP on cDNA synthesized from frog interrenal/kidney tissue. Sequences in bold show the degenerate forward primer and the degenerate reverse primer target sequences and identical regions are shaded gray. The expected product size was 185 base pairs. The amino acid alignment shows the region responsible for trafficking to the plasma membrane (PM) and the transmembrane spanning domain (TM).
Figure 10: PCR analysis of tissue distribution of *X. tropicalis* MC2R using cDNA synthesized from interrenal/kidney, heart, brain, liver, muscle, ovaries, and intestine. Ladder (L) sizes in base pairs listed on the right panel. Expected product size is 594 base pairs. Lane 1-Negative control of interrenal/kidney (no reverse transcriptase (RT)), Lane 2-interrenal/kidney cDNA (positive control), Lane 3-Negative control of heart (no RT), Lane 4-heart cDNA, Lane 5-Negative control of brain (no RT), Lane 6-brain cDNA, Lane 7-Negative control of liver (no RT), Lane 8-liver cDNA, Lane 9-Negative control of muscle (no RT), Lane 10-muscle cDNA, Lane 11-Negative control of ovaries (no RT), Lane 12-ovaries cDNA, Lane 13-Negative control of intestine (no RT), Lane 14-intestine cDNA.
**Figure 11:** PCR analysis of tissue distribution of *X. tropicalis* MC5R using cDNA synthesized from interrenal/kidney, heart, brain, liver, muscle, ovaries, and intestine. Ladder (L) sizes in base pairs listed on the right panel. Expected product size is 645 base pairs. *Lane 1*-Negative control of interrenal/kidney (no reverse transcriptase (RT)), *Lane 2*-interrenal/kidney cDNA (positive control), *Lane 3*-Negative control of heart (no RT), *Lane 4*-heart cDNA, *Lane 5*-Negative control of brain (no RT), *Lane 6*-brain cDNA, *Lane 7*-Negative control of liver (no RT), *Lane 8*-liver cDNA, *Lane 9*-Negative control of muscle (no RT), *Lane 10*-muscle cDNA, *Lane 11*-Negative control of ovaries (no RT), *Lane 12*-ovaries cDNA, *Lane 13*-Negative control of intestine (no RT), *Lane 14*-intestine cDNA.
Figure 12: PCR analysis of tissue distribution of *X. tropicalis* MRAP using cDNA synthesized from interrenal/kidney, heart, brain, liver, muscle, ovaries, and intestine. Ladder (L) sizes in base pairs listed on the right panel. Expected product size is 185 base pairs. *Lane 1*-interrenal/kidney cDNA (positive control, arrow points to expected band), *Lane 2*-Negative control of interrenal/kidney (no reverse transcriptase (RT)), *Lane 3*-Negative control of heart (no RT), *Lane 4*-heart cDNA, *Lane 5*-Negative control of brain (no RT), *Lane 6*-brain cDNA, *Lane 7*-Negative control of liver (no RT), *Lane 8*-liver cDNA, *Lane 9*-Negative control of muscle (no RT), *Lane 10*-muscle cDNA, *Lane 11*-Negative control of ovaries (no RT), *Lane 12*-ovaries cDNA, *Lane 13*-Negative control of intestine (no RT), *Lane 14*-intestine cDNA.
base pairs and was detected as expected from the interrenal/kidney tissue (Figure 13: Lane 2), in addition to the brain and muscle (Figure 13: Lane 6 and 9 respectively). No visible bands were detected in any of the negative controls (Figure 13: Lanes 1, 3, 5, 7, 10, 11, 13).

Since MC2R, MC5R, MRAP, and MRAP2 were found in the interrenal/kidney tissue of *X. tropicalis*, the next logical step was to analyze the pharmacological interaction of all of these proteins by expressing them in CHO cells. CHO cells are a good model system to study melanocortin receptors, because they do not endogenously express melanocortin receptors or the accessory proteins: MRAP and MRAP2 (Chung et al., 2008).

**Functional interaction of MC2R, MC5R, MRAP, and MRAP2**

CHO cells were transfected with different combinations of the receptors MC2R, MC5R, and the accessory proteins MRAP and MRAP2. To measure the amount of cAMP that was produced by MC2R and MC5R upon stimulation with either ACTH or α-MSH all transfections were done using a CRE-luciferase reporter gene. After being stimulated by the respective ligand, the amount of cAMP generated by the receptors was measured indirectly based on the amount of luminescence produced after the luciferase substrate was added.

**Ligand selectivity of MC2R and MC5R**

To characterize the ligand selectivity of frog MC2R, CHO cells were transiently transfected with xtMC2R in the presence and absence of (mouse) mMRAP, and
Figure 13: PCR analysis of tissue distribution of *X. tropicalis* MRAP2 using cDNA synthesized from interrenal/kidney, heart, brain, liver, muscle, ovaries, and intestine. Ladder (L) sizes in base pairs listed on the right panel. Expected product size is 312 base pairs. *Lane 1*-Negative control of interrenal/kidney (no reverse transcriptase (RT)), *Lane 2*-interrenal/kidney cDNA (positive control), *Lane 3*-Negative control of heart (no RT), *Lane 4*-heart cDNA, *Lane 5*-Negative control of brain (no RT), *Lane 6*-brain cDNA, *Lane 7*-Negative control of liver (no RT), *Lane 8*-liver cDNA, *Lane 9*-muscle cDNA, *Lane 10*-Negative control of muscle (no RT), *Lane 11*-Negative control of ovaries (no RT), *Lane 12*-ovaries cDNA, *Lane 13*-Negative control of intestine (no RT), *Lane 14*-intestine cDNA.
stimulated with ACTH or α-MSH. Activation of frog MC2R was only observed when the receptor was cotransfected with MRAP and stimulated by ACTH, but not in the absence of MRAP or if stimulated by α-MSH (Figure 14). Next, the ligand selectivity of frog MC5R was analyzed by transfecting CHO cells with xtMC5R and stimulating them with ACTH and α-MSH (Figure 15). Although it seemed that xtMC5R has a slight preference for ACTH (EC\(_{50}\) = 1.6 x 10\(^{-9}\) M) over α-MSH (EC\(_{50}\) = 8.3 x 10\(^{-9}\) M), the difference was only about 5-fold (p<0.05).

**Effect of MRAP on MC2R and MC5R**

As previously shown, MRAP is required by xtMC2R for activation by ACTH and in this assay was used as a control to compare the sensitivity of MC2R vs. MC5R (Figure 16). xtMC2R coexpressed with mMRAP (EC\(_{50}\) = 1.1 x 10\(^{-10}\) M) was about 10-fold more sensitive than xtMC5R (EC\(_{50}\) = 1.2 x 10\(^{-9}\) M) when stimulated with ACTH (Table 3, p<0.05). To test the hypothesis that MRAP will have a negative effect on MC5R trafficking to the plasma membrane and therefore activation of the receptor, xtMC5R was coexpressed with mMRAP and stimulated with ACTH (Figure 16). The coexpression of the frog MC5R with mMRAP made the receptor more sensitive to ACTH (EC\(_{50}\) = 8.7 x 10\(^{-11}\) M) and resulted in a 13-fold shift in the EC\(_{50}\) value of the receptor (Table 3, p<0.05). There was no significant difference in EC\(_{50}\) values for xtMC2R and xtMC5R when both receptors were coexpressed with mMRAP and stimulated with ACTH. Similarly, to assess whether activation of xtMC5R with α-MSH was affected by the presence of mMRAP, xtMC5R was coexpressed with mMRAP and stimulated with α-
Figure 14: Ligand selectivity of xtMC2R. Transient transfections of CHO cells with xtMC2R with and without mMRAP and stimulated with frog ACTH 1-24 and frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ value of xtMC2R/mMRAP stimulated with ACTH (squares) is $1.6 \times 10^{-10}$ M +/- $3.1 \times 10^{-11}$. Also shown are xtMC2R without MRAP stimulated with ACTH (circles), and xtMC2R with mMRAP stimulated with α-MSH (diamonds).
Figure 15: Ligand selectivity of xtMC5R. Transient transfections of CHO cells with xtMC5R and stimulated with frog ACTH 1-24 and frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R stimulated with ACTH (circles) $1.6 \times 10^{-9}$ M +/- $5.9 \times 10^{-10}$, and xtMC5R stimulated with α-MSH (squares) $8.3 \times 10^{-9}$ M +/- $2.9 \times 10^{-9}$, p=0.04.
Figure 16: Effect of mMRAP on xtMC2R and xtMC5R when stimulated with ACTH. Transient transfections of CHO cells with xtMC2R, xtMC5R, mMRAP and stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R/mMRAP (circles) $1.1 \times 10^{-10}$ M +/- $3.4 \times 10^{-11}$, xtMC5R (squares) $1.2 \times 10^{-9}$ M +/- $3.3 \times 10^{-10}$, and xtMC5R/mMRAP (diamonds) $8.7 \times 10^{-11}$ M +/- $2.5 \times 10^{-11}$. Statistical analysis shown in Table 3.
Table 3: Statistical analysis for Figure 16. Student’s t-test for the mean EC$_{50}$ values +/- S.E.M for the transfections shown in Figure 16. P-values listed and (*) indicates p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>xtMC2R/mMRAP 1.1 x 10$^{-10}$ M +/- 3.4 x 10$^{-11}$</th>
<th>xtMC5R 1.2 x 10$^{-9}$ M +/- 3.3 x 10$^{-10}$</th>
<th>P-value</th>
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<tr>
<td>xtMC2R/mMRAP</td>
<td>xtMC5R/mMRAP 1.1 x 10$^{-10}$ M +/- 3.4 x 10$^{-11}$</td>
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<td>xtMC5R/mMRAP 8.7 x 10$^{-11}$ M +/- 2.5 x 10$^{-11}$</td>
<td>0.02*</td>
</tr>
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</table>
MSH (Figure 17). There was no significant difference in the EC$_{50}$ values when xtMC5R (EC$_{50}$ = 1.0 x 10$^{-8}$ M) was expressed alone, or when coexpressed with mMRAP (EC$_{50}$ = 4.9 x 10$^{-9}$ M). Figure 18 summarizes the previous observations on the effect of mMRAP on the ligand selectivity of xtMC5R.

**Effect of MRAP2 on MC2R and MC5R**

The next step was to analyze the effect of xtMRAP2 on both xtMC2R and xtMC5R. Coexpression of xtMRAP2 with xtMC2R led to a 919-fold shift in the EC$_{50}$ value making the receptor significantly less sensitive to ACTH (Figure 19: EC$_{50}$ = 1.0 x 10$^{-7}$ M) when compared to xtMC2R coexpressed with mMRAP (Figure 16: EC$_{50}$ = 1.1 x 10$^{-10}$ M; p<0.05). In contrast, xtMRAP2 increased the sensitivity of xtMC5R to ACTH by about 10-fold. The EC$_{50}$ value for xtMC5R shifted from 1.2 x 10$^{-9}$ M when expressed alone to 1.3 x 10$^{-10}$ M when coexpressed with xtMRAP2 (Figure 19; p<0.01). The frog MC5R expressed alone or coexpressed with xtMRAP2 was significantly more sensitive to ACTH, when either transfection is compared to the sensitivity of xtMC2R coexpressed with xtMRAP2 (Table 4, p<0.05). xtMC5R coexpressed with xtMRAP2 was about 803-fold more sensitive to ACTH compared to xtMC2R with xtMRAP2. To test whether xtMRAP2 affected the affinity of xtMC5R to α-MSH, the receptor was also coexpressed with xtMRAP2 and stimulated with α-MSH (Figure 20). No significant difference was observed in the sensitivity of xtMC5R to α-MSH when expressed alone (EC$_{50}$ = 1.0 x 10$^{-8}$ M) or coexpressed with xtMRAP2 (EC$_{50}$ = 2.2 x 10$^{-8}$ M). The ligand selectivity of frog MC5R in the presence and absence of xtMRAP2 are summarized in Figure 21.
Figure 17: Effect of mMRAP on xtMC5R when stimulated with α-MSH. Transient transfections of CHO cells with xtMC5R, mMRAP and stimulated with frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $1.0 \times 10^{-8}$ M +/- $5.4 \times 10^{-9}$, and xtMC5R/mMRAP (squares) $4.9 \times 10^{-9}$ M +/- $1.9 \times 10^{-9}$, p=0.21.
Figure 18: Ligand selectivity of xtMC5R when coexpressed with and without mMRAP. Transient transfections of CHO cells with xtMC5R with and without mMRAP and stimulated with frog ACTH 1-24 and frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R stimulated with ACTH (circles) is $1.2 \times 10^{-9}$ M +/- $3.3 \times 10^{-10}$, xtMC5R/mMRAP stimulated with ACTH (squares) $8.7 \times 10^{-11}$ M +/- $2.5 \times 10^{-11}$, xtMC5R stimulated with α-MSH (diamonds) is $1.0 \times 10^{-8}$ M +/- $5.4 \times 10^{-9}$, xtMC5R/mMRAP stimulated with α-MSH (half-filled squares) $4.9 \times 10^{-9}$ M +/- $1.9 \times 10^{-9}$. 
Figure 19: Effect of xtMRAP2 on xtMC2R and xtMC5R when stimulated with ACTH. Transient transfections of CHO cells with xtMC2R, xtMC5R, xtMRAP2 and stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R/xtMRAP2 (circles) $1.0 \times 10^{-7}$ M +/- $3.2 \times 10^{-8}$, xtMC5R (squares) $1.2 \times 10^{-9}$ M +/- $2.2 \times 10^{-10}$, and xtMC5R/xtMRAP2 (diamonds) $1.3 \times 10^{-10}$ M +/- $5.0 \times 10^{-11}$. Statistical analysis shown in Table 4.
Table 4: Statistical analysis for Figure 19. Student’s $t$-test for the mean EC$_{50}$ values +/- S.E.M for the transfections shown in Figure 19. P-values listed and (*) indicates p<0.05, (**) indicates p<0.01.

<table>
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<td>$1.2 \times 10^{-9}$ M +/- $2.2 \times 10^{-10}$</td>
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</tr>
<tr>
<td>xtMC2R/xtMRAP2</td>
<td>xtMC5R/xtMRAP2</td>
<td>0.02*</td>
</tr>
<tr>
<td>$1.0 \times 10^{-7}$ M +/- $3.2 \times 10^{-8}$</td>
<td>$1.3 \times 10^{-10}$ M +/- $5.0 \times 10^{-11}$</td>
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<tr>
<td>xtMC5R</td>
<td>xtMC5R/xtMRAP2</td>
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<tr>
<td>$1.2 \times 10^{-9}$ M +/- $2.2 \times 10^{-10}$</td>
<td>$1.3 \times 10^{-10}$ M +/- $5.0 \times 10^{-11}$</td>
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Figure 20: Effect of xtMRAP2 on xtMC5R when stimulated with α-MSH. Transient transfections of CHO cells with xtMC5R, xtMRAP2 and stimulated with frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $1.0 \times 10^{-8}$ M +/- $5.4 \times 10^{-9}$, and xtMC5R/xtMRAP2 (squares) $2.2 \times 10^{-8}$ M +/- $8.3 \times 10^{-9}$, p=0.15.
Figure 21: Ligand selectivity of xtMC5R when coexpressed with and without xtMRAP2. Transient transfections of CHO cells with xtMC5R with and without xtMRAP2 and stimulated with frog ACTH 1-24 and frog α-MSH from 10^{-12} to 10^{-6} M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC_{50} values are the following: xtMC5R stimulated with ACTH (circles) is 1.2 x 10^{-9} M +/- 3.3 x 10^{-10}, xtMC5R/xtMRAP2 stimulated with ACTH (squares) 1.3 x 10^{-10} M +/- 7.7 x 10^{-11}, xtMC5R stimulated with α-MSH (diamonds) is 1.0 x 10^{-8} M +/- 5.4 x 10^{-9}, xtMC5R/xtMRAP2 stimulated with α-MSH (half-filled squares) 2.2 x 10^{-8} M +/- 8.3 x 10^{-9}.
Since the cloning experiments revealed a small region of MRAP that was highly similar to the chicken MRAP, it made sense to test the effect of chicken MRAP on the ligand selectivity of MC5R. Consistent with experiments using the mouse MRAP, the chicken MRAP also increased the sensitivity of xtMC5R to ACTH and caused a 383-fold shift in the $EC_{50}$ value from $1.5 \times 10^{-9} \text{ M}$ to $3.8 \times 10^{-12} \text{ M}$ (Figure 22; $p<0.05$). The effect that the chicken MRAP had on xtMC5R was comparable to the effect that xtMRAP2 had on xtMC5R when stimulated with ACTH. On the other hand, neither chicken MRAP or xtMRAP2 changed the sensitivity of xtMC5R to $\alpha$-MSH (Figure 23).

*Effect of both MRAPs on MC2R and MC5R*

Due to previous findings that have shown that MRAP and MRAP2 have the ability to form a heterodimer (Chan et al., 2009), both xtMC2R and xtMC5R were coexpressed with mMRAP and xtMRAP2. There was no change in the sensitivity of xtMC2R to ACTH when the receptor was coexpressed with both mMRAP and xtMRAP2 (Figure 24). However, there was about three times more cAMP produced when xtMC2R was coexpressed with both MRAPs ($p<0.001$). Both MRAPs coexpressed with xtMC5R increased the sensitivity of the receptor to ACTH (Figure 25; $p<0.05$). Coexpression of both MRAPs with xtMC5R caused a shift in the $EC_{50}$ value from $1.2 \times 10^{-9} \text{ M}$ to $8.0 \times 10^{-11} \text{ M}$, and resulted in a slight but significant increase in cAMP ($p<0.001$). There was no significant difference in the sensitivity of the receptor when xtMC5R was coexpressed with both MRAPs and stimulated with $\alpha$-MSH (Figure 26), but there was over twice as much cAMP produced ($p<0.001$).

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Figure 22: Effect of chicken MRAP and xtMRAP2 on xtMC5R when stimulated with ACTH. Transient transfections of CHO cells with xtMC5R, chicken MRAP, xtMRAP2 and stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $1.5 \times 10^{-9}$ M +/- 5.5 x $10^{-10}$, xtMC5R/chicken MRAP (squares) $3.8 \times 10^{-12}$ M +/- 3.4 x $10^{-12}$, and xtMC5R/xtMRAP2 (diamonds) $3.4 \times 10^{-11}$ M +/- 1.5 x $10^{-11}$. Statistical analysis shown in Table 5.
Table 5: Statistical analysis for Figure 22. Student’s t-test for the mean EC₅₀ values +/- S.E.M for the transfections shown in Figure 22. P-values listed and (*) indicates p<0.05.

<table>
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<th>xtMC5R/chicken MRAP</th>
<th>xtMC5R/xtMRAP2</th>
<th>P-value</th>
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<tr>
<td>xtMC5R</td>
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<td>3.8 x 10⁻¹² M +/- 3.4 x 10⁻¹²</td>
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<tr>
<td>xtMC5R/chicken MRAP</td>
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<tr>
<td>xtMC5R/xtMRAP2</td>
<td>3.4 x 10⁻¹¹ M +/- 1.5 x 10⁻¹¹</td>
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<td>0.06</td>
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Figure 23: Effect of chicken MRAP and xtMRAP2 on xtMC5R and stimulated with α-MSH. Transient transfections of CHO cells with xtMC5R, chicken MRAP, xtMRAP2 and stimulated with frog α-MSH from 10^{-12} to 10^{-6} M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC_{50} values are the following: xtMC5R (circles) 1.1 \times 10^{-8} M +/- 7.0 \times 10^{-9}, xtMC5R/chicken MRAP (squares) 2.2 \times 10^{-9} M +/- 1.3 \times 10^{-9}, and xtMC5R/xtMRAP2 (diamonds) 1.6 \times 10^{-8} M +/- 4.9 \times 10^{-9}. Statistical analysis shown in Table 6.
<table>
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<tr>
<th></th>
<th>xtMC5R/chicken MRAP</th>
<th>xtMC5R/xtMRAP2</th>
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</thead>
<tbody>
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<td>xtMC5R</td>
<td>1.1 x 10^{-8} M +/- 7.0 x 10^{-9}</td>
<td>2.2 x 10^{-9} M +/- 1.3 x 10^{-9}</td>
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<td>1.6 x 10^{-8} M +/- 4.9 x 10^{-9}</td>
<td>0.03*</td>
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Table 6: Statistical analysis for Figure 23. Student’s *t*-test for the mean EC_{50} values +/- S.E.M for the transfections shown in Figure 23. P-values listed and (*) indicates p<0.05.
**Figure 24:** Effect of mMRAP and xtMRAP2 on xtMC2R when stimulated with ACTH. Transient transfections of CHO cells with xtMC2R, mMRAP, xtMRAP2 and stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R/mMRAP (circles) $1.1 \times 10^{-10}$ M +/- $3.4 \times 10^{-11}$, and xtMC2R/mMRAP/xtMRAP2 (squares) $2.0 \times 10^{-10}$ M +/- $1.1 \times 10^{-10}$, p=0.24.
Figure 25: Effect of mMRAP and xtMRAP2 on xtMC5R when stimulated with ACTH. Transient transfections of CHO cells with xtMC5R, MRAP, xtMRAP2 and stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $1.2 \times 10^{-9}$ M +/- $3.3 \times 10^{-10}$, and xtMC5R/mMRAP/xtMRAP2 (squares) $8.0 \times 10^{-11}$ M +/- $3.1 \times 10^{-11}$, p=0.02.
Figure 26: Effect of mMRAP and xtMRAP2 on xtMC5R and stimulated with α-MSH. Transient transfections of CHO cells with xtMC5R, mMRAP, xtMRAP2 and stimulated with frog α-MSH from 10\(^{-12}\) to 10\(^{-6}\) M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC\(_{50}\) values are the following: xtMC5R (circles) 2.3 x 10\(^{-8}\) M +/- 1.0 x 10\(^{-8}\), and xtMC5R/mMRAP/xtMRAP2 (squares) 1.2 x 10\(^{-8}\) M +/- 4.1 x 10\(^{-9}\), p=0.20.
Interaction of MC2R, MC5R, MRAP, and MRAP2

After analyzing the effect of MRAP and MRAP2 on each individual receptor, the interaction of all these proteins was examined. CHO cells were transfected with xtMC2R, xtMC5R, mMRAP, and xtMRAP2 and stimulated with ACTH (Figure 27). The EC$_{50}$ value was 1.4 x 10$^{-10}$ M and was comparable to the control xtMC2R and mMRAP (1.1 x 10$^{-10}$ M); however, the level of cAMP was four times as much when all proteins were expressed (p<0.001). The contribution of xtMC2R/mMRAP was studied by using a mutant xtMC2R receptor in which the isoleucine at position 175 had been substituted by an alanine (xtMC2R I-175) (Figure 28). When compared to wild type MC2R, the mutant MC2R I-175 was not nearly as efficient as the wild type MC2R when stimulated by ACTH (p<0.01). There was a 526-fold shift in response to ACTH from an EC$_{50}$ of 3.1 x 10$^{-10}$ M from the wild type receptor to an EC$_{50}$ of 1.6 x 10$^{-7}$ M from the mutant receptor. Even if the mutant receptor xtMC2R I-175 was coexpressed with both MRAPs, the mutant receptor was not nearly as efficient as the wild type receptor (p<0.01).

Coexpression of mutant MC2R I-175 with xtMRAP2 completely abolished activation by ACTH. Mutant xtMC2R I-175 coexpressed with xtMC5R, mMRAP, and xtMRAP2 resulted in a similar EC$_{50}$ value compared to wild type xtMC2R, but did not result in a significant increase cAMP (p>0.05).

Likewise, expression of xtMC2R, xtMC5R, mMRAP, and xtMRAP2 were compared to xtMC5R (Figure 29). The receptor on its own was sixteen times less sensitive to ACTH and had an EC$_{50}$ of 1.2 x 10$^{-9}$ M versus an EC$_{50}$ of 7.2 x 10$^{-11}$ M when coexpressed with MC2R and both MRAPs (p<0.05). When both receptors and both
Figure 27: Transient transfection of CHO cells with xtMC2R, xtMC5R, mMRAP, xtMRAP2 compared to xtMC2R/mMRAP when stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R/mMRAP (circles) $1.1 \times 10^{-10}$ M +/- $3.4 \times 10^{-11}$, and xtMC2R/mMRAP/xtMRAP2/xtMC5R (squares) $1.4 \times 10^{-10}$ M +/- $4.9 \times 10^{-11}$, p=0.34.
Figure 28: Contribution of wild type xtMC2R/mMRAP when stimulated with ACTH. Transient transfection of CHO cells with mutant xtMC2R I-175, xtMC5R, mMRAP, and xtMRAP2 compared to wild type xtMC2R when stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R/mMRAP (filled circles) $3.1 \times 10^{-10}$ M +/- $2.8 \times 10^{-11}$, xtMC2R I-175/mMRAP (squares) $1.6 \times 10^{-7}$ M +/- $3.3 \times 10^{-8}$, xtMC2R I-175/mMRAP/xtMRAP2 (half-filled squares) $1.2 \times 10^{-7}$ M +/- $2.9 \times 10^{-8}$, xtMC2R I-175/mMRAP/xtMRAP2/xtMC5R (open circles) $2.0 \times 10^{-9}$ M +/- $1.9 \times 10^{-9}$. Also shown xtMC2R I-175/xtMRAP2 (triangles). Statistical analysis shown in Table 7.
Table 7: Statistical analysis for Figure 28. Student’s *t*-test for the mean EC$_{50}$ values +/- S.E.M for the transfections shown in Figure 28. P-values listed and (*) indicates p<0.05, (**) indicates p<0.01.

<table>
<thead>
<tr>
<th>xtMC2R/mMRAP 3.1 x 10$^{-10}$ M +/- 2.8 x 10$^{-11}$</th>
<th>xtMC2R I-175/mMRAP 1.6 x 10$^{-7}$ M +/- 3.3 x 10$^{-8}$</th>
<th>P-value</th>
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<tr>
<td>xtMC2R/mMRAP 3.1 x 10$^{-10}$ M +/- 2.8 x 10$^{-11}$</td>
<td>xtMC2R I-175/ mMRAP/xtMRAP2 1.2 x 10$^{-7}$ M +/- 2.9 x 10$^{-8}$</td>
<td>0.007**</td>
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<tr>
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<td>0.004**</td>
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<tr>
<td>xtMC2R I-175/ mMRAP/xtMRAP2/xtMC5R 2.0 x 10$^{-9}$ M +/- 1.9 x 10$^{-9}$</td>
<td>xtMC2R I-175/ mMRAP/xtMRAP2 1.2 x 10$^{-7}$ M +/- 2.9 x 10$^{-8}$</td>
<td>0.007**</td>
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**Figure 29:** Transient transfection of CHO cells with xtMC2R, xtMC5R, mMRAP, xtMRAP2 compared to xtMC5R when stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC2R (circles) $1.2 \times 10^{-9}$ M +/- 3.3 $\times 10^{-10}$, and xtMC2R/mMRAP/xtMRAP2/xtMC5R (squares) $7.2 \times 10^{-11}$ M +/- 4.0 $\times 10^{-11}$, p=0.01.
Accessory proteins were expressed together they produced almost five times more cAMP than xtMC5R by itself (p<0.001). To study this interaction further, a mutant xtMC5R with alanine substitutions at amino acid positions 92, 114, and 119 (xtMC5R A/92/114/119) was coexpressed with xtMC2R, mMRAP, and xtMRAP2 and compared to wild type xtMC5R (Figure 30). Mutant xtMC5R A92/114/119 alone or coexpressed with any of the MRAPs failed to be activated by ACTH. The coexpression of mutant xtMC5R A/92/114/119 with xtMC2R, mMRAP, and xtMRAP2 did not result in a significant shift in the EC$_{50}$ value when compared the wild type xtMC5R. However there was about five and a half times more cAMP produced when mutant xtMC5R A92/114/119 was coexpressed with xtMC2R, mMRAP, and xtMRAP2.

In contrast, expression of xtMC5R alone compared to expression of xtMC5R, xtMC2R, mMRAP, and xtMRAP2 did not change sensitivity to $\alpha$-MSH (Figure 31). The EC$_{50}$ value remained in the $10^{-8}$ M range.
Figure 30: Contribution of wild type xtMC5R when stimulated with ACTH. Transient transfection of CHO cells with mutant xtMC5R A92/114/119, xtMC2R, mMRAP, and xtMRAP2 compared to wild type xtMC5R when stimulated with frog ACTH 1-24 from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $9.8 \times 10^{-10}$ M +/- $5.4 \times 10^{-10}$, and xtMC5R A92/114/119/xtMC2R/mMRAP/xtMRAP2 (half-filled squares) $8.9 \times 10^{-10}$ M +/- $2.8 \times 10^{-10}$. Also shown are xtMC5R A92/114/119 (squares) and xtMC5R A92/114/119/xtMC2R/mMRAP/xtMRAP2 (triangles), P-value between xtMC5R and xtMC5R A92/114/119/xtMC2R/mMRAP/xtMRAP2, p=0.45.
Figure 31: Transient transfection of CHO cells with xtMC2R, xtMC5R, mMRAP, and xtMRAP2 compared to xtMC5R when stimulated with frog α-MSH from $10^{-12}$ to $10^{-6}$ M. The CRE-luciferase units correspond to the amount of cAMP produced upon stimulation at each concentration. Each transfection was done in triplicates and the mean +/- S.E.M. is plotted for each data point. The EC$_{50}$ values are the following: xtMC5R (circles) $1.0 \times 10^{-8}$ M +/- $5.4 \times 10^{-9}$, and xtMC5R/mMRAP/xtMRAP2/xtMC2R (squares) $7.7 \times 10^{-8}$ M +/- $3.4 \times 10^{-8}$, p=0.06.
Discussion

The primary aim of this study was to understand the role of melanocortin receptors (MC2R and MC5R) and the accessory proteins (MRAP and MRAP2) in the HPI-axis of the amphibian *Xenopus tropicalis*, and to test whether the function of this system is similar to the mammalian HPA-axis. It was hypothesized that *X. tropicalis* MC2R, MC5R, MRAP, and MRAP2 would be expressed in the interrenal gland. Additionally, it was predicted that the interaction of these proteins would be comparable to what Sebag and Hinkle (2009a) previously published for the mammalian MC2R, MC5R, MRAP, and MRAP2 in the adrenal gland. Based on their observations MRAP should facilitate trafficking of *X. tropicalis* MC2R to the plasma membrane and enable activation by ACTH, but MRAP and MRAP2 should inhibit cell surface expression of MC5R and no activation should be achieved by either ACTH or α-MSH. In this study RT-PCR performed on the interrenal/kidney tissue of the frog confirmed the expression of MC2R, MC5R, MRAP, and MRAP2. Furthermore, through testing the interaction of these four proteins *in vitro* it was verified that MRAP was required for MC2R activation by ACTH, but unlike the mammalian system MRAP and MRAP2 did not inhibit MC5R activation by ACTH or α-MSH.
Cloning and expression patterns of MC2R, MC5R, MRAP, MRAP2

The results obtained from PCR and sequencing verified the presence of MC2R, MC5R, MRAP, and MRAP2 in the frog interrenal/kidney tissue (See Figures 5-9). This fits in line with earlier studies that have shown coexpression of MC2R and MC5R in other organisms including the adrenals in mammals (Sebag and Hinkle, 2009a), and the head kidney of fish (Kobayashi et al., 2011). The significance of the coexpression of MC2R and MC5R in the same cell type could be the result of the proposed common origin of these two receptors (See Figure 32). It has been hypothesized that MC2R and MC5R are the result of a local gene duplication that occurred in an MC2R/MC5R gene (Dores, 2013). This could provide a possible explanation as to why both receptors were found in the interrenal tissue of the frog. The two accessory proteins MRAP and MRAP2 were also found in the interrenal/kidney tissue. Since MRAP has not yet been identified in the *X. tropicalis* genome, degenerate primers designed from conserved regions of MRAP were used in the PCR reaction. From the sequencing results, a 185 base pair region of MRAP was obtained that matched the chicken MRAP sequence (Figure 9). This region contains the domain that is responsible for facilitating MC2R trafficking to the plasma membrane and the transmembrane spanning domain (Liang et al., 2011). The sequenced portion of *X. tropicalis* MRAP was highly similar to the chicken MRAP both at the nucleotide and amino acid level. For MRAP the N-terminal region and transmembrane spanning domain are highly conserved and the region cloned from the frog interrenal was almost identical to the chicken MRAP. Currently the sequencing of
Figure 32: Proposed outline for the evolution of melanocortin receptors (MCRs). This diagram shows the prediction of how two genome duplication events and one local gene duplication could have given rise to the five known MCRs. This schematic predicts that MC4R was the ancestral MCR. Receptors in a dashed box have not yet been identified and the dashed lines indicate the hypothesis that melanocortin receptors 2 and 5 are the result of a local gene duplication from an MC2R/MC5R gene. (Figure adapted from Dores, 2013).
MRAP from the interrenal tissue is being repeated to ensure that the results obtained here were not due to contamination.

One caveat to the results obtained from the RT-PCR experiments is that due to the difficulty of extracting the interrenal tissue, a small portion of the kidney had to be extracted along with it; therefore, the possibility that some of these genes were cloned from the small portion of the kidney cannot be ruled out. Another factor that must be considered is that although both receptors and accessory proteins were cloned from the interrenal tissue, these results do not provide definitive proof that all four genes are being expressed in the glucocorticoid producing cells.

After determining that the primers for MC2R, MC5R, MRAP, and MRAP2 had amplified the target sequence, the primers were tested on other frog tissues to look at the expression pattern of these genes (Figures 10-13). The tissues analyzed were interrenal/kidney, heart, brain, liver, muscle, ovaries, and intestine. Expression of MC2R was positive for the interrenal/kidney, heart, ovaries, and a very faint band was observed in muscle (Figure 10). Expression in the interrenal tissue and ovaries of the frog is consistent with tissue expression studies of MC2R in mice (Nimura et al., 2006) and in fish (Aluru and Vijayan, 2008) that have shown that MC2R is also found in the adrenal/interrenal tissue and the ovaries/testis of these organisms. The next gene to be analyzed in the frog was MC5R and expression was detected in the interrenal/kidney, brain, muscle, ovaries, and intestine (Figure 11). These findings are in agreement with several studies in mammals that have shown that MC5R has the widest range of tissue
distribution (Gantz et al., 1994; Fathi et al., 1995; Chen et al., 1997; Nimura et al., 2006). These studies have found varying levels of expression of MC5R in the mouse adrenal gland, exocrine glands, RT2-2 retinal neuronal cells, muscle, skin, spinal cord, brain stem, kidney, testis, heart, liver, lung, spleen, and adipose tissue. To this date, the only known function that has been determined for MC5R in mammals is its role in exocrine gland secretion (Chen et al., 1997), but its role in the other tissues and other species is still unknown. One important observation from this earlier study was that the HPA-axis of MC5R knock-out mice was intact, suggesting that in mammals MC5R is not involved in glucocorticoid secretion despite being found in the adrenal gland. For MRAP, other than the interrenal/kidney tissue, the degenerate primers failed to detect expression in the other frog tissues that were analyzed (Figure 12). Failure to detect MRAP in the other frog tissues could be due to lack of expression, lower expression, or nonspecific binding of the degenerate primers. To increase the specificity of this screening method, the full-length sequence of the frog MRAP must be obtained so that gene specific primers can be designed and these tissues can be screened again. Lastly MRAP2 primers were tested in the various frog tissues and it was found in interrenal/kidney, brain, and muscle (Figure 13). The team of researchers that discovered MRAP2 showed that it was expressed in the human brain and adrenal gland (Chan et al., 2009). This group of researchers also showed that both MRAP and MRAP2 could interact with all five members of the melanocortin receptor family to regulate their function. A relevant example of this is a recent study done in which it was shown that MRAP2 proteins alter the function of MC4R in the brain to regulate energy homeostasis during the development of zebrafish (Sebag et al., 2013).
Therefore, finding MRAP2 in the frog brain was not surprising and it is likely that MRAP2 could also involved in regulating the function of the frog MC4R in the brain.

**Modeling the interaction of frog MC2R, MC5R, MRAP, MRAP2 in CHO cells**

Another objective of the present study was to recreate the internal environment of the frog interrenal cell. This was implemented through transient transfections of CHO cells with varying combinations of xtMC2R, xtMC5R, mMRAP, and xtMRAP2 and stimulating the cells with ACTH or α-MSH. This was done to enable the characterization of the functional interaction of the proteins involved in glucocorticoid secretion during activation of the HPI-axis. Collectively, the observations from the transfection experiments give indication that the interaction of MC2R, MC5R, MRAP, and MRAP2 in *Xenopus tropicalis* differs from the mammalian system, specifically with respect to the function of MC5R.

First, it was shown that similar to mammals, functional activation of the frog MC2R by ACTH requires coexpression with MRAP, and that despite coexpression of xtMC2R with MRAP no activation was observed when the cells were stimulated with α-MSH (Figure 14). The ligand selectivity of MC2R for ACTH and requirement for MRAP has been well established for mammals, birds, reptiles, and amphibians (Barlock et al., 2014; Agulleiro et al., 2010; Liang et al., 2011; Davis et al., 2013). In addition, the HFRW and KKRRP motifs on ACTH have been shown to be important for proper activation of MC2R (Costa et al., 2004; Davis et al., 2013). This means that MC2R must have a binding site not only for the HFRW domain of ACTH, but also for KKRRP, which
is not present in any of the MSH-ligands. Another similarity to the mammalian MC2R is that MRAP2 did not completely abolish activation of xtMC2R to ACTH, but greatly reduced the sensitivity of the receptor (Figure 19). This is consistent with the finding that MRAP2 can interact with MC2R, but it is not nearly as efficient as MRAP for trafficking to the plasma membrane or functional activation by ACTH (Chan et al., 2009).

Subsequently it was determined that unlike the mammalian MC5R, the function of the amphibian MC5R was not inhibited by the presence of MRAPs. When xtMC5R was coexpressed with MRAP, MRAP2, or a combination of both MRAPs, the receptor became significantly more sensitive to ACTH (See Figures 16, 19, 22, 25). From the sequencing results, it was not surprising that the chicken MRAP had the greatest effect on xtMC5R and increased the sensitivity to ACTH by three orders of magnitude from $1.5 \times 10^{-9}$ M (alone) to $3.8 \times 10^{-12}$ M (with MRAP) (Figure 22). Interestingly, when either MC2R or MC5R were coexpressed with both MRAPs and stimulated with ACTH, the amount of cAMP produced was 2-3 times as much (See Figures 24 and 25). This can be an indication that the receptor might be interacting with an MRAP-MRAP2 heterodimer, but further studies must be done to confirm this interaction. Then, even though xtMC5R had a slight preference for ACTH over $\alpha$-MSH (Figure 15), the effect of MRAPs on MC5R was also tested when the cells were stimulated with $\alpha$-MSH (See Figures 17, 20, 23, 26). The coexpression of xtMC5R with any of combination of MRAPs did not change the sensitivity of the receptor to $\alpha$-MSH in either a positive or a negative manner. The EC$_{50}$ value of the receptor remained virtually the same regardless of the presence of MRAPs when xtMC5R was stimulated with $\alpha$-MSH.
Finally, the interaction of MC2R, MC5R, MRAP, and MRAP2 was analyzed by transfecting CHO cells with equal amounts of each cDNA construct and stimulating with ACTH or α-MSH. When stimulated with ACTH, the EC$_{50}$ value obtained when both receptors and both accessory proteins were present was comparable to xtMC2R/mMRAP (Figure 27), but when compared to xtMC5R (Figure 29) the EC$_{50}$ value of all four proteins was significantly more sensitive. Under both circumstances the amount of cAMP produced was 4-5 times higher in the presence of both receptors and both accessory proteins, compared to each receptor on its own. This indicates that both receptors may be contributing to the overall response when stimulated with ACTH, and that the combination of both accessory proteins does not have a negative effect on the function of the receptors. On the other hand, there was no significant difference when the cells were transfected with xtMC5R alone or when transfected with a combination of xtMC2R, xtMC5R, mMRAP, and xtMRAP2 and stimulated with α-MSH (Figure 31). This is due to the fact that xtMC2R cannot be activated by α-MSH, and that the MRAPs do not have an effect on MC5R when the receptor is stimulated with α-MSH. Therefore, the response to α-MSH comes only from the presence of xtMC5R.

To further understand the interaction of MC2R, MC5R, MRAP, and MRAP2 and to assess the contribution of each receptor, studies were done using a mutant xtMC2R I-175 receptor (Figure 28) and a mutant xtMC5R A92/114/119 receptor (Figure 30). The mutant xtMC2R I-175 has an alanine substitution at isoleucine position 175. This mutant MC2R receptor coexpressed with mMRAP (EC$_{50}$= 1.6 x 10$^{-7}$ M) has a greatly reduced sensitivity to ACTH compared to the wild type MC2R with mMRAP (Figure 28; EC$_{50}$= 1.6 x 10$^{-7}$ M) and xtMC5R alone.
$3.1 \times 10^{-10}$ M; p<0.01). The interaction of xtMC2R I-175 with xtMRAP2 completely eliminated activation by ACTH, and coexpression with both MRAPs did not change the sensitivity of the mutant receptor. When the mutant MC2R I-175 is coexpressed with MC5R and both MRAPs, the response to ACTH was similar to wild type MC2R with MRAP. This was interesting since mutations can occur in the frog MC2R where ACTH no longer activates this receptor, but the presence of MC5R and the interaction with MRAPs almost restores functional activation by ACTH similar to wild type MC2R/mMRAP. Then, studies were done using a mutant MC5R. The mutant xtMC5R A92/114/119 has alanine substitutions at amino acid positions 92, 114, 119. These mutations lie in the transmembrane 2 and transmembrane 3 domains (TM2/TM3) of MC5R, which are amino acid residues involved in the binding pocket of the receptor (Yang, 2011). This mutant MC5R did not respond at all to ACTH, alone or in the presence of MRAPs (Figure 30). However, addition of xtMC2R restores activation by ACTH and this response was only from activation of xtMC2R, since the mutant xtMC5R A92/114/119 does not respond to ACTH. When the interaction of xtMC2R, MRAP, MRAP2, and mutant xtMC5R A92/114/119 is compared to what the wild type xtMC5R would contribute, it becomes evident that the majority of the cAMP response to ACTH comes from activation of xtMC2R. Additional studies, such as Co-Immunoprecipitation (Co-IP), must be performed in order to confirm that this mutant MC5R does interact with MC2R and the MRAPs, and that this receptor is not being degraded or retained in the ER due to improper folding.


Conclusion

Taken together, the results obtained here confirm the hypothesis that all four proteins MC2R, MC5R, MRAP, and MRAP2 are found in the frog interrenal tissue. Also, it was confirmed that the interaction of the frog MC2R with MRAP would allow functional activation of the receptor by ACTH. However, the hypothesis that MRAP and MRAP2 would have a negative effect on MC5R must be rejected based on the observations that MRAP and MRAP2 increased the sensitivity of MC5R to ACTH, and did not change the sensitivity of the receptor to α-MSH. When all four proteins were coexpressed in CHO cells and stimulated with ACTH, the response observed seemed to be a combined effect of both MC2R and MC5R interacting with MRAP and MRAP2. From these observations it can be said that at least in vitro, the frog MC5R contributes to the overall production of cAMP. If this holds true in vivo it means that MC5R also contributes to glucocorticoid secretion during activation of the HPI-axis.

Nevertheless, the results obtained in this study still leave unresolved the observation that α-MSH does not induce glucocorticoid secretion in vivo (Schwyzer, 1977; Aluru and Vijayan 2008; Kobayashi et al., 2011). Although in mammals the second highest concentration of the antagonist AGRP has been found in the adrenal gland, MC2R and MC5R have no affinity for this antagonist (Gantz and Fong, 2003). A possible mechanism for the selectivity of ACTH over α-MSH in the mammalian adrenal cell could be due to the negative effect that MRAPs have on the cell surface expression of MC5R. If MC5R cannot reach the cell surface, then it cannot be functionally activated by α-MSH to induce glucocorticoid secretion during the stress response. This does not seem
to be the case for regulating the ligand selectivity in the frog interrenal cell. It is possible that in the frog, the levels of $\alpha$-MSH required for activation of MC5R in the interrenal cell are extremely high, ($10^{-8}$ M range) which begins to get above physiological levels. Even if the concentration of $\alpha$-MSH were to reach these levels in vivo, this ligand would preferentially bind to and activate MCRs that are more sensitive to $\alpha$-MSH, such as MC1R.

**Future directions**

The studies presented here only begin to examine the interactions between MC2R, MC5R, MRAP, and MRAP2, but future studies should be done to further understand the interaction of these proteins in the frog interrenal cells. First, the sequence of MRAP should be verified and the full-length MRAP gene should be sequenced. This can be done through performing 3’ and 5’ RACE (Rapid amplification of cDNA ends) reactions on the interrenal cDNA to obtain the 3’ and 5’ ends of the frog MRAP gene. Next, Quantitative RT-PCR (qRT-PCR) can be done to measure the expression levels of MC2R, MC5R, MRAP, and MRAP2 in the frog interrenal cells. It could be that MC2R has higher expression levels than MC5R and this could explain why $\alpha$-MSH does not induce cortisol secretion in vivo. An earlier study showed that ACTH upregulates expression levels of MC2R in mice and human cell lines (Mountjoy et al., 1994). Therefore, the frog MC2R and MC5R expression levels could be measured by qRT-PCR before and after stimulation by ACTH. Additionally, in situ hybridization using RNA probes could be done on the frog interrenal tissue to determine if MC2R, MC5R, MRAP,
and MRAP2 are all expressed in the glucocorticoid producing cells. Another experiment that can be performed is cell surface ELISA to measure the amount of receptor (MC2R or MC5R) that reaches the cell surface in the presence and absence of MRAPs. Then another method for analyzing these interactions can be applied such as fluorescent immunocytochemistry to show the co-localization of the receptors and accessory proteins. Moreover, it has been shown that other members of the melanocortin receptor family can form homo- and heterodimers (Mandrika et al., 2005) and Co-IP can be done to determine whether frog MC2R/MC5R can form heterodimers. In this study, the pharmacological properties of xtMC2R and xtMC5R when stimulated with ACTH or α-MSH were also tested, but in either case the response was similar to xtMC5R being expressed alone (data not shown); however it is possible that MC2R/MC5R do form a heterodimer. Co-IP can also be used to show that MC2R, MC5R, MRAP, and MRAP2 have the potential to form a complex in vitro, and that this complex might also be formed in vivo. The work presented here and future work that remains to be done, will help to provide a further understanding of how the HPA/I-axis has evolved, and the role of melanocortin receptors and accessory proteins in regulating the function of this system.
References


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