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EVOLUTION OF *XENOPUS TROPICALIS* MC2R: EXPRESSION AND  
ACTIVATION

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A Thesis

Presented to

The Faculty of Natural Sciences and Mathematics

University of Denver

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Perry Victoria Davis

August 2013

Advisor: Dr. Robert Dores

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Author: Perry Victoria Davis

Title: EVOLUTION OF *XENOPUS TROPICALIS* MC2R: EXPRESSION AND ACTIVATION

Advisor: Dr. Robert Dores

Degree Date: August 2013

### **Abstract**

The structure and functional relationship between the melanocortin-2 receptor (MC2R) and adrenocorticotrophic hormone (ACTH) is the most complex of the melanocortin gene family. Prior studies had been done on amniote tetrapod MC2Rs (e.g., mammals); this study analyzed the expression and activation of MC2R by an anamniote tetrapod, *Xenopus tropicalis* (xtMC2R). An immunofluorescence approach, done on the expression of xtMC2R in Chinese Hamster Ovary cells (CHO cells), indicated that the trafficking of xtMC2R to the plasma membrane required co-expression with a tetrapod MRAP1 (melanocortin-1 receptor accessory protein). A cAMP-reporter assay was used to show that xtMC2R can be activated by human ACTH(1-24), but not by  $\alpha$ -MSH. These two properties are also observed for human MC2R, and are common for tetrapod MC2Rs in general. Alanine-substitution analogs of hACTH(1-24) were used to deduce a possible mechanism for the activation of xtMC2R. These studies showed that alanine substitutions to the HFRW motif in hACTH(1-24) eliminated activation of the receptor. Furthermore, the alanine-substitution analysis revealed that positions 15 and 16 in the KKRRP motif are more important for the activation of xtMC2R than positions 17 through 19. Finally, the alanine-substitution assays coupled with analysis of internally truncated analogs of the GKPVG motif resulted in decreased or complete elimination of xtMC2R activation. These data were used to construct a proposed three step model for the activation of

MC2R. The final goal of this thesis was to identify the region of the receptor involved in the docking of the KKRRP motif of ACTH. Based on a model of MC2R, these experiments used alanine substitution site-directed mutagenesis to analyze the transmembrane 4 (TM4), extracellular loop 2 (EL2), and transmembrane 5 (TM5) region of xtMC2R. These experiments revealed that the following mutations had the greatest effect on the sensitivity ( $EC_{50}$  value) of xtMC2R: I/A<sup>175</sup>, F/A<sup>178</sup>, and I/A<sup>184</sup>. These results were compared to site-directed mutagenesis studies done on human and rainbow trout MC2Rs. Collectively, these analyses revealed that all three MC2Rs have docking sites for the KKRRP motif of ACTH that are similar in general location, and mostly similar in 3-dimensional structure, but that are not identical. The evolutionary implications of these observations are discussed.

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## **Introduction**

### *Melanocortin Receptor Family: G-Coupled Protein Receptors*

The melanocortin receptors (MCRs) are a family of hormone-activated receptors that influence a number of physiological functions in mammals. The melanocortin receptor family consists of five different receptors, which were named in the numerical order in which they were cloned from the human genome (Cone, 2006). More so, each of these receptors is regulated by its own gene, and these receptors are expressed in different cells and tissues (Cone, 2006) throughout an organism. Melanocortin receptors are G protein-coupled receptors (GPCRs) that belong to the rhodopsin/ $\beta_2$ -adrenergic-like family of GPCRs. G protein-coupled receptors are the largest group of cell surface receptors, and all these receptors use guanine nucleotide-binding proteins (G proteins) as transducers. All GPCRs have seven transmembrane domains that are linked by extracellular and intracellular protein loops. More specifically, the extracellular domains allow for the binding of specific ligands, which causes a conformational change in the receptor. In turn, this conformational change causes the intracellular G protein to be activated. Finally, a subunit of the G protein interacts with either an ion channel or an enzyme to illicit a biological response in the target cell. In the case of the melanocortin receptors the enzyme that is activated is adenylyl cyclase, and this enzyme generates the second messenger cyclic AMP (cAMP). The increase in the intracellular concentration of

cAMP results in the activation of protein kinase A, and this enzyme in turn, can activate transcription factors, interact with channels, or affect the activity of enzymes. The end result is a biological response within a cell (Cooper & Hausman, 2009).

The MCRs appear to be the smallest GPCRs in terms of amino acid length, and have relatively short N- and C- terminal ends (Cooray & Clark, 2011). In terms of the location and function of the melanocortin receptors (Figure 1; Cone, 2006), MC1R is located on melanocytes, in areas of the brain, and on macrophages. This MCR plays a role in pigmentation (melanocytes), body temperature regulation (CNS), and has anti-inflammatory properties. MC3R is predominantly expressed in the brain, but can be found in the placenta, stomach and pancreas; its main function lies in energy metabolism. MC4R is expressed mainly in the brain, as well as the autonomic nervous system, and spinal cord where it plays a role in the regulation of food consumption and energy output. MC5R is expressed in many different tissues including skin, adrenal and exocrine glands. It is thought to play a role in the production of types of lipids, as well as some regulation of the immune system. Finally, MC2R is located in the adrenal cortex, and is involved in the initiation of steroidogenesis; the production of the glucocorticoid, cortisol. However, this receptor is also expressed in the skin (melanocytes), as well as, in adipocytes. MC2R is unlike any of the other melanocortin receptors because of its unique intracellular trafficking properties and ligand selectivity.

**Figure 1: Melanocortin Receptor Expression, Function, and Ligand Selectivity**

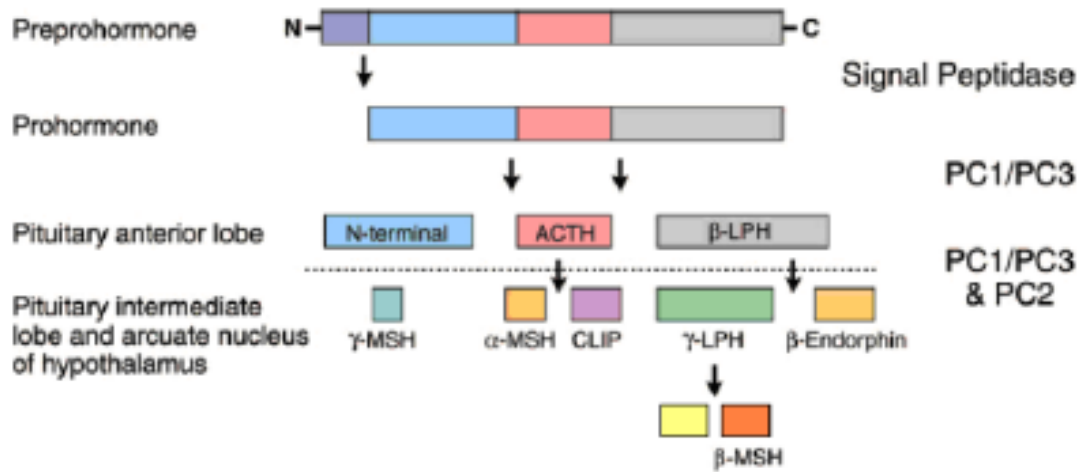
| Melanocortin receptor | Main site of expression                  | Ligand preference                                       | Main physiological function                                |
|-----------------------|--|---|--|
| MC1R                  | Melanocytes, melanoma, macrophage, brain | $\alpha$ -MSH = $\beta$ -MSH = ACTH $\ll$ $\gamma$ -MSH | Pigmentation, anti-inflammatory action                     |
| MC2R                  | Adrenal cortex                           | ACTH only   | Adrenal steroidogenesis                                    |
| MC3R                  | Brain, placenta, pancreas, stomach       | $\gamma$ -MSH = $\alpha$ -MSH = $\beta$ -MSH = ACTH     | Energy homeostasis, inflammatory action                    |
| MC4R                  | Brain, spinal cord                       | $\alpha$ -MSH = $\beta$ -MSH = ACTH $>$ $\gamma$ -MSH   | Appetite regulation, energy homeostasis, erectile function |
| MC5R                  | Lymphocytes, exocrine glands             | $\alpha$ -MSH $>$ $\beta$ -MSH/ACTH $>$ $\gamma$ -MSH   | Regulation of exocrine gland secretion                     |

(Figure adapted from Cone, 2006)

### *Melanocortin Peptides Derived from Proopiomelanocortin*

The melanocortin receptors are activated by hormones derived from the proopiomelanocortin (POMC) gene, which is a member of the opioid/orphanin gene family (Dores & Baron, 2010). This gene is expressed in the pituitary gland, and is responsible for the production of POMC proproteins in two different types of pituitary cells: corticotropic and melanotropic cells. Located at the anterior pituitary, the corticotropic cells are responsible for the production of the hormone, adrenocorticotropin (ACTH), by the specific and selective posttranslational cleavage involving proprotein convertase 1/3 (PC 1/3) (Cone, 2006). At the intermediate pituitary, the melanotropic cells produce various hormones including:  $\gamma$ -MSH,  $\alpha$ -MSH, CLIP,  $\beta$ -MSH, and  $\beta$ -endorphin by posttranslational events involving PC1/3, as well as, PC2 (Dores & Baron, 2010). An illustration of these specific endoproteolytic cleavage events can be seen in Figure 2.

**Figure 2: Illustration of POMC Post-Translational Cleavage Products**

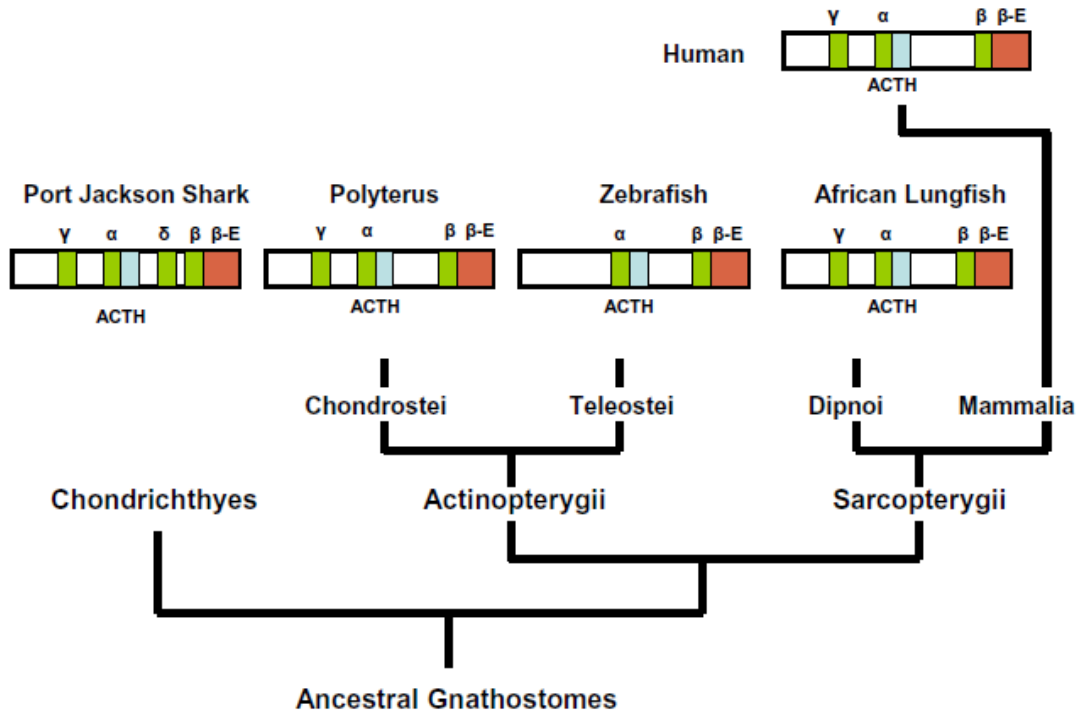


(Figure adapted from Cone, 2006)

A striking feature with respect to the evolution of the POMC gene is the remarkable degree of conservation in the organization of the precursor and the number of melanocortin-related sequences in organisms ranging from the jawless fishes to mammals as illustrated in Figure 3 (Vallarino et al., 2012).

Note also that  $\alpha$ -MSH is the first 13 amino acids of ACTH (Figure 4). These peptides have the HFRW motif which is an essential feature of melanocortin-related peptides (Schwyzer, 1977).  $\beta$ -MSH and  $\gamma$ -MSH are located at completely different cleavage sites within POMC. These polypeptides share the HFRW motif with ACTH and alpha-MSH as well.

**Figure 3: POMC Gene Phylogenic Tree**



(Vallarino et. al, 2012)

**Figure 4: Human & *Xenopus tropicalis* POMC Sequence and Peptides**

**A) Vertebrate POMC Sequences**

**Human POMC:**

MPRSCCSRSGALLLALLLQASMEVIRGWCLESSQCQDLTTESNLLECIRACKPDLAETPMFPNGN  
 DEQPLTENPRKYVMGHFRWDRFGRRNSSSSGSSGAGQKREDVSAGEDCGPLPEGGPEPRSDGAKP  
 GPREGKRSYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEPKRELTGQRLREGDGPDPGA  
 DDGAGAQADLEHSLLVAAEKKDEGPYRMEHFRWGSPPKDKRYGGFMTSEKSQTPLVTLFKNAIIK  
 NAYKKGE

**Xenopus tropicalis POMC:**

MFRPLWGCSLAILGAFIFHVGEVQGCWESSRCADLSSDGVLECIKACKMDLSAESPVFPNGNH  
 LQPLSEIRKYVMTHFRWNKFGRRNSTGNDGSSSGYKREDISNYPVFNLFVSDNMEQNAQGDNM  
 EGEPLDRQENKRAYSMEHFRWGKPVGRKRRPIKVYPNGVEEESSENYPMELRRELSLELDYPDID  
 LDEDIEDNEVESALTKKNGNYRMHFRWGSPPKDKRYGGFMTPEPERSQTPMLTLFKNAIIKNTTHK  
 GQ

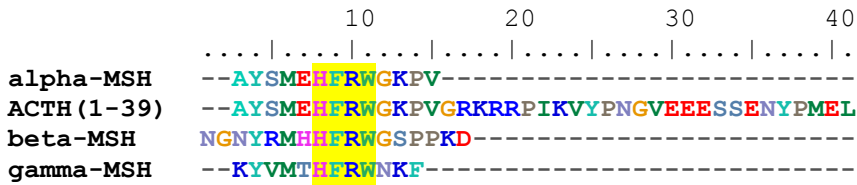
Alpha-MSH

ACTH

Beta-MSH

Gamma-MSH

**B) Alignment of Conserved HFRW Domain for *X. Tropicalis* POMC Melanocortin Peptides**



As shown in Figure 1, the MCRs can be activated by different peptide hormones derived from POMC. Based on studies on mammals, the MC1R, MC4R, and MC5R, all have a higher affinity for  $\alpha$ -MSH, than ACTH,  $\beta$ -MSH, or  $\gamma$ -MSH, while MC3R interacts with  $\gamma$ -MSH,  $\alpha$ -MSH,  $\beta$ -MSH, and ACTH with equal favorability. Although these different receptors seem to prefer one hormone or ligand over the other melanocortin peptides, they all respond to ACTH in varying degrees of efficacy (Dores & Lecaude, 2005). However, MC2R can only be activated by ACTH, and therefore, a closer look at this polypeptide is warranted.

*A Highly Conserved Melanocortin Peptide: Adrenocorticotropin Hormone (ACTH)*

Human ACTH is composed of 39 amino acids and can be found in all vertebrates because of the important role this hormone plays in glucocorticoid biosynthesis. However, it should be noted that within the ACTH peptide, only a certain number of amino acid residues are needed for functionality; the critical domains lie within the first 24 amino acid residues (Dores & Lecaude, 2005). In Figure 4, the POMC amino acid products are designated by different colors. Both human and *Xenopus tropicalis* (amphibian) ACTH sequences are strikingly similar with only a few differences lying within the 39 amino acid residues, which suggests that the conservation in the melanocortin peptide sequence is of importance for the fitness of the organism. As noted, all four melanocortin peptides shown in Figure 4.b, have the four amino acid motif, histidine-phenylalanine-arginine-



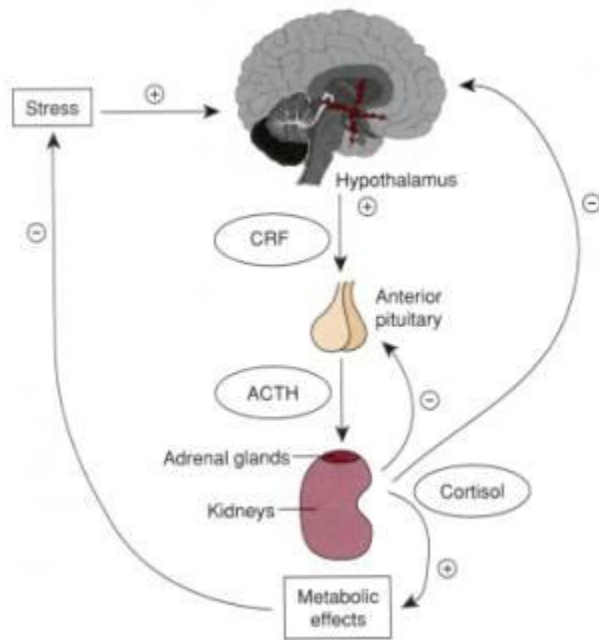
tryptophan (HFRW). The HFRW domain within a melanocortin peptide is required for the activation of all of the melanocortin receptors. This relationship was established by studies done in the 70's on the MSH receptor (i.e., MC1R) and the ACTH receptor (i.e., MC2R) as summarized in the review article by Robert Schwyzer (Schwyzer, 1977).

In Schwyzer's review (1977), he suggests that the location of the "activation motif" within ACTH was within the first 24 amino acids of the polypeptide because of *in vivo* experiments carried out with elongated or shortened sequences of human ACTH(1-39). He demonstrated that high corticotropic activity was observed with human ACTH N-terminal amino acid residues 1-19, 1-20, 1-23, and 1-24. Thus, he found that H<sup>6</sup> F<sup>7</sup> R<sup>8</sup> W<sup>9</sup> motif located within amino acid residues 1-10 of ACTH was essential for activation of the ACTH receptor. Furthermore, he proposed that another site within ACTH(1-24) acted as a type of "address" domain to direct or to position the HFRW stimulatory domain in the proper position on the receptor so that activation of the ACTH receptor would occur. He proposed that this important address sequence is located at residues 15-18 at the Lysine-Lysine-Arginine-Arginine or KKRR motif (Schwyzer, 1977). At this stage it would desirable to focus on the nature of the ACTH (aka MC2R) receptor.

### *The Physiological Relationship between Glucocorticoid Production, ACTH, & MC2R*

In terms of the MC2R receptor and glucocorticoid production, the hypothalamus-pituitary-adrenal axis (HPA) should be mentioned to understand how this receptor is part of a neuroendocrine circuit that can respond to stress. As shown in Figure 5, Corticotropin-releasing factor (CRF) is a 41-amino acid polypeptide that is found in the hypothalamus, and was first isolated from a sheep; CRF plays an important role in regulating the stress response. When stress is introduced to an organism, CRF mRNA levels elevate in parts of the brain, such as the paraventricular nucleus (PVN), *locus coeruleus*, Barrington's nucleus and bed nucleus of *stria terminalis*. As CRF levels increase within the hypothalamus, CRF receptor type 1 (CRF<sub>1</sub> receptor) is expressed on the anterior pituitary. CRF is released from the hypothalamus into a capillary bed, the median eminence, where it binds to the CRF<sub>1</sub> receptor (Kageyama & Suda, 2009). CRF<sub>1</sub> receptor stimulation activates the synthesis and secretion of ACTH from the corticotropic cells located within the anterior pituitary. In turn, ACTH secretion activates glucocorticoid production by binding to the MC2R receptor located on the adrenal cortex (Kageyama & Suda, 2009). As illustrated in Figure 5, the HPA axis is regulated by negative feedback loops, which turn off the production of ACTH or CRF in response to elevated levels of circulating cortisol.

**Figure 5: Hypothalamus-Pituitary Axis Involving Cortisol Release**



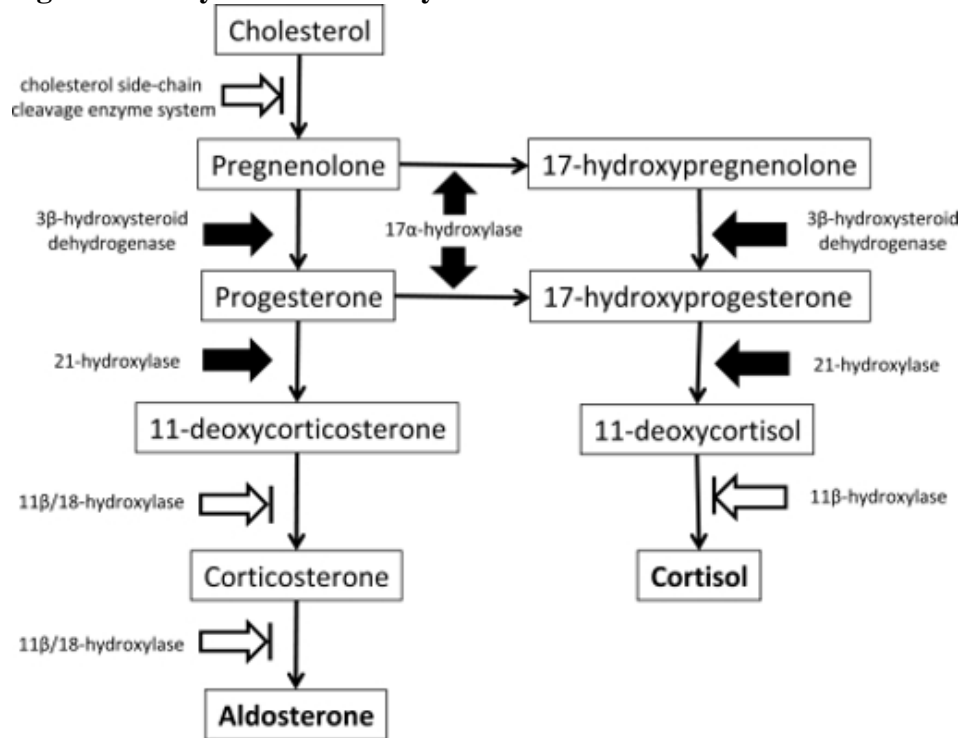
<http://www.montana.edu/wwwai/imsd/alcohol/Vanessa/vwhpa.htm>

When ACTH is present, the MC2R is specifically expressed to activate a biosynthetic pathway to release cortisol from the target cell of the adrenal cortex. As seen in Figure 6, the biosynthesis of this hormone occurs within the *zona fasciculata* and *zona reticularis* of the adrenal cortex. The steroid, cholesterol, is broken down by enzymes into an intermediate, progesterone, and then more specific enzymatic reactions produce cortisol (Barrett, 2003). Cortisol's functions span throughout the physiological system, and therefore, it is considered an important glucocorticoid hormone. Mainly, it functions to regulate stress and restore homeostasis within the body. Although its primary targets involve metabolism, it can be involved in ion transport and the physiology of the immune system as well. Additionally, when acute stress is introduced to the body, cortisol stimulates gluconeogenesis, the synthesis of glucose, in the liver (Cooper & Hausman,

2009). Prior to 1992, while there was no doubt that an ‘ACTH’ receptor was present on adrenal cortex cells, the biochemical structure of that receptor had not been determined.

This discovery will be discussed next.

**Figure 6: Biosynthetic Pathway of Adrenal Steroid Hormones**



[http://openi.nlm.nih.gov/detailedresult.php?img=2908652\\_wjem-11-161f1&req=4](http://openi.nlm.nih.gov/detailedresult.php?img=2908652_wjem-11-161f1&req=4)

### *MC2R: A Unique Melanocortin Receptor*

In 1992, Mountjoy et. al, knew that ACTH and β-endorphin were co-expressed in the anterior pituitary in response to stimulation by CRF. Also, they acknowledged the relationship between ACTH and α-MSH in that both these neuropeptides bind to G protein-coupled receptors found in the brain, as well as, melanocytes, and the adrenal cortex. However, the sequence and site of expression of these specific G protein-coupled receptors was yet to be discovered. Therefore, Mountjoy’s group hypothesized that these specific receptors would share sequence similarity with other G protein-coupled

receptors. First, they determined by DNA sequencing, two PCR fragments that encoded G protein-coupled receptors one of which was a substantial part of the MSH-R, or the MC1R receptor. By carrying out a Northern hybridization assay, this group discovered that more than one fragment held specific sites of expression in melanocytes and the adrenal cortex. Furthermore, they screened two sequences against the human genomic library, and isolated the gene sequences of the MC1R and MC2R receptor, which they referred to as the MSH-R and ACTH-R respectively. To further support their findings of these genomic sequences, they wanted to functionally test these genes with their corresponding peptide, and therefore, carried out functional assay experiments. In these experiments, specific cell lines were used to express the MSH-R and ACTH-R. The MSH-R and ACTH-R were then stimulated with different concentrations of their corresponding neuropeptides, and increased cAMP levels were detected in the cells. Therefore, these assays represent compelling evidence that they had discovered both MSH-R and ACTH-R genes. However, the location of these two receptors was still lacking in experimental data. First, they carried out a Northern Blot to test how much mRNA could be detected in different types of tissues. They found that an abundance of MSH-R mRNA could be found in human melanocyte samples. Also, this group found the presence of ACTH-R mRNA in monkey adrenal gland tissue. Overall, Mountjoy's group provided strong evidence two separate genes code for MC1R and MC2R, as well as, proving that these genes are expressed in specific tissues within an organism (Mountjoy et al., 1992).

Although Mountjoy's group was able to express and functionally test the human MC2R, they were limited to using a mammalian cell line, Cloudman S91 melanoma cells. This cell line endogenously expressed MC1R, thus making analysis of the unique properties of MC2R difficult to interpret in this particular cell line. However, Rached et al. (2005) successfully expressed the human MC2R in two different eukaryotic cell lines, M3 melanoma and HEK293, in 2004. In this study Rached et al. (2005) first stably expressed the human MC3R and MC4R genes in HEK293 cells. In these experiments each melanocortin receptor was tagged at the C-terminus with an enhanced green fluorescent protein (EGFP). Thus the receptors could be visualized using fluorescent microscopy. The EGFP-tagged human MC3R and MC4R fluoresced along the plasma membrane of the HEK 239 cells, and this was to be expected because of previous research in 2000 and 2003 (Rached et al. (2005). However, when EGFP- tagged human MC2R was expressed in HEK293 cells, the labeled receptor could be detected within the cytosol of these cells, not on the plasma membrane. These results suggested that the human MC2R required some chaperone to facilitate trafficking of the receptor to the plasma membrane (Rached et al., 2005).

Clinical observations also indicated that some chaperone may be needed to facilitate the activation of MC2R. Familial glucocorticoid deficiency (FGD) is a genetic, autosomal recessive disease where individuals become insensitive to ACTH levels. In turn, this ACTH resistance causes alarmingly low glucocorticoid output from the adrenal cortex, accompanied by high levels of ACTH. The deficient levels of cortisol in the circulatory system interrupt the development of some organ systems in newborns and this

condition can be fatal. Mutations within the MC2R receptor are responsible for at least one fourth of FGD cases and this condition is designated as Type I FGD (Hinkle & Sebag, 2009). Several different mutations within the human MC2R have been shown to cause Type 1 FGD, and in turn, individuals become insensitive to ACTH (Chan et al., 2008). Another 50% of FGD cases are caused by errors in the glucocorticoid biosynthetic pathway. However, in approximately 25% of FGD cases there is no evidence of mutation in MC2R, and there are no errors in the expression of the enzymes that make cortisol. Clearly in these patients a protein that interacts with MC2R is the problem.

*MRAP: An Essential Accessory Protein for MC2R Trafficking & Functionality*

Melanocortin Receptor Accessory Protein (MRAP) was discovered by clinical researchers, Metherell et al, (2005) who studied a group of individuals diagnosed with FGD, but who had no mutations within their MC2R receptor. Therefore, they mapped a region of the human genome that was connected to FGD, and studied the expression of 30 susceptible genes in the adrenal cortex, while comparing genes from the liver and brain (Metherell et al., 2005). By comparing and contrasting these specific genes, the group was able to identify a gene that encoded for protein with a single transmembrane spanning domain. Tissue analysis observed that the protein's gene, C21orf61, was expressed in adrenal tissue, but not in the brain or liver (Webb & Clark, 2010). As it turned out, this small protein played a major role in the successful expression of a functional MC2R receptor in adrenal tissue. Interestingly, MRAP is the first GPCR accessory protein to be implicated in causing a disease, and 9 different mutations within MRAP have been found in FGD patients. Because the mutations causing FGD were

found in MRAP and not the MC2R receptor, this form of the disease was designated as Type II FGD. Additionally, MRAP was previously identified as fat cell-specific low molecular weight protein (Falp) because of the appearance of its transcript only when differentiation occurred within adipocytes (Hinkle & Sebag, 2009).

As mentioned above, MRAP is a small protein consisting of one transmembrane domain. MRAP mRNA can be found in many parts of the mammalian body, such as the adrenal cortex, lymph nodes, brain, testis, breast, thyroid, and adipose tissue. The gene that encodes for this mammalian accessory protein consists of 6 exons. Alternate splicing of these exons can create two different MRAP products: Human MRAP $\alpha$  and MRAP $\beta$  (Hinkle & Sebag, 2009). The exons 3 and 5 encode for MRAP $\alpha$ , which is made up of 172 amino acids. On the other hand, exons 4 and 6 give rise to the 102 amino acid isoform, MRAP $\beta$ . However, the crucial transmembrane domain of MRAP is encoded by exon 4, and therefore, the two isoforms of MRAP have identical N-termini, as well as, transmembrane domains (Webb and Clark, 2010). Specifically, the 37 amino acid N-terminal sequence, the 23 amino acid transmembrane domain, and the first 9 residues of the C-terminal are identical in MRAP $\alpha$  and  $\beta$  (Hinkle & Sebag, 2009). However, MRAP $\alpha$  and  $\beta$  differ by many residues in their C-termini (Webb and Clark, 2010). Therefore, these findings suggest a level of conservation in the amino acid sequence of MRAP; particularly at the N-terminal and transmembrane domain. Although these isoforms of MRAP differ slightly in their C-termini, it is interesting to find that Roy et al. (2007) discovered cAMP production occurring within a heterologous cell line while coexpressing the MC2 receptor and the MRAP isoforms.



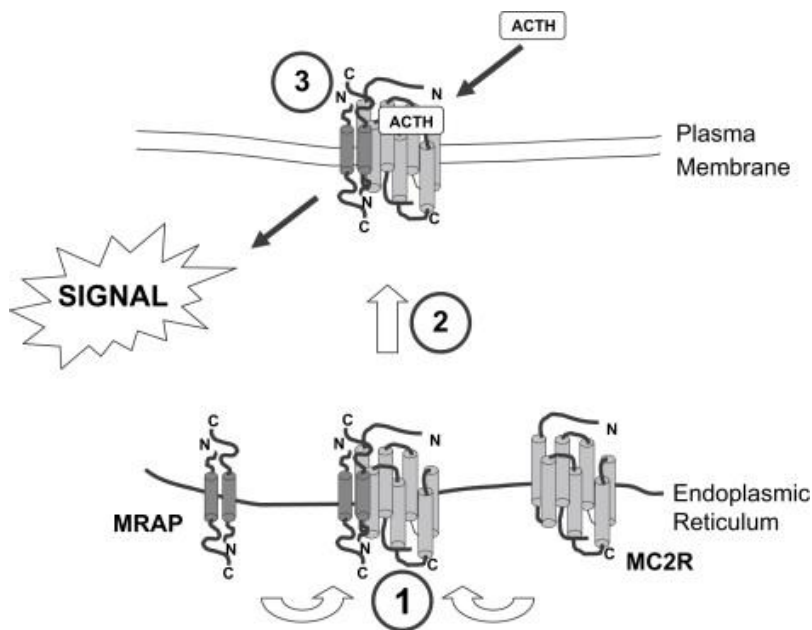
In terms of MRAP's structural properties, it is necessary to mention the importance of MRAP's orientation in the endoplasmic reticulum (ER) and the plasma membrane because of its complex interaction with the MC2R. Through topology analysis programming, Viklund & Elofsson (2004) predicted that MRAP was a type II integral membrane protein with a C-terminal that faced the inside of the ER and Golgi apparatus. On the other hand, it was hypothesized that this same C-terminus of MRAP was exposed to the exterior of the cell when MRAP was expressed on the cell membrane. Interestingly, Sebag and Hinkle (2008) found that both mouse MRAP's N- and C- termini were oriented extracellularly while being expressed on the surface of CHO cells. Therefore, a dual topology of MRAP was discovered, and found to be completely independent of the MC2R receptor (Hinkle & Sebag, 2009). Therefore, to confirm MRAP's dual topology, Hinkle and Sebag performed two critical experiments where endogenous MRAP was expressed in adrenocortical cells. By tagging MRAP's N- and C-termini, they found that both ends of MRAP were found on the endoplasmic reticulum as well as the plasma membrane. In another experiment, they used an adrenal cell line, OS3, where the MC2 receptor is not endogenously expressed, and found that the tagged MRAP produced the same results on the surface of the cell. Therefore, this suggests that MRAP's dual topology occurs independently of the MC2R. Furthermore, studies have generated convincing data that MRAP's dual topology implies formation of a homodimer. Corray et al. (2008) found that MRAP's size was comparable to that of dimers by using electrophoresis and mass spectrometry to confirm molecular weight. Furthermore, antibodies were used to test against two differently tagged MRAPs which

were coexpressed in CHO cells. Immunoprecipitation found that both MRAPs coprecipitated, indicating the presence of dimer formation (Corray et al., 2008). In addition, Hinkle and Sebag (2008) performed a set of experiments where they tagged both N- and C-termini, and found that the C-terminus of MRAP existed in both glycosylated and unglycosylated forms suggesting that MRAP structure forms an antiparallel homodimer. Therefore, this evidence of MRAP's dimeric properties suggests that this accessory protein would form a complex with the MC2 receptor, and in turn, facilitate proper function of the receptor.

The direct interaction of MRAP with the MC2 receptor is required for proper trafficking, and activation of MC2R. Webb et al. (2009) suggests that the functional domain of MRAP lies within the N-terminus and the transmembrane domain because of high level of conservation within these two regions of the accessory protein. By using truncation constructs of MRAP at both regions they were able to provide strong evidence that the transmembrane domain was responsible for the direct interaction between human MRAP and the MC2 receptor. Furthermore, they discovered that the N-terminus of MRAP is required for the MC2 receptor's surface expression, as well as, the receptor's affinity to be activated by ACTH (Webb et al., 2009). In addition, multiple research studies showed that cAMP production is affected by the presence or absence of MRAP. If MRAP was not expressed with the MC2 receptor, then cAMP production was insignificant. On the other hand, cAMP production increased significantly when MRAP and the MC2 receptor were co-expressed in mammalian cells lines (Metherell et al., 2005; Roy et al., 2007; Hinkle & Sebag, 2008). Figure 7 illustrates how MRAP

interaction and MC2R activation can be broken down in three steps. First, the MC2 receptor and MRAP form a complex at the endoplasmic reticulum. Second, MRAP facilitates the trafficking of the MC2R to the plasma membrane. Third, MRAP not only enables the receptor to traffic to the membrane, but also increases the binding affinity of the receptor for ACTH. Finally, a signal is transduced into the intracellular compartment of the cell to produce cAMP.

**Figure 7: Interaction of MC2 Receptor and MRAP**



<http://www.sciencedirect.com/science/article/pii/S1521690X08001048>

### *Evolution of the Melanocortin-2 Receptor*

Comparative studies have proposed that the evolution of the melanocortin receptor family is complex. What seems to be even more perplexing is the evolution of one melanocortin receptor in particular: the MC2R. This receptor's evolution creates an interesting story because of its intimate relationship with POMC products, ACTH, and MRAP. While MC2R depends on these two components for proper functionality and trafficking, their evolutionary trends seem to parallel that of the MC2 receptor. Therefore, a comparative analysis on the origin of the melanocortin receptors is necessary to understand the occurrence of the MC2R, as well as, observe any similar trends in POMC and MRAP evolution. It should be mentioned that genomic databases have revealed the absence of orthologous genes in protostomes, as well as, many deuterostomes. On the other hand, genomic comparisons have revealed MCR-related genes in hagfish, lamprey, cartilaginous fish, teleost, and tetrapod genomes. Therefore, these genomic data suggests that the MCR family is only found in chordates (Dores, 2013).

In 1994, comparative studies suggested that the melanocortin receptor family evolved from an ancestral gene found in protochordates (Holland et al., 1994). This single gene was subjected to two separate genomic duplication events, in which the first created two paralogous genes, and the second yielded four paralogous genes (Holland et al, 1994). The first duplication event seems likely because of two MCR genes that have been cloned from the lamprey genome (Haitina et al., 2007; Baron et al., 2008). Also, evidence of the second genomic duplication comes from bony fish in which MC1R, MC2R, MC4R and MC5R genes have been observed in the fugu genome while an

ortholog of the MC3R gene is found in zebrafish (Klovins et al., 2004; Baron et al., 2008; Ringholm et al., 2002). Furthermore, it was proposed that the MC2R and the MC5R receptors resulted from a local gene duplication dating back to the early gnathostomes because these receptors' genes are found on the same chromosome (Fredriksoon et al, 2003; Klovins et al., 2004; Baron et al, 2008). Therefore, a phylogenic tree of the melanocortin receptors was established due to sequence alignments of the receptors from different chordates. Figure 8 illustrates an early rendition of melanocortin receptor evolution as a result of the genome duplication events that lead to five distinct melanocortin receptors (Baron et al, 2008). Although this seems to be a logical hypothesis, the evolution of the melanocortin receptor family proved to be more complex because of recent findings involving the MC2R, MC4R, and MC5R.

Although the MC2R and MC5R genes are located in close proximity to one another on the same chromosome, their evolutionary origins have been called in question. Alignment of the MC2R and MC5R amino acid sequences reveals that these two receptors differ greatly from one another. Therefore, this would suggest that a local gene duplication of the MC2R and MC5R genes proves to be a weak hypothesis without further investigation. Therefore, another evolutionary scenario is introduced to include the MC4R because of its similarity in amino acid sequence with that of the MC5R. Therefore, these data implies that the MC5R gene resulted from a local duplication of the MC4R gene (Vastermark and Schioth, 2011).

In turn, a new evolutionary phylogeny hypothesis of the melanocortin receptors could be established to include the MC2R/MC5R local duplication and the MC4R/MC5R

relationship. Figure 9 illustrates this new explanation of melanocortin evolution where the MC4R gene is considered the ancestral gene (Dores, 2013). If the MC4R gene is the “original” melanocortin gene, then genome and local gene duplications would result in the paralog genes containing a conserved motif of amino acids from the ancestral MC4R gene. Synteny studies have shown that it is possible for the MC2R/MC5R gene to undergo a local duplication dating back to ancestral gnathostomes (Schiøth et al., 2003; Dores, 2013). Therefore, it has been suggested that this duplication event selected for the MC5R gene, and therefore, retained sequence similarities with its ancestral gene. Consequently, its “partner” gene, the MC2R, underwent a different fate. The MC2R gene was subjected to mutations, and in turn, its functionality would be forever different from the rest of the melanocortin family (Dores, 2013).

#### *Main Objectives*

These observations have focused on the structure, functionality, and evolution of the mammalian MC2 receptor. Also, the mammalian MC2R’s activation by ACTH has been carefully analyzed in past studies. More specifically, past research has asked these questions: Does the mammalian MC2 receptor have specific binding sites for ACTH? Do these binding sites rely on crucial ACTH amino acid motifs to ensure proper function of the MC2 receptor? (Liang, 2013) has tried to answer these questions about the human MC2R. The data have suggested that key ACTH motifs exist within the first 24 amino acids, and therefore, corresponding binding sites on the mammalian MC2 receptor can be established within the receptor’s sequence. Therefore, could these same questions be

asked of a different species of chordate to gain a better understanding of the MC2 receptor's evolution?

Based on these findings of the mammalian MC2R, this study has two different objectives, which involve the amphibian, *Xenopus tropicalis*, MC2 receptor. The first objective was to answer this question: what regions of ACTH are responsible for the activation of *X. tropicalis* MC2 receptor? Our hypothesis suggests that there are three important regions of ACTH that are required for the successful activation of the MC2 receptor. As mentioned above, ACTH is a polypeptide chain consisting of 39 amino acids. However, only the first 24 amino acids are required for activation of the receptor because of the high level of conservation within this area of the peptide (Schwyzer, 1977). Although the wild type *X. tropicalis* MC2R is utilized in the following experiments, we used the mammalian melanocortin, human ACTH(1-24). We were able to use the mammalian ACTH(1-24) because of its high level of sequence similarity with the amphibian ACTH peptide (Figure 3A). The first zone of ACTH(1-24), Zone A, consists of amino acids 6-9, which are HFRW (Histidine-Phenylalanine-Arginine-Tryptophan). This crucial motif is found in several of the melanocortin peptides (Cone, 2006). The second region we analyzed was Zone B, which was made up amino acids 10-14, GKPVG (Glycine-Lysine-Proline-Valine-Glycine). The third region of ACTH(1-24), Zone C, is made up of amino acids KKRRP (Lysine-Lysine-Arginine-Arginine-Proline). To test the importance of these three regions in ACTH(1-24), we replaced individual amino acids within each zone, and stimulated the wild type *X. tropicalis* MC2 receptor with these ACTH(1-24) analogs to produce a dose response curve. By analyzing these

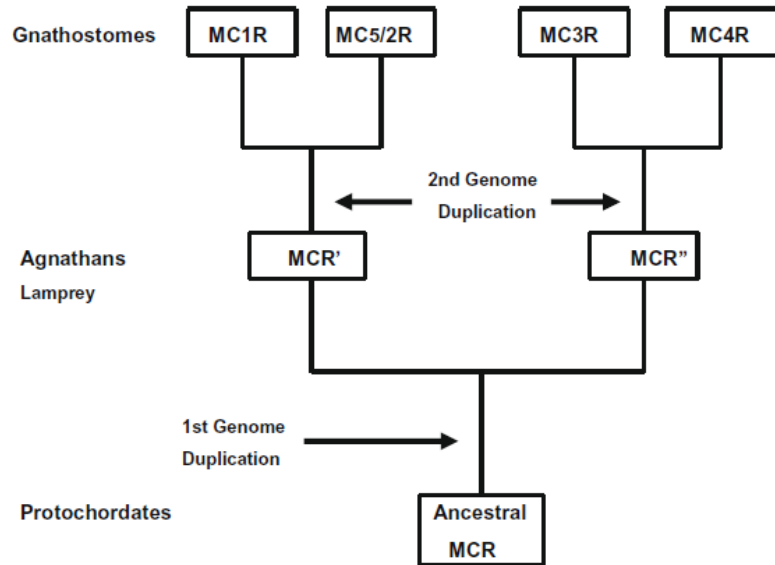
zones of ACTH(1-24), we believe that the activation of the *X. tropicalis* MC2R could be quite similar to that of human MC2R. Therefore, the hypothesized mechanism for the *X. tropicalis* MC2R can be broken down into three major steps. First, the C-terminus region of ACTH(1-24), Zone C, would make contact with its corresponding binding site on the *X. tropicalis* MC2R. In turn, this specific binding induces a conformational change in the receptor, and the second binding site is exposed. Third, Zone A, HFRW, is able to bind to the receptor's second site, and activate the G-protein of the receptor (Baron et al. 2008; Liang, 2013).

The second objective of this study focused on this question: what regions of the amphibian MC2 receptor bind to ACTH? Since we believe that the amphibian MC2R activation is similar to that of the mammalian MC2R, it would be logical to suggest that these receptors would have a similar binding complex model. The theoretical model illustrates the amphibian receptor having two binding pockets like that of the human receptor. The first binding pocket is utilized as a docking pocket for the C-terminal amino acids of ACTH(1-24), Zone C. The second binding pocket is responsible for the binding of the HFRW motif, Zone A, so that activation of the receptor may occur. It has been hypothesized that the second binding pocket consists of the receptors transmembrane regions 4 (TM4) and 5 (TM5), as well as, extracellular loop 2 (EC2). To test this second binding pocket hypothesis, we replaced single amino acids within TM4, TM5, and EC2 regions with alanine, and then stimulated these mutants with ACTH(1-24) to produce a dose response. Additionally, it is important to note that Zone B is utilized as an adapter

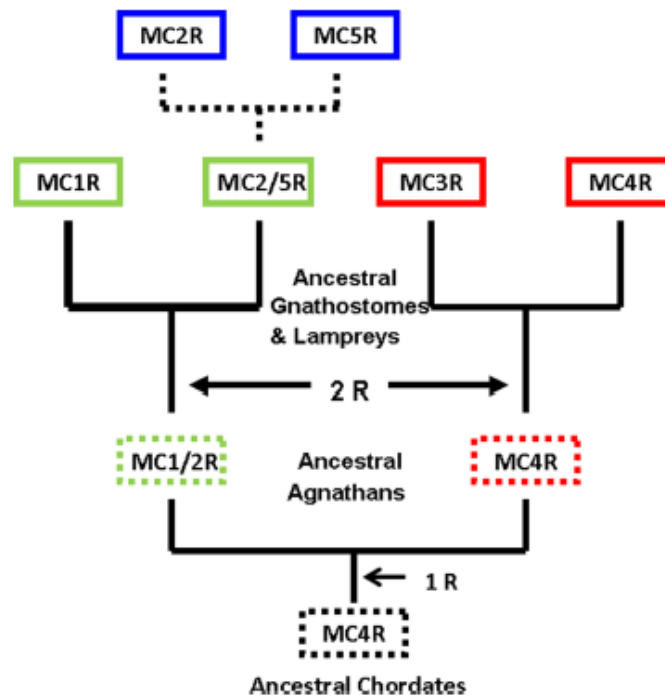


region of ACTH(1-24), and without this sequence of residues Zone C and Zone A would not be properly situated within the binding pockets of the receptor (Liang, 2013).

**Figure 8: “Early” Melanocortin Receptor Phylogenic Tree**



**Figure 9: Revised Melanocortin Receptor Phylogenic Tree**



(Both Figures are adapted from Dores, 2013)

## Materials and Methods

### *Tissue Culture*

Experiments were done utilizing Chinese Hamster Ovary (CHO) cells (ATCC, VA). The cells were grown in Kaighn's Modification of Ham's F12K media supplied by ATCC. Media was supplemented with 10% fetal bovine serum, 10 unit/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml normacin (CHO media) The cells were grown in a 25 cm<sup>3</sup> tissue culture flask with vent cap by CELLTREAT™, and maintained in an incubator with 95% air, 5% CO<sub>2</sub> at 37°C, and exposed to humidity. When the CHO cells reached 70% confluence, cells were split into new culture flasks using 0.05% trypsin/0.53 mM EDTA produced by CORNING cellgro™.

### *DNA Constructs*

The amphibian, *Xenopus tropicalis*, MC2R (xtMC2R; Accession No.: XP\_003215733) cDNA construct was synthesized by GenScript (Piscataway, NJ). This receptor was tagged at the N-terminus with a V-5 epitope, and inserted into a pcDNA3.1+ vector. In addition, the mammalian, *Mus musculus* (mouse), MRAP1 (mMRAP1; Accession No.: NM\_029844) was synthesized by GenScript, tagged at its N-terminus with a FLAG epitope, and inserted into a pcDNA3.1+ vector as well. The cAMP reporter, CRE-Luc (Chepurny and Holz, 2007), was provided by Dr. Patricia Hinkle (University of Rochester, NY). A set of alanine-substituted mutants of the wild

type *Xenopus tropicalis* MC2R was also made by GenScript. Each of these cDNA constructs had a V5 epitope tag and was inserted into the pcDNA3.1+ vector.

Individually, mutations were introduced to the *Xenopus tropicalis* MC2R by site-directed mutagenesis. Single alanine substitutions were made at TM4, EC2, and TM5 are illustrated in Figure 10.

#### *ACTH Analog Peptides*

The melanocortin peptide used in the experiments was human ACTH(1-24), and this synthetic hormone was purchased from Sigma-Aldrich Inc. located in Saint Louis, MO. Additionally, the human ACTH(1-24) analogs include alanine substitutions, as well as, truncated forms of ACTH(1-24) which were made by New England Peptide Inc. in Boston, MA. The amino acid sequence of hACTH(1-24), the analogs, and truncated peptides used in this study can be found in Table 1.

#### *Immunocytochemistry*

For immunocytochemical experiments, the CHO cells were plated in 8-well chamber slides at  $2.5 \times 10^4$  cells per well. After 24 hours, the cells were transfected with 1  $\mu$ g of the cDNA constructs by using Lipfectamine 2000 (Invitrogen, Carlsbad, CA) in an OptiMEM medium (Madiatech Inc., Herndon, VA). Experiments were done on cells transfected with xtMC2R V5 tagged cDNA alone or xtMC2R V5 tagged cDNA and mMRAP1 Flag tagged cDNA. Transfected cells were incubated for 24 hours at 37°C. Next, the transfected CHO cells were fixed with a 4% PFA solution for 15 minutes, and then all wells were washed with a 1xPBS solution. At this stage, half of the wells (4

wells) were permeablized with a 0.3% Triton X-100 for 10 minutes, while the other 4 wells were left in the 1xPBS solution (unpermeablized). The primary antibodies, mouse anti-V5 and the rabbit anti-FLAG, were used to detect the receptor and MRAP respectively. It should be noted that both primary antibodies were diluted to 1:500, and applied to the cells for 1 hour at 37°C. After three washes with the 1xPBS solution, cells were incubated with the respective secondary antibodies, donkey anti-mouse conjugated with Alexa388 and donkey anti-rabbit conjugated with Alexa555, for the receptor and MRAP respectively. These secondary antibodies were applied for 45 minutes at a 1:800 dilution. After another three washes with 1xPBS, chambers were removed from slides. Coverslips were applied to the slides with Vecta-Shield from Vector Laboratories Inc., Burlingame, CA. Additionally, the nuclei were stained with DAPI (blue), and coverslips were sealed with clear nail polish. The slides were imaged using a fluorescent Zeiss Axioplan 2 microscope with a Hamamatsu digital camera. Finally, all slides were observed using a 100x oil immersion objective. The immunocytochemical images were analyzed using Slidebook software ([www.slidebook.com](http://www.slidebook.com)). The negative control for the immunocytochemical staining slides were non-transfected CHO cells that did not include the cDNAs of interest to make sure that the CHO cells did not express receptor or accessory protein endogenously.

*cAMP Reporter Assay (Luciferase Assay)*

In the cAMP Reporter Assay,  $2.5 \times 10^6$  cells/reaction were used (24 wells of a white 96 wells plate = one reaction). It should be noted that 4 reactions could be performed on a white 96 well plate. Cells were co-transfected with 2 $\mu$ g of the following cDNA constructs: xtMC2R, mMRAP1, and CRE-Luciferase (Chepurny & Holz, 2007). Transfections were done utilizing the Amaxa Cell Line Nucleofector II system (Lonza Group, LTD, MD), 100  $\mu$ l Solution T/reaction, and program U-23. After a 10 minute period of recovery in 500  $\mu$ l of CHO media, the transfected cells were diluted in 7.5 mL of CHO media. It should be noted that the 600 $\mu$ L of reaction mixture should be mixed well in the dilution media, so that the mixture is homogenous to plate  $1 \times 10^5$  cells per well (300 $\mu$ L per well). After about 24 hours of the transfection, cells were fed with 300  $\mu$ L of fresh CHO media.

On the third day of the Luciferase Assay, the transfected cells were stimulated with hACTH(1-24) or hACTH(1-24) analogs. Using serum-free CHO Media (does not contain FBS), serial dilutions were carried out with the wild type peptide or the analogs, at concentrations ranging from  $10^{-6}$  to  $10^{12}$  M. In addition, each dose was tested in triplicate. Then, the stimulated plate was incubated at 37°C incubator for 4 hours. After the 4 hour stimulation period, the plate was allowed to cool to room temperature, and the stimulation solution was removed.. Next, a 1:1 ratio of serum free CHO media and Luciferase substrate reagent Bright GLO (Promega, WI) was gently mixed in a 15 mL conical tube. The Luciferase substrate solution was applied to each well (100  $\mu$ L/well),

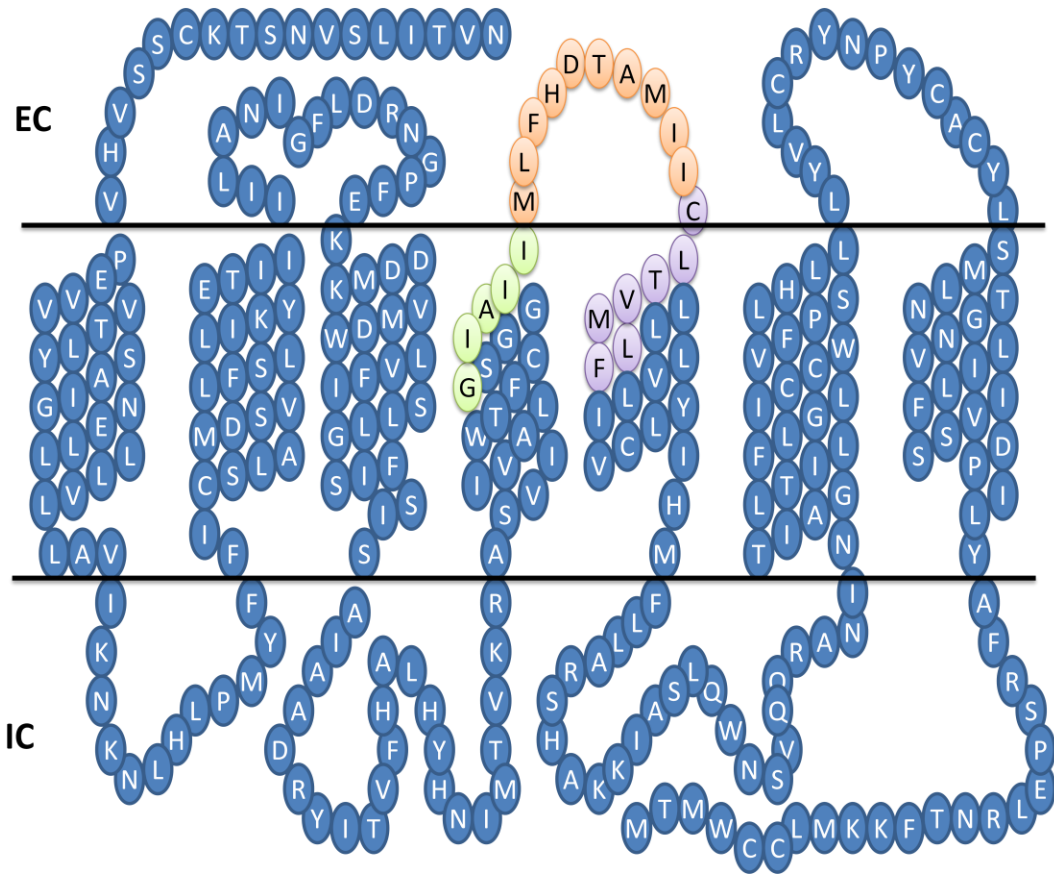
and allowed to incubate at room temperature for 5 minutes. Finally, luminescence of each well was measured using the Bio-Tek Synergy HT plate reader (Winooski, VT).

Additionally, basal levels of cAMP production needed to be determined to produce an accurate activation curve. Therefore, a negative control was included in each assay where the transfected CHO cells were not stimulated with wild type ACTH(1-24) or the analog peptides used in the experiment. The negative control was subtracted from each data point, and the corrected data points for each dose response curve were fit to the Michaelis-Menton equation to produce an EC<sub>50</sub> value for each activation curve. These activation curves were analyzed by using Kaleidograph software ([www.syngery.com](http://www.syngery.com)).

#### *Statistical Analysis*

Data points were calculated as a mean with standard error values that were obtained from experiments performed in a triplicate. To determine statistical significance between experimental treatments and their corresponding controls, an unpaired two-tailed Student's t-test for equal variance was calculated; significance was set at  $P \leq 0.05$ .

**Figure 10: Two Dimensional Structure of *Xenopus tropicalis* MC2R**



- = normal amino acid sequence of xt.MC2R
- = TM4 alanine substitution sites
- = EC2 alanine substitution sites
- = TM5 alanine substitution sites

**Table 1: Human ACTH(1-24) Wild Type Peptide and Analogs.** The alanine substitutions that have replaced amino acids in the peptide are underlined.

| ACTH Analogs |             | Amino Acid Sequences                       |
|--------------|-------------|--|
| Wild Type    | ACTH (1-24) | SYSMEHFRWGKPVGKKRRPVKVYP                   |
| Zone A       | A4/HFRW     | SYSME <u>AAA</u> AGKPVGKKRRPVKVYP          |
|              | HFAW        | SYSMEHF <u>A</u> WGKPVGKKRRPVKVYP          |
|              | HARW        | SYSMEH <u>A</u> RWGKPVGKKRRPVKVYP          |
|              | AFRW        | SYSME <u>A</u> FRWGKPVGKKRRPVKVYP          |
|              | HFRA        | SYSMEHF <u>R</u> AGKPVGKKRRPVKVYP          |
| Zone B       | A10-14      | SYSMEHFRW <u>AAAA</u> AKRRPVKVYP           |
|              | A10/14      | SYSMEHFRW <u>A</u> KPV <u>A</u> KKRRPVKVYP |
|              | KP          | SYSMEHFRW <u>GAA</u> VGKKRRPVKVYP          |
|              | P12         | SYSMEHFRWG <u>KAV</u> GKKRRPVKVYP          |
|              | ACTH (1-22) | SYSMEHFRW-KPV-KRRPVKVYP                    |
|              | ACTH (1-21) | SYSMEHFRWG---GKKRRPVKVYP                   |
| Zone C       | A5/Tetra    | SYSMEHFRWGKPVG <u>AAAA</u> AVKVYP          |
|              | AARRP       | SYSMEHFRWGKPVG <u>AARR</u> PVKVYP          |
|              | KKAAA       | SYSMEHFRWGKPVGK <u>KAAA</u> VKVYP          |
|              | AAW         | SYSMEHFRWGKPVGK <u>RAAW</u> VKVYP          |



## Results

### *Immunocytochemistry of the *Xenopus tropicalis* MC2 Receptor and Mouse MRAP1*

Several studies had shown that functional expression of mammalian MC2Rs in heterologous mammalian cells, such as CHO cells, required co-expression with the accessory protein, MRAP1 (Webb and Clark, 2010, Hinkle and Sebag, 2010). Would this same restriction apply to other tetrapod MC2Rs? To initially address this question, an immunofluorescence approach was used to determine whether a V5-epitope tagged xtMC2R cDNA construct also required MRAP1 for trafficking to the plasma membrane. As a control, non-transfected CHO cells were reacted with the V5 primary antiserum and the V5 secondary antiserum (Figure 11A), and the cells were left non-permeabilized. Note that no reaction was observed. This outcome indicated that the V5 antiserum did not react with any polypeptides on the surface of non-transfected cells. In the next experiment (Figure 11B), CHO cells were transfected with the V5-tagged xtMC2R cDNA construct alone, and the immunofluorescence reaction was once again done on non-permeabilized cells. No reaction was observed. This outcome could indicate a problem with the expression of the xtMC2R cDNA construct, hence the experiment was repeated, but the transfected cells were permeabilized (Figure 11C). Note the intense reaction in the cytoplasm of the transfected cells. Collectively, Figures 11B and 11C

indicate that the CHO cells could express the V5-tagged xtMC2R cDNA construct, but the receptor was not moving to the plasma membrane of the transfected cells.

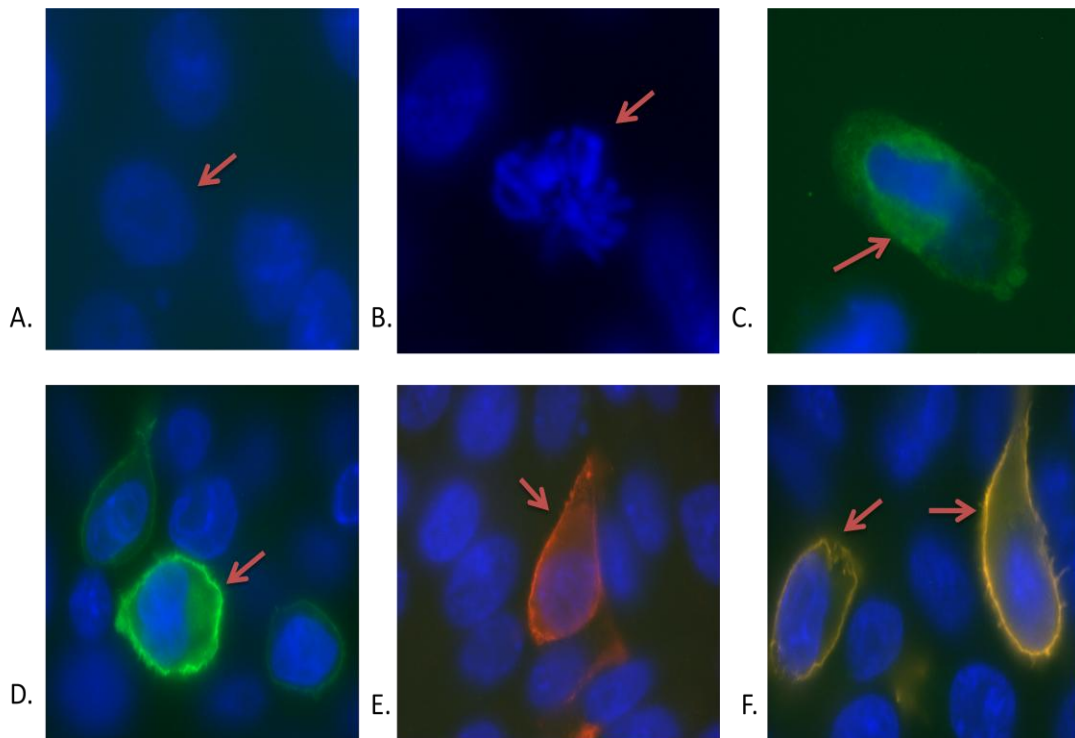
In the next series of experiments (Figure 11D, E, F), CHO cells were co-transfected with a V5-tagged xtMC2R cDNA construct and a Flag-tagged mouse MRAP1 (mMRAP1) cDNA construct. In these experiments the immunofluorescence reaction was performed on non-permeabilized cells. Figure 11D indicates that the V5-tagged xtMC2R can be clearly detected on the plasma membrane. Figure 11E indicates that the Flag-tagged mMRAP1 could also be detected on the plasma membrane. Figure 11F shows the immunofluorescence images merged, which is an indication that xtMC2R and mMRAP1 are in close proximity on the plasma membrane. Collectively, these experiments provided the first evidence that an amphibian MC2R requires the presence of an MRAP1 to facilitate trafficking to the plasma membrane. As a result, in all subsequent experiments the xtMC2R cDNA construct was co-expressed with mMRAP1. Mouse MRAP1 was used for functional assay experiments due to the fact that a *X. tropicalis* MRAP1 ortholog had not been detected in the *X. tropicalis* genome project (web site for xt genome project). The operating assumption was that tetrapod MC2Rs should be able to interact with tetrapod MRAP1s. The following experiments supported this assumption.

The immunocytochemistry images (Figure 11) showed that the xtMC2R required co-expression with an MRAP to facilitate trafficking to the plasma membrane. The next question to address was whether xtMC2R could be functionally expressed in CHO cells; that is, was it possible to show cAMP production when xtMC2R was co-expressed with

mMRAP1? The CHO cell system was also used to determine whether MRAP2, a paralog of MRAP1 had any effect on the activation of xtMC2R. These experiments utilized a cAMP reporter gene (CRE/Luciferase construct; see Methods) to measure the amount of cAMP produced following stimulation with human ACTH(1-24). The rationale for using hACTH(1-24) was presented in the Introduction.

**Figure 11: *Xenopus Tropicalis* MC2R and Mouse MRAP1 Immunocytochemistry**

**Images**



Immunocytochemical analysis of the *Xenopus tropicalis* MC2 receptor. For these experiments the xtMC2R receptor was tagged with a V-5 epitope and the mMRAP1 was tagged with a FLAG epitope. In all of the experiments nuclei were stained with DAPI. Permeabilized cells were pretreated with 0.3% Triton X-100 prior to application of the

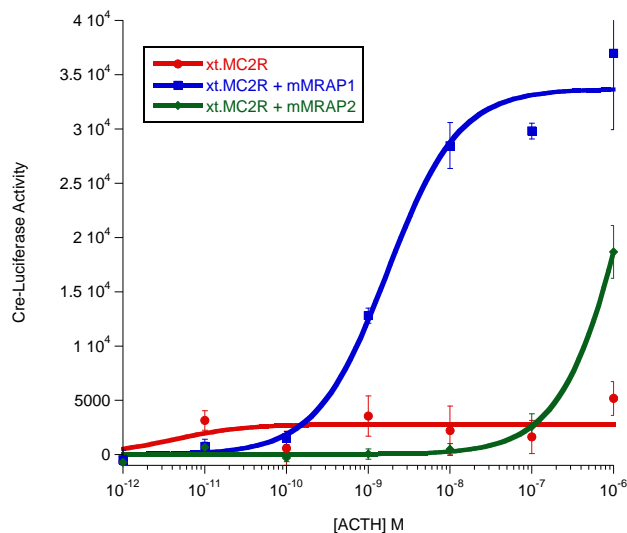
V5 primary antiserum. A) Non-permeabilized, non-transfected CHO cells were reacted with the V5 antiserum. The arrow points to a DAPI stained nucleus. B) Non-permeabilized CHO were transfected with xtMC2R cDNA construct only. The arrow points to a DAPI stained nucleus. C) Permeabilized CHO cells were transfected with the xtMC2R cDNA construct only. The arrow points to fluorescence detected in the ER/Golgi complex. D) Non-permeabilized CHO cells were co-transfected with the xtMC2R cDNA construct and mMRAP1 cDNA construct. This section was only reacted with the V5 antiserum. The arrow points to FITC fluorescence on the surface of the transfected cells. E) The same sections in “D” were reacted with Flag antiserum. The arrow points to CY3 fluorescence on the surface of the transfected cells. F) Images from “D” and “E” were merged and the co-localization of xtMC2R and mMRAP1 can be visualized as a neon-orange color on the cell membrane (arrow).

#### *Functional Expression of xtMC2R: Interactions with Mouse MRAP1 & 2*

Figure 12 represents a collection of xtMC2R activation curves following stimulation with hACTH(1-24). When xtMC2R was expressed alone (red circles) there was no evidence of a dose dependent increase in cAMP following stimulation with hACTH(1-24). However, when xtMC2R and mMRAP1 were co-expressed, stimulation with hACTH(1-24) resulted in a dose dependent increase in cAMP production (blue squares). The  $EC_{50}$  for this dose response curve was  $1.7 \times 10^{-9}$  M; a value very similar to the  $EC_{50}$  value reported by Liang et al. (2011).

Although an ortholog of MRAP1 has not been found in the *X. tropicalis* genome, an ortholog of MRAP2 has been found in this genome. Prior studies have shown that co-expression of mammalian MC2Rs with mammalian MRAP2 resulted in a weak response when stimulated with ACTH(1-24) (Webb and Clark, 2010, Hinkle and Sebag, 2010). It seemed worthwhile to repeat this experiment using xtMC2R. As shown in Figure 12 (green diamonds), co-expression of xtMC2R with mouse MRAP2 resulted in a weak stimulation of the transfected cells only at a concentration of  $10^{-6}$ M. These results are consistent with experiments done using mammalian MC2Rs (Hinkle and Sebag, 2010) and experiments done by Liang et al. (2011) using *X. tropicalis* MRAP2. Collectively these experiments indicate that tetrapod MRAP2s do not facilitate the activation of tetrapod MC2Rs.

**Figure 12: xtMC2R Co-Transfected with Mouse MRAP1 & 2**

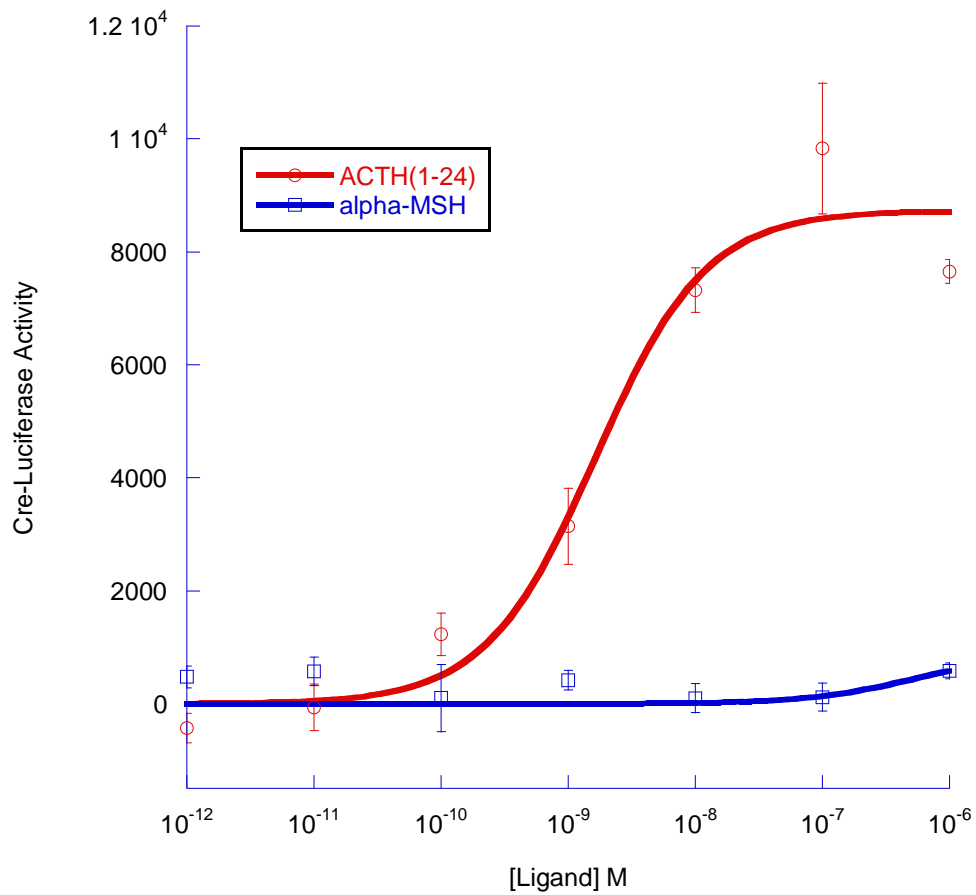


This figure represents a collection of xtMC2R activation curves following stimulation with hACTH(1-24); concentrations ranged from  $10^{-6}$ - $10^{-12}$  M. CRE/Luciferase activity was measured as reported in Methods. The red circles in the response of xtMC2R expressed alone. Co-expression of xtMC2R and mMRAP1 (blue squares) resulted in a dose dependent increase in activation curve ( $EC_{50}$  of  $1.7 \times 10^{-9}$  M). However, co-expression of xtMC2R and mMRAP2 (green diamonds) only showed a minimal response at a ligand concentration of ( $10^{-6}$ M). N = 3 for all experiments.

#### *xtMC2R Stimulation with Human ACTH(1-24) or NDP-MSH*

Previous studies had shown that mammalian MC2Rs could be activated by mammalian ACTH(1-24) , but not by mammalian  $\alpha$ -MSH (Schwyzer, 1977). This outcome is puzzling given that both ACTH(1-24) and  $\alpha$ -MSH have the HFRW motif (see Introduction). These experiments were conducted to determine whether xtMC2R was also exclusively selective for ACTH, but not  $\alpha$ -MSH. In Figure 13, xtMC2R was stimulated with NDP-MSH, an analog of  $\alpha$ -MSH that is more potent than the native hormone. In this experiment, the positive control was stimulation with hACTH(1-24) (red circles;  $EC_{50}$  of  $7.23 \times 10^{-9}$  M). On the other hand, stimulation with NDP-MSH ( $\alpha$ -MSH) resulted in no activation (blue squares). Based on these observations, it would appear that all tetrapod MC2Rs are exclusively selective for ACTH, but not  $\alpha$ -MSH.

**Figure 13: Wild Type *Xenopus tropicalis* MC2R Stimulated with hACTH(1-24) or  $\alpha$ -MSH**



Stimulation of xtMC2R with hACTH(1-24) (red circles) or NDP-MSH (blue square) at concentrations ranging from  $10^{-6}$ - $10^{-12}$  M. Following hACTH(1-24) stimulation the  $EC_{50}$  value was  $7.2 \times 10^{-9}$  M. N = 3.

### *XtMC2R: Human ACTH(1-24) Analog Studies*

Prior to the characterization of the melanocortin-2 receptor as the “ACTH” receptor on mammalian adrenal cortex cells (Mountjoy et al., 1992), a considerable number of studies had been done on the structure/function relationship between mammalian ACTH and the “ACTH” adrenal cortex receptor (i.e., melanocortin-2 receptor). As noted previously, studies done in the 1970’s had established that the first twenty-four residues of mammalian ACTH(1-39) had full biological activity (Schwyzer, 1977). In addition, two domains in ACTH(1-24) were required for activation of the ACTH receptor (MC2R); the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif (Eberle and Schwyzer, 1975; Schwyzer, 1977) and the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif (Schwyzer, 1977; Costa et al., 2004; Liang et al., 2011). Finally, several studies had demonstrated that  $\alpha$ -MSH [NAc-ACTH(1-13)NH<sub>2</sub>] cannot activate the “ACTH” receptor on mammalian adrenal cortex cells (Schwyzer, 1977; Buckley and Ramachandran, 1981; Mountjoy et al., 1992).

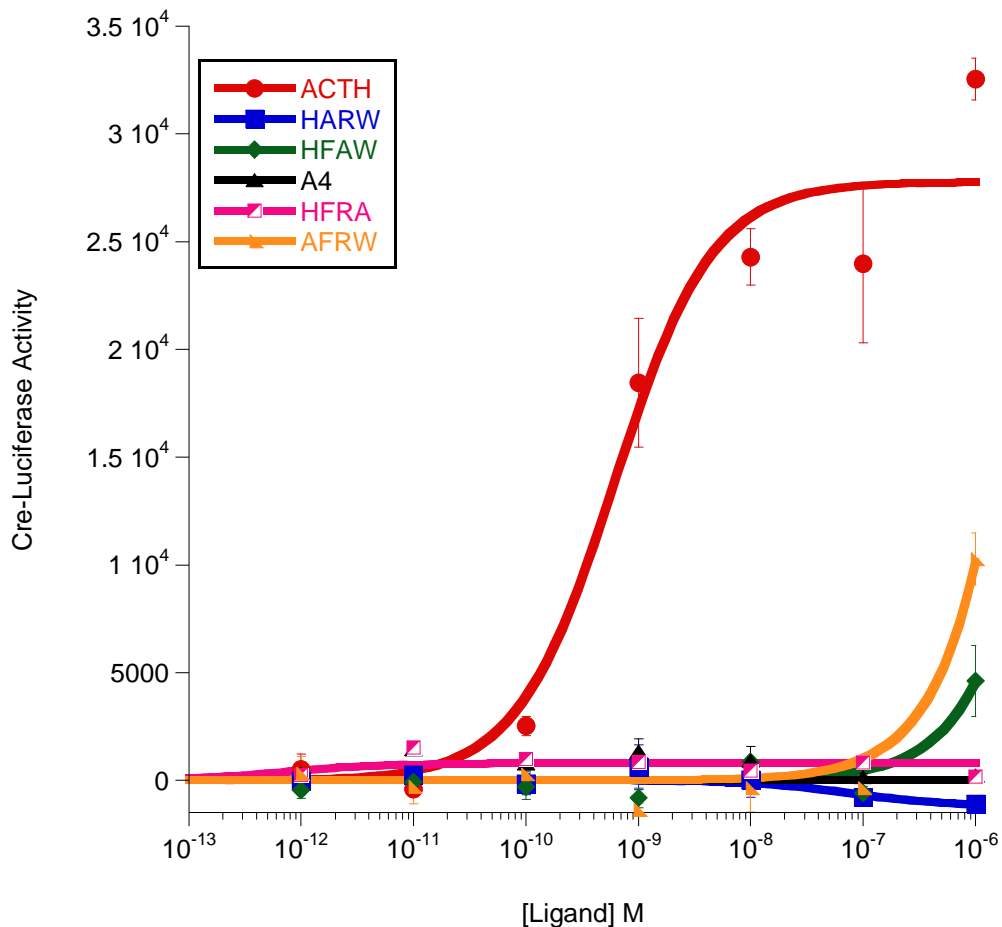
More recently, the HFRW and KKRRP motifs in hACTH(1-24) were examined in greater detail (Liang et al., 2013). In this study, hACTH(1-24) was divided into three functional zones: A – H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup>; B – G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup>; C - K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> and single alanine or multiple alanine analogs of hACTH(1-24) were synthesized (Table 1 in Methods). The ability of these analogs to stimulate xtMC2R was tested by co-transfecting CHO cells with xtMC2R and mMRAP1. Activation was measured using the CRE/Luciferase cAMP reporter Assay. The positive control for these analog activation studies was stimulation of xtMC2R by human ACTH(1-24).



#### *A) ACTH(1-24) Zone A Analogs: cAMP Assays*

First, Zone A analogs (Table 1) were used to address the question of the relative importance of each amino acid in the HFRW motif with respect to the activation of the receptor. The results of these experiments are presented in Figure 14. The dose response curve for the positive control of this experiment is represented by red circles. As expected, the A4 (AAAA) analog was unable to activate the receptor (black triangle). However, what was not expected was that incubation with either the AFRW, HARW, HRAW, or the HFRA analogs also resulted in no stimulation at ligand concentrations of  $10^{-7}$ M and lower. The AFRW and the HRAW analogs did produce a slight stimulation at the  $10^{-6}$ M concentration. These results are summarized in Table 2. Given the dose response curves presented in Figure 14, it was not possible to calculate  $EC_{50}$  values for any of the Zone A. analogs. It would appear that all of the positions in the HFRW motif are equally crucial for activation of xtMC2R. It should be noted that the Zone A stimulation results for xtMC2R are in sharp contrast to an earlier study on the human MC2 receptor (Liang et al. 2013). The differences in the responses of the two receptors to the Zone A analogs will be evaluated in the Discussion.

**Figure 14: Wild Type *Xenopus tropicalis* MC2R & Human ACTH(1-24) Zone A Analogs**



This figure represents a collection of activation curves that resulted when xtMC2R was incubated with various Zone A analogs of human ACTH(1-24). Dilutions of analogs ranged from 10<sup>-6</sup>-10<sup>-12</sup> M. Activation curves for the wild type hACTH(1-24) (red circles), the AFRW analog (orange right triangles), the HARW analog (blue squares), the HFAW

analog (green diamonds), the HFRA analog (pink squares), and the A4 analog (black triangles) are presented. N = 3.

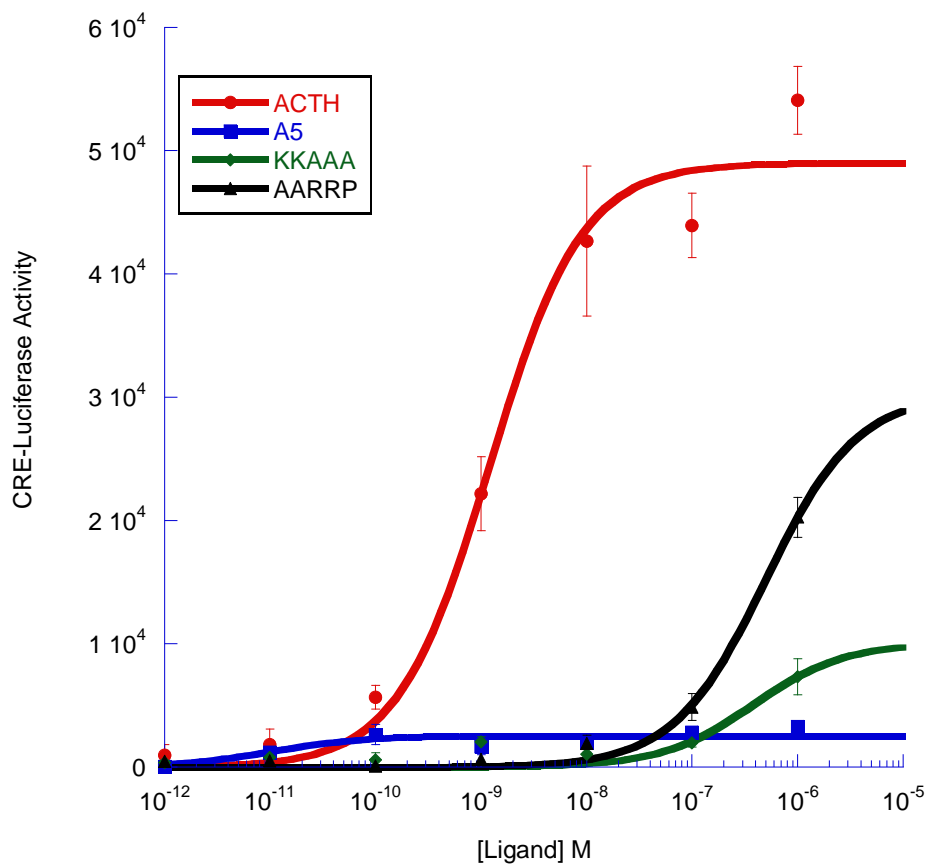
*B) ACTH(1-24) Zone C Analogs: cAMP Assays*

Past studies on the human MC2 receptor have indicated that the binding of KKRRP motif of hACTH(1-24) is a critical first step in the activation of hMC2R (Liang et al., 2013). In order to determine whether this same motif in hACTH(1-24) is also essential for the activation of xtMC2R, three Zone C analogs were analyzed (Figure 15). The positive control for these experiments was xtMC2R stimulated with hACTH(1-24).

The Zone C analogs were: A5 (A<sup>15</sup> A<sup>16</sup> A<sup>17</sup> A<sup>18</sup> A<sup>19</sup>), KKAAA, and AARRP, and the EC<sub>50</sub> values for these analogs are presented in Table 2. As shown in Figure 15, there was no stimulation following incubation of xtMC2R with the A5 analog at any of the concentrations tested (black triangles). In addition, incubation of xtMC2R with either the KKAAA analog or the AARRP analog did not result in any stimulation at analog concentrations of 10<sup>-7</sup>M or less. However, there was minor stimulation at the 10<sup>-6</sup>M concentration. As a result of the weak activation at the highest concentration of ligand it was possible to generate estimates of the EC<sub>50</sub> values for the KKAAA and AARRP experiments. The estimated EC<sub>50</sub> value for the KKAAA analog was 3.7 x 10<sup>-6</sup> M; a 512 fold shift in activation as compared to the positive control (Table 2). The estimated EC<sub>50</sub> value for AARRP analog was 5.0 x 10<sup>-6</sup> M; a 692 fold shift in stimulation as compared to the positive control (Table 2). It would appear that KKRRP motif of ACTH(1-24) is

required for the activation of xtMC2R. The role this motif may play in the activation process will be considered in the Discussion.

**Figure 15: Wild Type *X. tropicalis* MC2R & Human ACTH(1-24) Zone C Analogs**



This figure represents a collection of activation curves that resulted when xtMC2R was incubated with various Zone C analogs of human ACTH(1-24). Dilutions of analogs ranged from 10<sup>-6</sup>-10<sup>-12</sup> M concentrations. For the positive control xtMC2R was stimulated

with hACTH(1-24) (red dots). The results of stimulating with the A5 analog (blue squares), KKAAA analog (green diamonds), and the AARRPP analog (black triangles) are presented. N = 3.

*C) ACTH(1-24) Zone B Analogs: cAMP Assays*

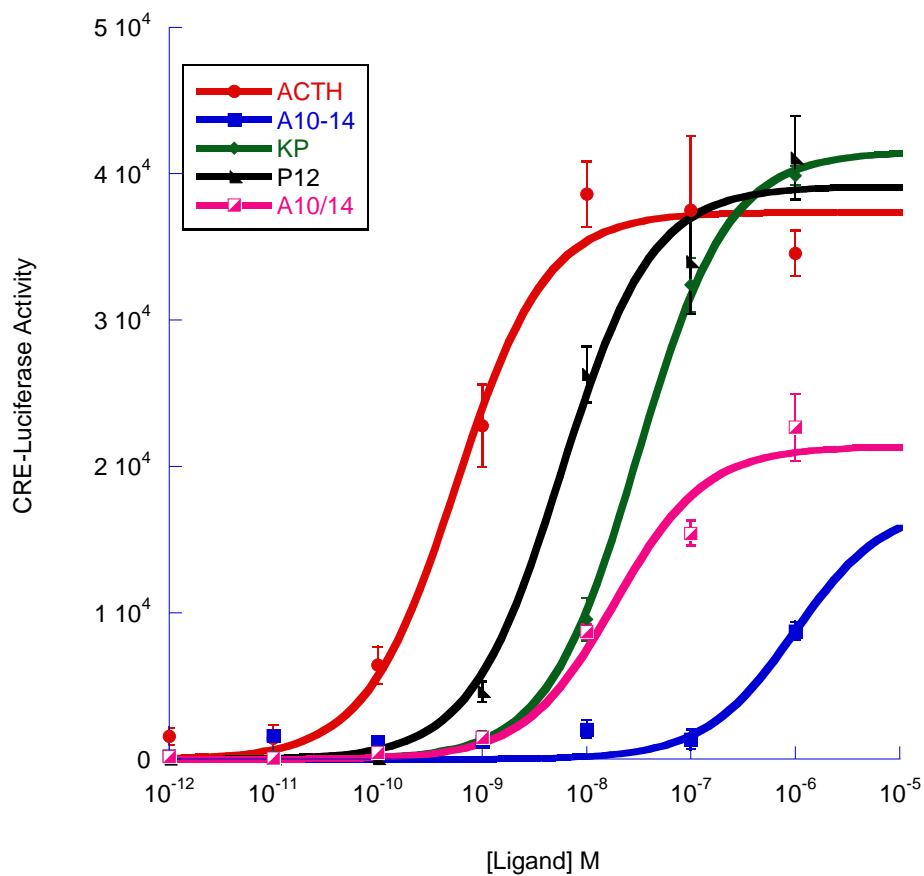
While it was expected that both Zones A and C in hACTH(1-24) have roles in the activation of xtMC2R, it seemed appropriate to determine whether the Zone B motif (GKPVG) also has a role in the activation process. The GKPVG motif in ACTH(1-24) is highly conserved among the gnathostome vertebrates, and only a single amino acid difference (M<sup>13</sup> for V<sup>13</sup>) has been observed in gnathostomes ranging from the cartilaginous fishes to mammals (Dores and Baron, 2011). A recent study on human MC2R indicated that the Zone B motif may play a role in the positioning of the HFRW and KKRRP motifs of ACTH(1-24) into their proposed binding sites on the receptor (Liang et al, 2013). That study indicated that while single or double alanine substitutions in the Zone B motif had no effect on activation, replacement of all residues in this motif with alanines had a significant effect on activation (Liang et al, 2013). The following experiments were done to determine if xtMC2R responded in a similar manner to the Zone B alanine analogs.

Figure 16 presents a collection of activation curves that evaluated the effects of Zone B analogs on the stimulation of xtMC2R. For the positive control for this experiment (red circles), xtMC2R was stimulated with hACTH(1-24). Incubation with the A10-14 (AAAAA; blue squares), did not result in any stimulation of the receptor at

ligand concentrations tested at  $10^{-7}$ M or less. There was slight stimulation at a ligand concentration of  $10^{-6}$ M. Similar results were observed when this analog was used to stimulate human MC2R (Liang et al., 2013). Although this analog shows some cAMP production, the response proved to be too weak, and therefore, no  $EC_{50}$  could be calculated for A10-14 (Table 2). For the A10/14 analog the two glycines at

**Figure 16: Wild Type *Xenopus tropicalis* MC2R & Human ACTH(1-24) Zone B**

**Analogs**



This figure represents a collection of activation curves based on cAMP production due to the stimulation of xt.MC2R with Zone B analogs of human ACTH(1-24). Dilutions of analogs ranged from  $10^{-6}$ - $10^{-12}$  M. The positive control, xtMC2R, was stimulated with hACTH(1-24) (red dots). The results of stimulating with A10-14 analog (blue squares),

the KP (green diamonds), the P12 (black right-triangles), and the A10/14 analog (pink squares) are shown, N = 3.

positions 10 and 14 were each replaced with an alanine residue (Table 1). While a dose response curve was generated by this analog (pink-white squares), there was a significant decrease in cAMP production relative to the positive control ( $p = 0.01$ ), and a 25 fold change in the  $EC_{50}$  value ( $1.8 \times 10^{-7}$  M;  $p < 0.02$ ) relative to the positive control (Table 2).

For the KP analog, alanine substitutions were done at K<sup>11</sup> and P<sup>12</sup> (green diamonds). The KP analog generated a dose response curve with an  $EC_{50}$  value of  $3.1 \times 10^{-7}$  M (Table 2). This is a 43 fold shift in sensitivity for the ligand (Table 2). The t-test for this  $EC_{50}$  value relative to the control was  $p < 0.00003$  (Table 2). Therefore, these results indicate that K<sup>11</sup> and P<sup>12</sup> play a role in the activation of the receptor.

The P12 analog (alanine substitution at position 12) generated a dose response curve (black right-triangles) with an  $EC_{50}$  value of  $5.6 \times 10^{-8}$  M (Table 2). This is an 8-fold shift in  $EC_{50}$  value relative to the positive control, and this shift was statistically significant ( $p < 0.007$ ). Overall, the hierarchy of crucial amino acid positions at Zone B for activation of xtMC2R based on  $EC_{50}$  values was : A10-14>A10/14>KP>P.

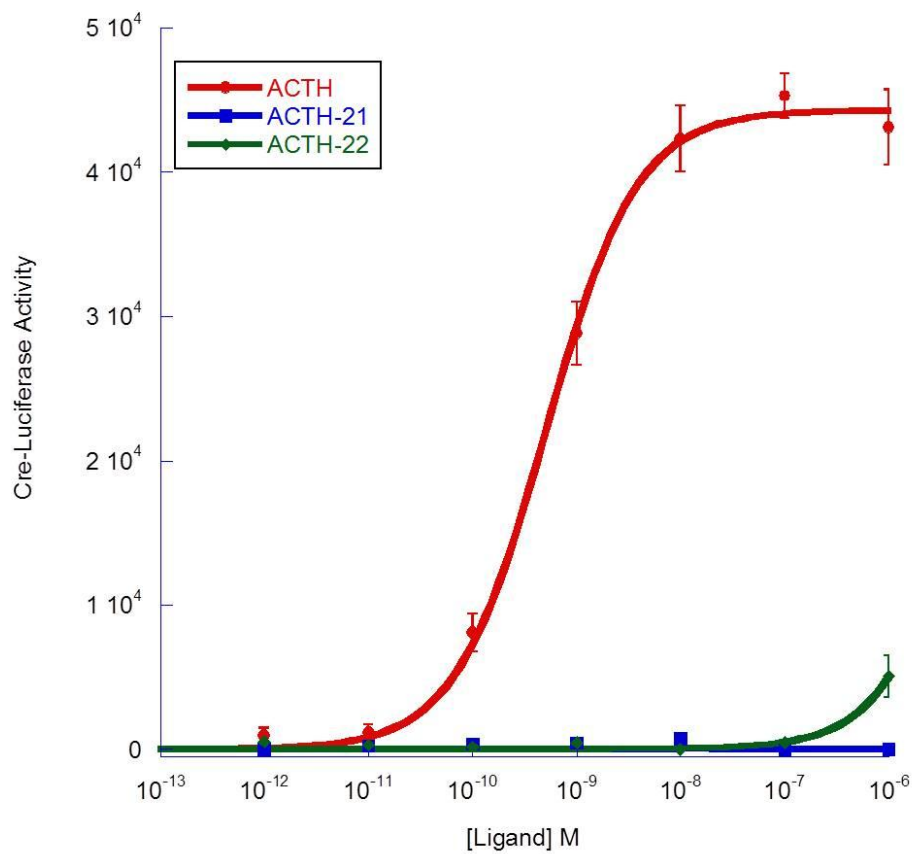
#### *D) ACTH(1-24) Zone B Truncated Analogs: cAMP Assays*

The working hypothesis is that the Zone B motif is required for the proper positioning of the HFRW motif and the KKRRP motif of ACTH(1-24) into



corresponding binding sites on the receptor. The next experiments asked the question of whether analogs of ACTH(1-24) shortened in the Zone B motif can stimulate the receptor. The analogs, ACTH(1-21) and ACTH(1-22), were made to address this question.

**Figure 17: Wild Type *Xenopus tropicalis* MC2R & Human ACTH Truncated Analogs**



This figure represents a collection of activation curves based on cAMP production due to the stimulation of xt.MC2R with Zone B analogs of human ACTH(1-24). Dilutions of analogs ranged from  $10^{-6}$ - $10^{-12}$  M. The positive control, xtMC2R was stimulated with hACTH(1-24) (red dots). The results of stimulating with ACTH(1-21) analog (blue squares) and ACTH(1-22) analog (green diamonds) are shown, N = 3.

The first analog, ACTH(1-21) was shortened by removing amino acid positions 11, 12, and 13 (Lysine-Proline-Valine) from Zone B (Table 2). The second analog, ACTH(1-22), was shortened by removing amino acid positions 10 and 14, which were glycine residues at positions 10 and 14 (Table 2). Figure 17 presents the results of stimulating xtMC2R with these truncated analogs. As indicated in the figure neither analog was able to stimulate the receptor at ligand concentrations ranging from  $10^{-12}$ M to  $10^{-6}$ M (Table 2). Clearly, the length of ACTH(1-24) plays a role in the activation of the receptor.

The results of the alanine analog studies have been used to create a proposed mechanism for the activation of xtMC2R. This model will be presented in the Discussion section.

**Table 2: Human ACTH(1-24) Analog EC<sub>50</sub> and P Values**

| Analogs    | Amino Acid Sequence   | EC <sub>50</sub> ± SEM<br>(10 <sup>-9</sup> M) | Fold<br>Change | P-Value |
|------------|---|--|----------------|---------|
| ACTH(1-24) | SYSM <b>E</b> HFRWGK <b>P</b> VG <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b>                            | 7.23 ± 3.2                                     | ---            | ---     |
| A4         | SYSM <b>E</b> AAAA <b>G</b> K <b>P</b> VG <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b>                   | NA   | ---            | ---     |
| AFRW       | SYSM <b>E</b> A <b>F</b> RWGK <b>P</b> VG <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b>                   | NA   | ---            | ---     |
| HARW       | SYSM <b>E</b> H <b>A</b> RWGK <b>P</b> VG <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b>                   | NA   | ---            | ---     |
| HFAW       | SYSM <b>E</b> H <b>F</b> A <b>W</b> GK <b>P</b> VG <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b>          | NA   | ---            | ---     |
| HFRA       | SYSM <b>E</b> H <b>F</b> R <b>A</b> G <b>K</b> P <b>V</b> G <b>G</b> KRR <b>P</b> V <b>K</b> V <b>P</b> | NA   | ---            | ---     |
| A10-14     | SYSM <b>E</b> HFRW <b>AAAA</b> AKRR <b>P</b> V <b>K</b> V <b>P</b>                                      | NA   | ---            | ---     |
| AKPVA      | SYSM <b>E</b> HFRW <b>AKPV</b> AKRR <b>P</b> V <b>K</b> V <b>P</b>                                      | 180 ± 61*                                      | 24.8           | 0.02    |
| GAAVG      | SYSM <b>E</b> HFRW <b>AAV</b> GKRR <b>P</b> V <b>K</b> V <b>P</b>                                       | 310 ± 17***                                    | 42.8           | 0.00003 |
| GKAVG      | SYSM <b>E</b> HFRW <b>GKAV</b> GKRR <b>P</b> V <b>K</b> V <b>P</b>                                      | 56 ± 12*                                       | 7.7            | 0.007   |
| ACTH(1-22) | SYSM <b>E</b> HFRW <b>-KPV</b> -KRR <b>P</b> V <b>K</b> V <b>P</b>                                      | NA   | ---            | ---     |
| ACTH(1-21) | SYSM <b>E</b> HFRW <b>---</b> GKRR <b>P</b> V <b>K</b> V <b>P</b>                                       | NA   | ---            | ---     |
| A5         | SYSM <b>E</b> HFRWGK <b>PV</b> AAAA <b>A</b> V <b>K</b> V <b>P</b>                                      | NA   | ---            | ---     |
| AARRP      | SYSM <b>E</b> HFRWGK <b>PV</b> AA <b>RRP</b> V <b>K</b> V <b>P</b>                                      | 3700 ± 3900                                    | 511.7          | 0.2     |
| KKAAA      | SYSM <b>E</b> HFRWGK <b>PV</b> GK <b>AAA</b> V <b>K</b> V <b>P</b>                                      | 5000 ± 1400*                                   | 691.6          | 0.01    |

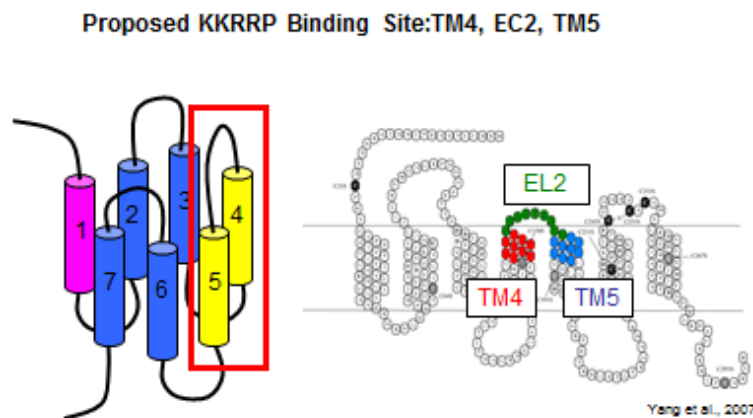
\*\*\*Human ACTH(1-24) Analog Zones = **Zone A**, **Zone B**, **Zone C** This table represents

human ACTH(1-24) analogs in Zones A, B, and C. Each ligand is shown with its alanine substitution(s) at the respective position(s) while the wild type hACTH(1-24) is shown as the first ligand. It should be noted that the EC<sub>50</sub> of the standard curve is a mean of all wild type ACTH(1-24) standard activation curves. Experimental EC<sub>50</sub> values are reported, and were standardized at 10<sup>-9</sup> Molar. If a ligand did not receive a calculated EC<sub>50</sub> value, the ligand was reported as causing no activation (NA). The fold change of ACTH(1-24) analogs was reported for experimental EC<sub>50</sub> values that were found to be of significance. Fold change = (Experimental EC<sub>50</sub> value ÷ Standard EC<sub>50</sub> value). If the analog's EC<sub>50</sub> value was found to be significantly different than the mean positive standard EC<sub>50</sub> value, p-values were reported to suggest statistical significance (\*: p-value < 0.05; \*\*: p-value < 0.005; \*\*\*: p-value < 0.0005).

*xtMC2R Alanine Mutant Studies: TM4, EC2, and TM5 Regions*

As noted, there is general agreement that the HFRW binding site in all melanocortin receptors involved amino positions located close to the surface in TM regions 2, 3, 6, and 7 (Pogosheva et al. 2005; Baron et al. 2008; Dores, 2009; Dores, 2013; Davis et al., 2013). The model presented in Figure 18 shows the relative position of HFRW binding site (TM regions in blue) and served as a reference point for the operating assumption that the KKRRP binding site would involve amino acid positions possibly in TM 4, extracellular loop 2 (EL2) or TM5.

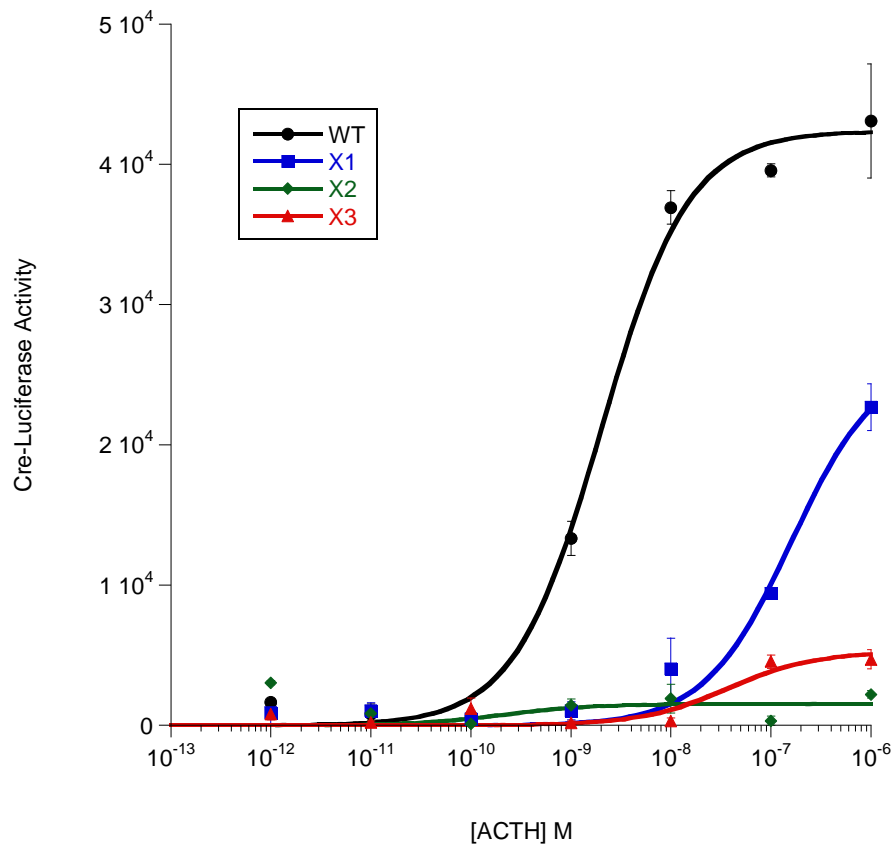
**Figure 18: MC2R: Proposed KKRRP binding pocket**



(Lisa Liang 2013 Ph.D. Thesis) On the “barrel” diagram the blue shaded areas indicate the location of the HFRW binding site, and the yellow shaded areas indicated the proposed location of the KKRRP binding site. In the linear diagram the positions that were targeted for alanine substitution are in color (red – TM2; green EL2; blue TM5).

In the initial experiments, all the amino acid positions in the respective colored regions (Figure 18; linear image of the receptor) were substituted with alanine residues. This resulted in three mutant receptors that were labeled X<sub>1</sub> (TM4 mutant), X<sub>2</sub> (EL2 mutant), and X<sub>3</sub> (TM5 mutant), respectively. These mutant receptors were individually stimulated with hACTH(1-24). In these experiments (Figure 19), the wild type xtMC2R (black circles) was also stimulated with hACTH(1-24) and served as the positive control. Stimulation of the X<sub>1</sub> mutant receptor (TM4 region; blue squares) resulted in a significant decrease in cAMP production. The estimated EC<sub>50</sub> value for this dose response curve was 1.60 x 10<sup>-7</sup>M and resulted in a 168-fold shift in the EC<sub>50</sub> value relative to the positive control (Table 3; p < 0.01). The X<sub>2</sub> mutant (EL2 region; green diamonds) did not show any signs of activation. Therefore, an EC<sub>50</sub> value for this mutant receptor could not be calculated (Table 3). Stimulation of the X<sub>3</sub> mutant receptor (TM5 region; red triangles) resulted in a slight production of cAMP. The X<sub>3</sub> mutant receptor had an estimated EC<sub>50</sub> value of 3.7 x 10<sup>-8</sup> M; a 34-fold shift in EC<sub>50</sub> value as compared to the positive control (Table 2). Although these results were suggestive, the presence of so many alanine residues in each of these mutants could have altered confirmation, and as a result affected trafficking of the mutant receptor to the plasma membrane. Hence, the next approach was to do single alanine substitutions in each targeted region.

**Figure 19: *Xenopus Tropicalis* MC2R TM4, EC2, and TM5 (X<sub>1</sub>, X<sub>2</sub>, and X<sub>3</sub>) Regions with Complete Alanine Substitutions**



This figure represents a collection of mutant xtMC2R activation curves with multiple alanine substitutions in the TM4, EL2, and TM5 regions of the receptor. The TM4, EL2, and TM5 were abbreviated as X<sub>1</sub>, X<sub>2</sub>, and X<sub>3</sub> respectively. The X<sub>1</sub>, X<sub>2</sub>, and X<sub>3</sub> mutant were all stimulated with hACTH(1-24). Dilutions of hormone ranged from 10<sup>-6</sup>-10<sup>-12</sup> M. The wild type xtMC2R is shown in black circles. The X<sub>1</sub> mutant receptor (TM4: blue

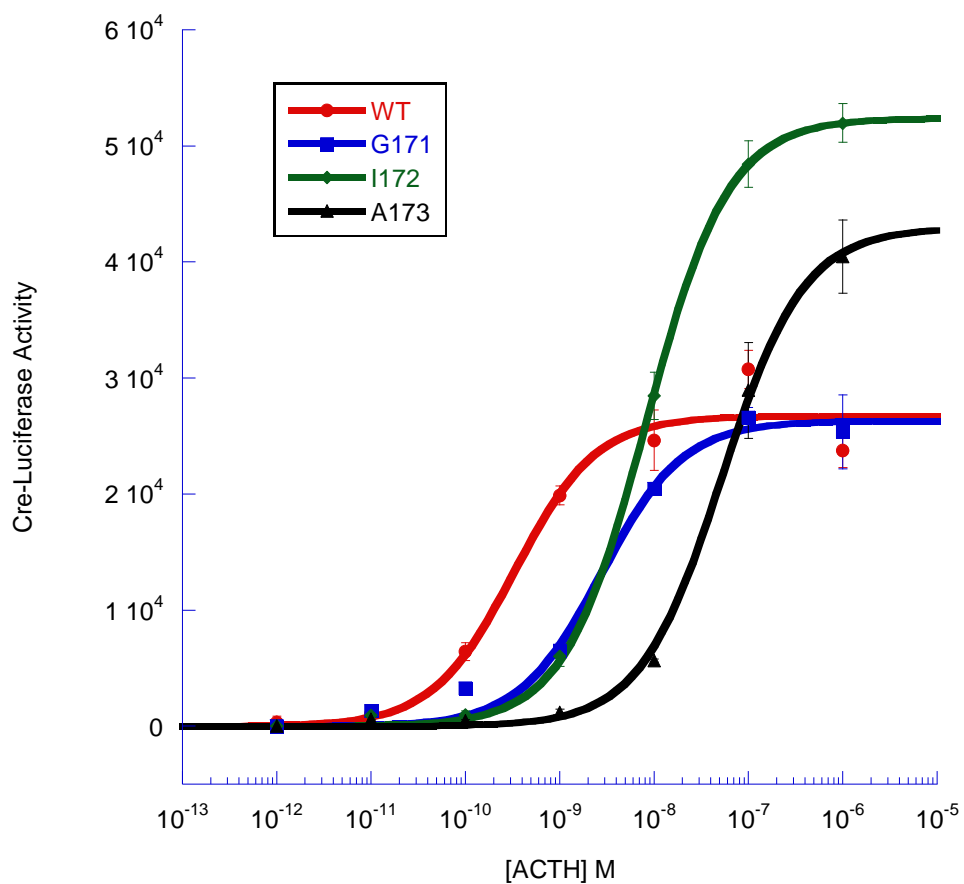
squares) had a decreased response to hormone stimulation, and the estimated EC<sub>50</sub> value for this dose response curve was  $1.6 \times 10^{-8}$  M (Table 3). The X<sub>2</sub> mutant receptor (EC2; green diamonds) did not show any activation. The X<sub>3</sub> mutant receptor (TM5; red triangles) showed a slight activation at  $10^{-7}$  M and  $10^{-6}$  M. The estimated EC<sub>50</sub> value for X<sub>3</sub> mutant receptor was  $3.7 \times 10^{-8}$  M (Table 3).

#### *A) Transmembrane 4 Domain (TM4): Single Alanine Mutant Assays*

The activation dose response curves for the single alanine mutants of the TM4 region are presented in Figures 20 and 21. The EC<sub>50</sub> values and the fold shift in EC<sub>50</sub> values relative to the positive control for these single alanine mutant receptors are presented in Table 3. In Figure 20, the dose response curves for the single alanine mutants at amino acid positions G<sup>171</sup>, I<sup>172</sup>, and A<sup>173</sup> are presented. For the A<sup>173</sup> mutant a glutamine residue was used. All three receptors had EC<sub>50</sub> values that were statistically different from the positive control (Table 3). However, there was only a threefold change for the G/A<sup>171</sup> mutant, and this change is not considered significant. There was a 8 fold change for the I/A<sup>172</sup> and this is a mild, but not dramatic shift in EC<sub>50</sub>. However, the A/Q<sup>173</sup> mutant had a 55 fold shift in EC<sub>50</sub> value, and this outcome was initially unexpected. Alanine substitution is the most common approach used in this type of study because alanine residues are generally considered to be place holder residues. The R-group for this amino does not interact with other amino acid R-groups, hence we assumed this position would not be involved in the receptor activation process. However, by substituting a glutamine residue, an amino acid with a much larger and more reactive R-

group, we suspect that we have interfered with the role that neighboring amino acids may play in the activation process.

**Figure 20: *Xenopus Tropicalis* TM4 Mutant Receptors G171, I172, & A173**



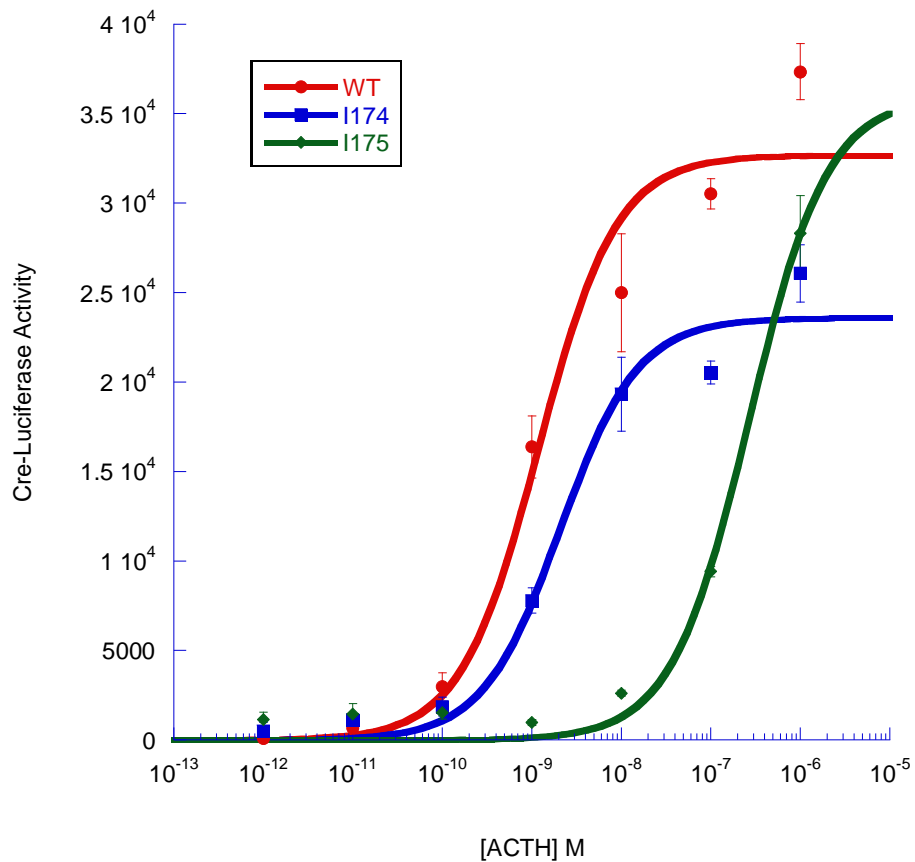
Dose response curves for G<sup>171</sup>, I<sup>172</sup>, and A<sup>173</sup> mutants in the TM4 region of the receptor.

The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown in red circles.



The dose response curves for the G<sup>171</sup> mutant (blue squares), the I<sup>172</sup> mutant (green diamonds), and the A<sup>173</sup> mutant (black triangles) are presented. N = 3.

**Figure 21: *Xenopus Tropicalis* TM4 Mutant Receptors I174 & I175**



Dose response curves for the I<sup>174</sup> and I<sup>175</sup> mutants in the TM4 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown in red circles. The

dose response curves for the I<sup>174</sup> mutant (blue squares) and the I<sup>174</sup> mutant (green diamonds) are presented. N = 3.

In Figure 21 the dose response curves for the single alanine mutants at amino acid positions I<sup>174</sup> and I<sup>175</sup> are presented. The EC<sub>50</sub> values for this mutant receptor are presented in Table 3. The EC<sub>50</sub> value for the I/A<sup>174</sup> was only three fold and not considered significant. However, the I/A<sup>175</sup> mutant had an EC<sub>50</sub> value that resulted in a 284.2 fold shift relative to positive control. This amino acid position is clear important for the activation of xtMC2R. In addition it is possible that the A/Q<sup>173</sup> mutant may have partially interfered with I<sup>174</sup>.

#### *B) Extracellular Loop 2 (EL2): Single Alanine Mutants Assays*

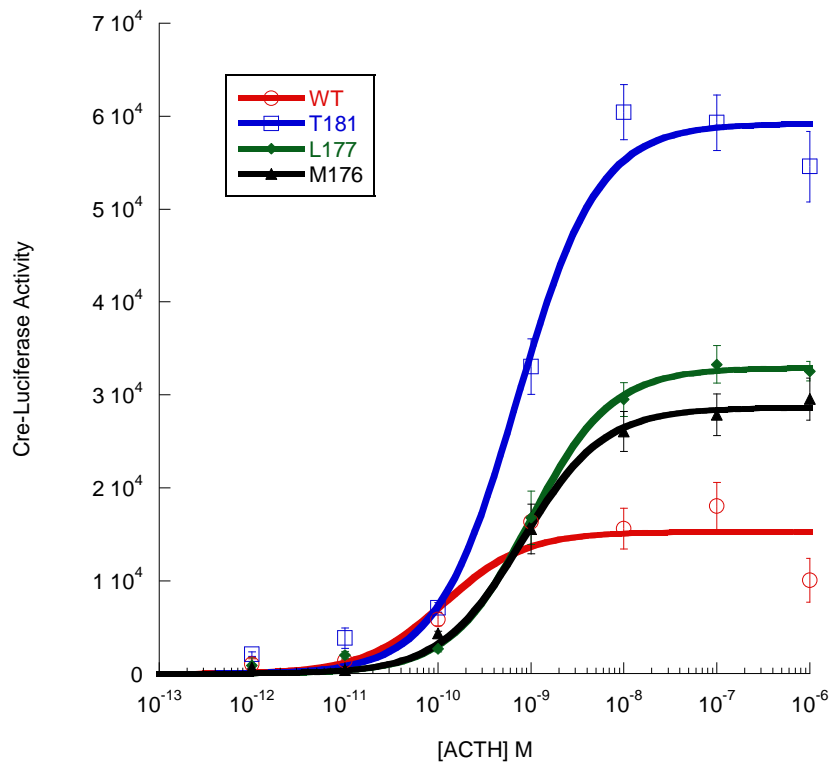
The activation dose response curves for the single alanine mutants of the EL2 region are presented in Figures 22, 23, 24, and 25. The EC<sub>50</sub> values and the fold shift in EC<sub>50</sub> values relative to the positive control for these single alanine mutant receptors are presented in Table 3.

In Figure 22 the dose response curves for the single alanine mutants at amino acid positions M<sup>176</sup>, L<sup>177</sup>, and T<sup>181</sup> are presented. The EC<sub>50</sub> values for the M<sup>176</sup>, L<sup>177</sup>, and T<sup>181</sup> mutants were not significantly different from the positive control (Table 3); hence these positions do not appear to be involved in the activation of the receptor.

In Figure 23 the dose response curves for the single alanine mutants at amino acid positions F<sup>178</sup> and A<sup>182</sup> are present. For the A<sup>182</sup> mutant a glutamine residue replace A<sup>182</sup>. Activation of the F/A<sup>178</sup> mutant was affected as a result of the alanine substitutions, there

was an 83 fold shift in the  $EC_{50}$  value (Table 3) as a result. Not only does the  $EC_{50}$  value indicate receptor insensitivity, the  $V_{max}$  for F/A<sup>178</sup> mutant indicates a significant decrease in cAMP production. In addition, the A/Q<sup>182</sup> mutant also clearly interfered with the

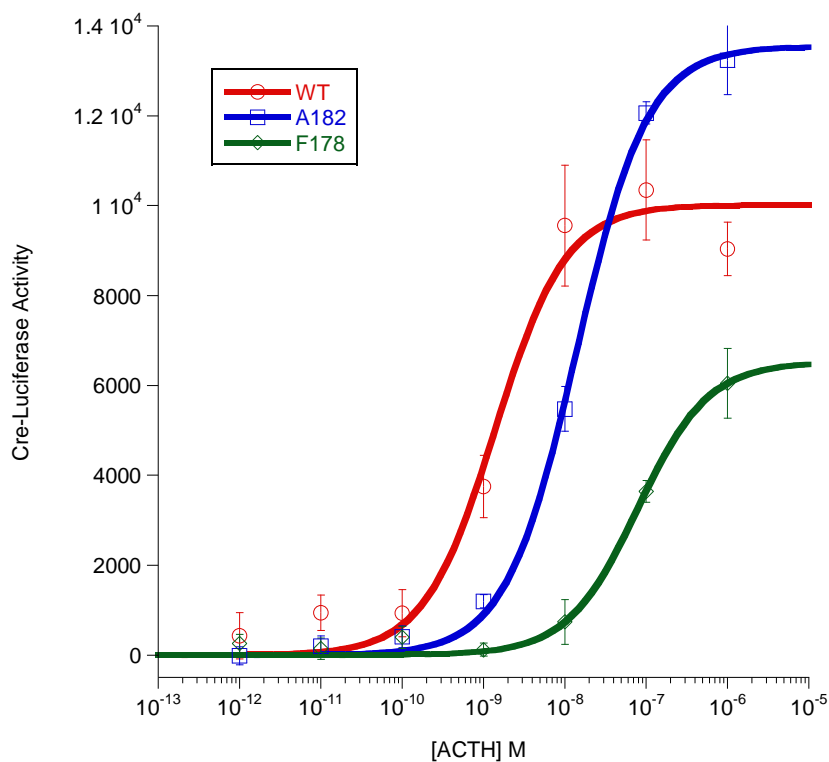
**Figure 22: *Xenopus Tropicalis* EC2 Mutant Receptors T181, L177, and M176**



Dose response curves for the M<sup>176</sup>, L<sup>177</sup>, and T<sup>181</sup> mutants in the EL2 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown

in red circles. The dose response curves for the M<sup>176</sup> mutant (black triangles), L<sup>177</sup> (green diamond), and the T<sup>181</sup> mutant (blue squares) are presented. N = 3.

**Figure 23: *Xenopus Tropicalis* EC2 Mutant Receptors A182 & F178**



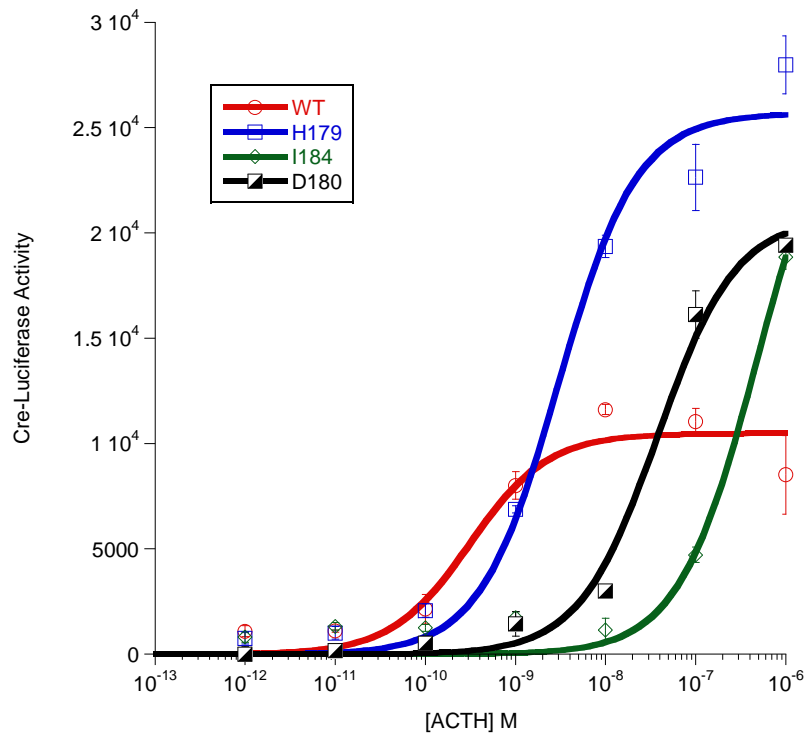
Dose response curves for the F<sup>178</sup> and A<sup>182</sup> mutants in the EL2 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown in red circles. The dose response curves for the F<sup>178</sup> mutant (green triangles) and the A<sup>182</sup> mutant (blue squares) are presented. N = 3.

activation of the receptor. It is possible that substitution at this site may have affected either H<sup>179</sup> or M<sup>183</sup> (see Table 3).

In Figure 24 the dose response curves for the single alanine mutants at amino acid positions H<sup>179</sup>, D<sup>180</sup>, and I<sup>184</sup> are presented. Substitution at H<sup>179</sup> had a minimal impact on the activation of the receptor (Table 3). However, the dose response curve for the D/A<sup>180</sup> mutant resulted in an EC<sub>50</sub> value with a 40 fold shift relative to the positive control (Table 3). The dose response curve for the I/A<sup>184</sup> mutant was even more dramatic. This mutant had an EC<sub>50</sub> value with 495 fold shift relative to the positive control (Table 3). The I<sup>184</sup> position is clearly the most important site for interaction with the KKRRP region of ACTH(1-24).

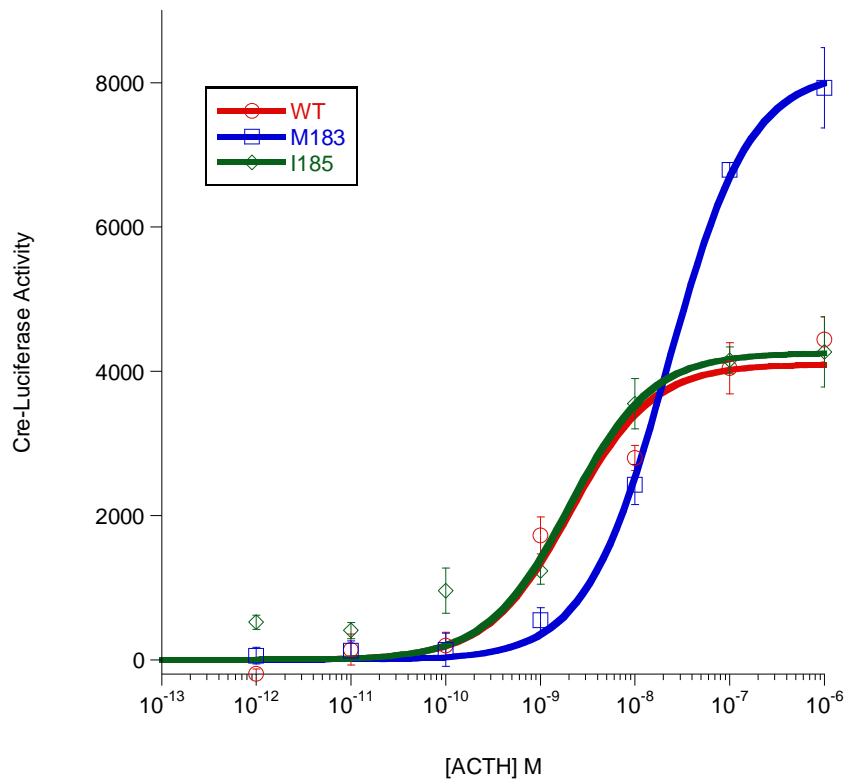
In Figure 25 the dose response curves for the single alanine mutants at amino acid positions M<sup>183</sup> and I<sup>185</sup> are presented. The I/A<sup>185</sup> mutant generated a dose response curve with an EC<sub>50</sub> value that resulted in a 2 fold shift relative to the positive control (Table 3). Substitution at this site does not appear to significantly affect the activation of the receptor. However, the M/A<sup>183</sup> mutant generated a dose response curve with an EC<sub>50</sub> value that resulted in a 22 fold shift relative to the control (Table 1), and this position is considered important for the activation of the receptor.

**Figure 24: *Xenopus Tropicalis* EC2 Mutant Receptors H179, I184, & D180**



Dose response curves for the H<sup>179</sup>, D<sup>180</sup>, and I<sup>184</sup> mutants in the EL2 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from  $10^{-6}$ - $10^{-12}$  M. The dose response curve for the wild type xtMC2R is shown in red circles. The dose response curves for the H<sup>179</sup> mutant (blue squares), D<sup>180</sup> (black squares), and the I<sup>184</sup> mutant (green squares) are presented. N = 3.

**Figure 25: *Xenopus Tropicalis* EC2 Mutant Receptors M183 & I185**



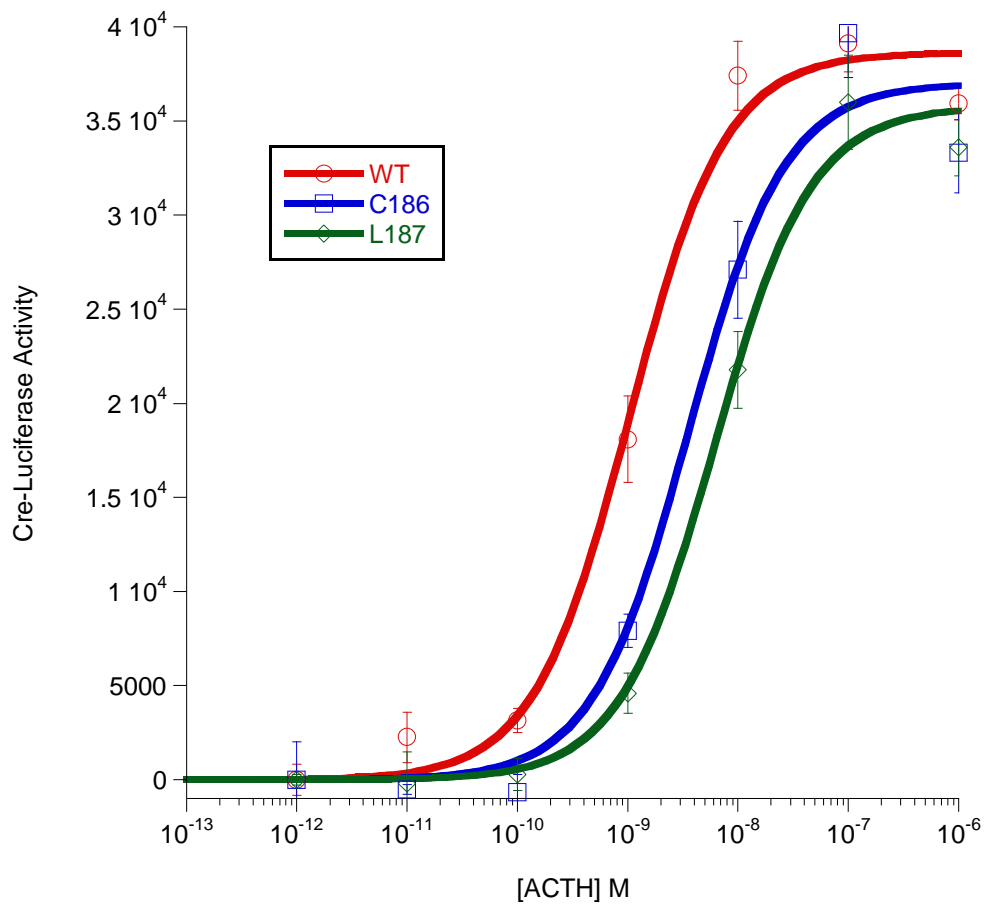
Dose response curves for the M<sup>183</sup> and I<sup>185</sup> mutants in the EL2 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from  $10^{-6}$ - $10^{-12}$  M. The dose response curve for the wild type xtMC2R is shown in red circles. The dose response curves for the M<sup>183</sup> mutant (blue squares) and the I<sup>185</sup> mutant (green diamonds) are presented. N = 3.

*C) Transmembrane 5 Domain: Single Alanine Mutant Assays*

The activation dose response curves for the single alanine mutants of the TM5 region are presented in Figures 26, 27, and 28. The EC<sub>50</sub> values and the fold shift in EC<sub>50</sub> values relative to the positive control for these single alanine mutant receptors are presented in Table 3. In Figure 26 the dose response curves for the C<sup>186</sup> and L<sup>187</sup> mutants are presented. In Figure 27 the dose response curves for the T<sup>188</sup>, V<sup>189</sup>, and M<sup>190</sup> mutants are presented. In Figure 28 the dose response curves for the F<sup>191</sup> and L<sup>192</sup> mutants are presented. As indicated in Table 3, none of these mutants had dose response curves with EC<sub>50</sub> values greater than 4 fold relative to the positive control with the exception of L/A<sup>187</sup> mutant (6.5 fold change). Based on these observations it appears that region of the receptor is not important for the activation of the receptor.



**Figure 26: *Xenopus Tropicalis* TM5 Mutant Receptors C186 & L187**



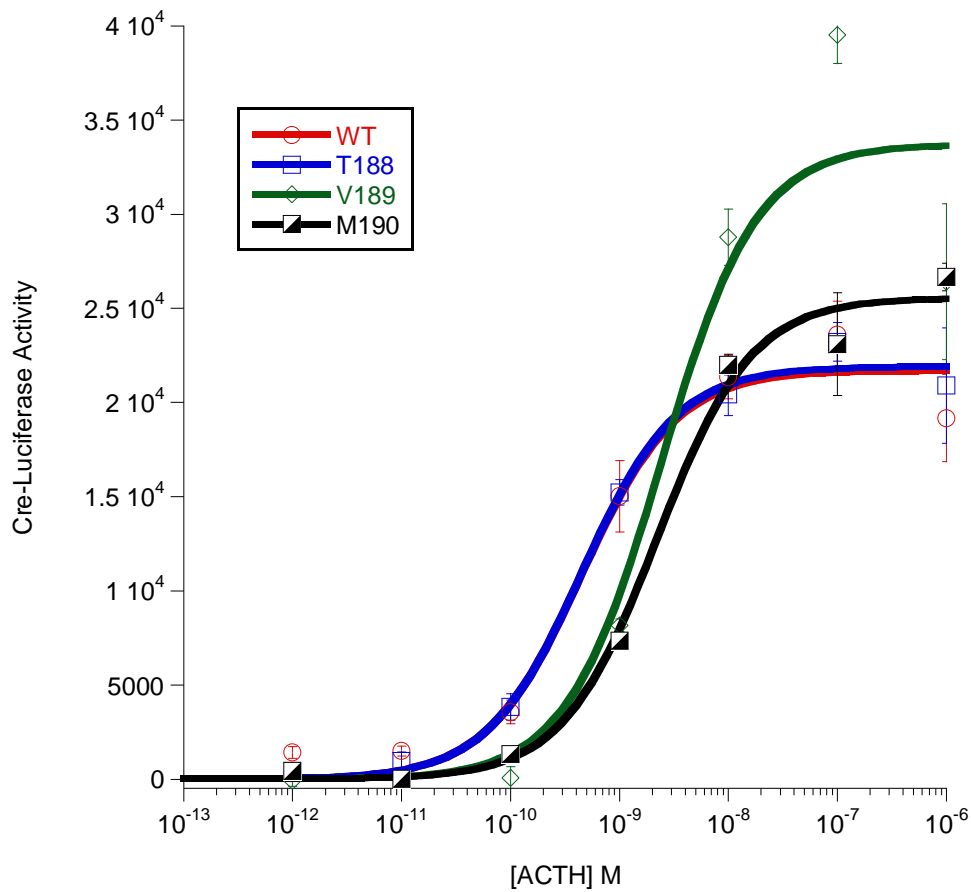
Dose response curves for the C<sup>186</sup> and L<sup>187</sup> mutants in the TM5 region of the receptor.

The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from

$10^{-6}$ - $10^{-12}$  M. The dose response curve for the wild type xtMC2R is shown in red circles.

The dose response curves for the C<sup>186</sup> mutant (blue squares) and the L<sup>187</sup> mutant (green diamonds) are presented. N = 3.

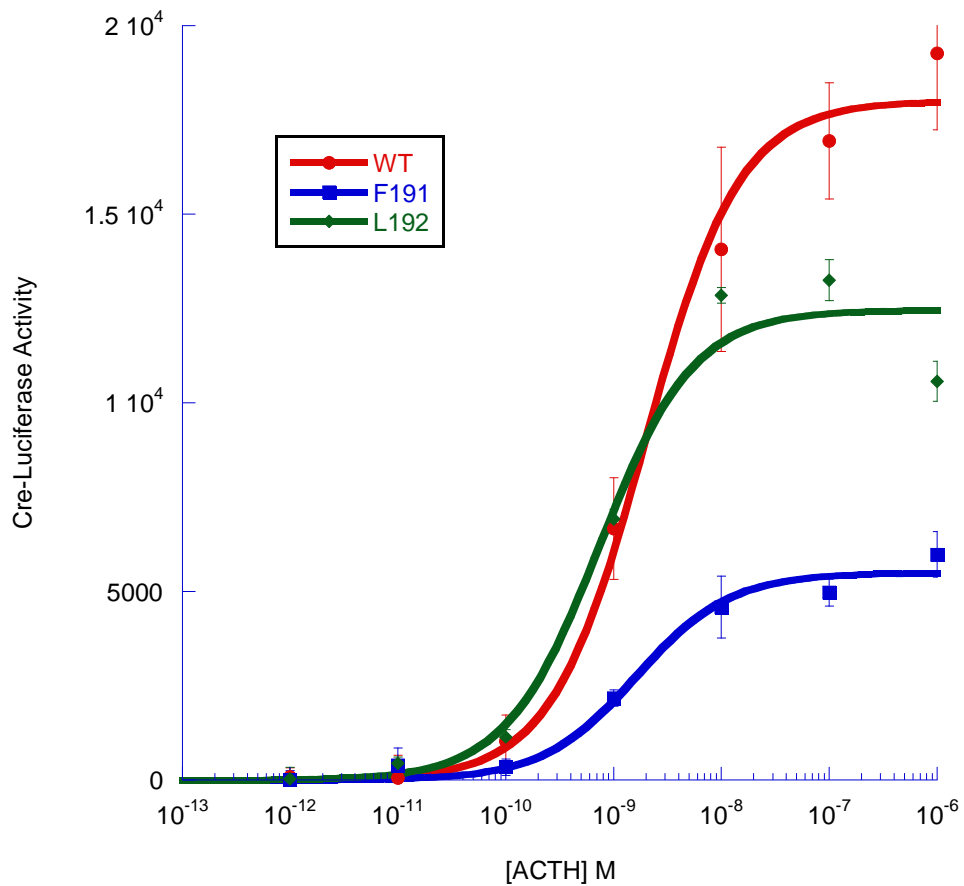
**Figure 27: *Xenopus Tropicalis* TM5 Mutant Receptors T188, V189 & M190**



Dose response curves for the T<sup>188</sup>, V<sup>189</sup>, and M<sup>190</sup> mutants in the TM5 region of the receptor. The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown

in red circles. The dose response curves for the T<sup>188</sup> mutant (blue squares), V<sup>189</sup> mutant (green diamonds), and the M<sup>190</sup> mutant (black squares) are presented. N = 3.

**Figure 28: *Xenopus Tropicalis* TM5 Mutant Receptors F191 & L192**



Dose response curves for the F<sup>191</sup> and L<sup>192</sup> mutants in the TM5 region of the receptor.

The mutant receptors were all stimulated with hACTH(1-24) at dilutions ranging from 10<sup>-6</sup>-10<sup>-12</sup> M. The dose response curve for the wild type xtMC2R is shown in red circles.

The dose response curves for the F<sup>191</sup> mutant (blue squares) and L<sup>192</sup> mutant (green diamonds), are presented. N = 3.

**Table 3: *Xenopus tropicalis* TM4, EC2, and TM5 Mutant Receptor EC<sub>50</sub> Value and****P-Values**

| Amino Acid     | EC <sub>50</sub> (10 <sup>-9</sup> M) | Fold Change | P-Value |
|----------------|---------------------------------------|-------------|---------|
| Wild Type      | 0.95 ± 0.44                           | ---         | ---     |
| X <sub>1</sub> | 160 ± 49*                             | 168.4       | 0.01    |
| X <sub>2</sub> | NA                                    | ---         | ---     |
| X <sub>3</sub> | 37 ± 29                               | 34          | 0.1     |
| G171           | 2.8 ± 0.62**                          | 3.0         | 0.0008  |
| I172           | 8.3 ± 0.31***                         | 8.7         | 0.00008 |
| A173           | 52.4 ± 4.76***                        | 55.2        | 0.0002  |
| I174           | 2.8 ± 0.66**                          | 3.0         | 0.001   |
| I175           | 270.0 ± 72***                         | 284.2       | 0.0002  |
| M176           | 0.8 ± 0.09                            | 0.8         | 0.8     |
| L177           | 0.98 ± 0.11                           | 1.0         | 0.4     |
| F178           | 79.0 ± 14***                          | 83.2        | 0.00004 |
| H179           | 2.9 ± 0.87**                          | 3.1         | 0.002   |
| D180           | 38.0 ± 8.6***                         | 40          | 0.0001  |
| T181           | 0.72 ± 0.17                           | 0.7         | 0.9     |
| A182           | 14.0 ± 1.1***                         | 14.7        | 0.0002  |
| M183           | 22.0 ± 1.8***                         | 23.2        | 0.0002  |
| I184           | 470.0 ± 240**                         | 494.7       | 0.002   |
| I185           | 2.0 ± 0.93*                           | 2.1         | 0.02    |
| C186           | 3.5 ± 1.1**                           | 3.7         | 0.001   |
| L187           | 6.2 ± 1.04***                         | 6.5         | 0.00008 |
| T188           | 0.5 ± 0.07                            | 0.5         | 0.9     |
| V189           | 2.4 ± 1.4*                            | 2.5         | 0.02    |
| M190           | 2.2 ± 0.44**                          | 2.3         | 0.002   |
| F191           | 1.62 ± 0.42*                          | 1.1         | 0.01    |
| L192           | 0.75 ± 0.3                            | 0.8         | 0.8     |

This table includes all EC<sub>50</sub> values for the xt.MC2R **TM4**, **EC2**, and **TM5** region mutant receptors where single alanines substituted corresponding amino acid positions of the xt.MC2R. Also, the EC<sub>50</sub> for complete alanine substitution of TM4, EC2, and TM5 regions were inserted at the top of the table. The fold change of the mutant receptors compared to the mean EC<sub>50</sub> value of the standard curve was calculated as Fold Change = mutant receptor EC<sub>50</sub> value ÷ standard EC<sub>50</sub> value. Also, a Student's T Test was used to

observe if mutant receptor  $EC_{50}$  value was different than standard  $EC_{50}$  value. If the comparison of the two  $EC_{50}$  values differed drastically, the mutant receptor would be designated as significant. Therefore, significance would of the experimental  $EC_{50}$  value would be assigned a p-value  $\leq 0.05$  (\*: p-value  $< 0.05$ ; \*\*: p-value  $< 0.005$ ; \*\*\*: p-value  $< 0.005$ )

## Discussion

### *Tetrapod and Teleost MC2Rs: MRAP1 Requirement and Ligand Selectivity for ACTH*

This study on the melanocortin-2 receptor of the amphibian, *Xenopus tropicalis* (xtMC2R), adds to the growing literature on teleost and tetrapod MC2Rs, and provides additional evidence that among teleost and tetrapod MC2Rs there are a number of universal features. First, teleost and tetrapod MC2Rs can only be activated by ACTH (for review see Does, 2013). In this regard, only the ACTH(1-24) sequence is required for full activation of teleost or tetrapod MC2R. Second, teleost and tetrapod MC2Rs require an intimate interaction with MRAP1 in order to not only move from the ER to the plasma membrane, but also for activation following an ACTH binding event. The corollary to this statement is that it appears that tetrapod MC2Rs require interaction with tetrapod MRAP1, and teleost MC2Rs require interaction with a teleost MRAP1 to achieve functional activation (Liang et al., 2011).

With regard to the MC2R expression requirements, the results of this study showed that xtMC2R cannot be functionally expressed without interaction with a mammalian MRAP, (i.e., mouse MRAP1; Figure 12). Although it is hypothesized that an xtMRAP1 homolog exists in the *Xenopus tropicalis* genome, this accessory protein gene has yet to be identified. Therefore for the studies presented in this thesis, mouse MRAP1 (mMRAP1) was used to functionally express xtMC2R on the plasma membrane of CHO



cells. However, genomic screening has detected a homolog of the MRAP2 gene in the *X. tropicalis* genome. Past studies have shown that xtMC2R can be activated by xtMRAP2 in the CHO cell functional assay system. However, activation was only shown at the highest concentration of hACTH(1-24) tested (i.e.,  $10^{-6}$ M; Liang et al., 2011). Similar results were observed in Figure 12 when an ortholog of MRAP2 (mMRAP2) was co-expressed with xtMC2R.

With regard to ligand selectivity, several studies have shown that mammalian MC2Rs cannot be activated by  $\alpha$ -MSH ((Buckley and Ramachandran, 1981; Mountjoy et al., 1992; Schwyzer, 1997). As previously noted, the  $\alpha$ -MSH amino acid sequence is positioned within the ACTH(1-39) amino acid sequence (Figure 4B). This thesis showed that while xtMC2R can be activated by ACTH(1-24) (Figure 13), this receptor cannot be activated by  $\alpha$ -MSH (Figure 13). Given the sequence relationship between  $\alpha$ -MSH and ACTH, these observations support the hypothesis that tetrapod MC2Rs interact not only with the HFRW motif in ACTH. But also with another site in ACTH (Schwyzer, 1977), and correspondingly, the melanocortin-2 receptor must have multiple binding sites for ACTH. In support of these conclusions a recent study indicated that the MC2R ortholog of the reptile, *Anolis carolinensis*, also required co-expression with mMRAP1 when expressed in CHO cells, and the reptile receptor could be activated by ACTH(1-24), but not by  $\alpha$ -MSH (Davis et al., 2013).

The arguments for expanding the ligand selectivity and MRAP1 requirements of MC2R to include teleost MC2Rs, come from studies on the zebrafish, *Danio rerio*

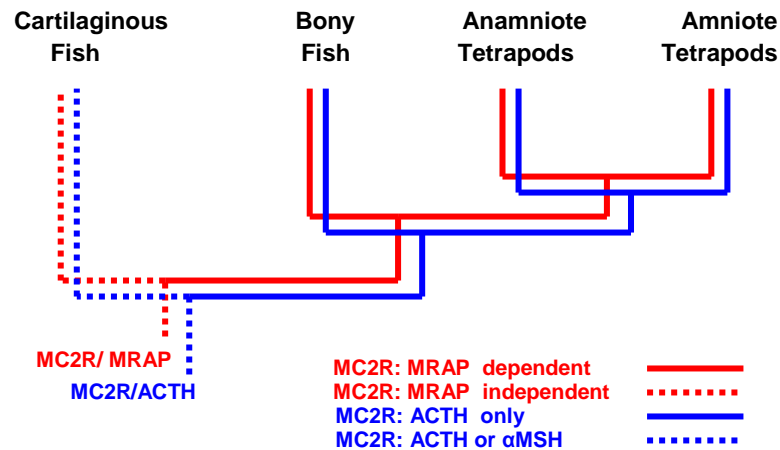
(Agulleiro et al., 2010), the rainbow trout, *Oncorhynchus mykiss* (Aluru and Vijayan, 2008; Liang et al., 2011), and the sea bass, *Dicentrarchus labrax*, (Aguilleiro et al., 2013). In these studies, the functional expression of the teleost MC2R in heterologous mammalian cell lines required co-expression with a teleost MRAP1. In addition, ligand selectivity studies confirmed that while the teleost MC2Rs could be activated by hACTH(1-24), these melanocortin receptors did not respond to stimulation by  $\alpha$ -MSH. Hence, it appears that among the bony vertebrates (i.e., modern bony fishes, amphibians, reptiles, birds, and mammals), MC2R is MRAP1 dependent, and insensitive to  $\alpha$ -MSH. These conclusions are summarized in Figure 29.

Do these conclusions apply to all gnathostomes? The cartilaginous fishes, together with the bony vertebrates constitute Superclass Gnathostoma (Nelson, 1994). Recently an ortholog of MC2R has been detected in the genome of the cartilaginous fish, *Callorhynchus milii* (the elephant shark; Vastermark and Schiøth, 2011). Studies on the elephant shark MC2R indicate that this receptor is MRAP1 independent and the receptor can be activated by both ACTH(1-24) and  $\alpha$ -MSH (Reinick et al., 2012). Similar results have been observed for the MC2R ortholog in the genome of the sting ray, *Dasyatis akajei* (R.M. Does, unpublished data).

Collectively, these observations have led to the following evolutionary hypothesis (Figure 29; Does, 2013). During the emergence of the ancestral gnathostomes (approximately 480 MYA), the melanocortin-2 receptor was MRAP1 independent, and the receptor could be activated by both ACTH and  $\alpha$ -MSH. These properties appear to be

retained in the cartilaginous fishes. Following the divergence of the ancestral cartilaginous fishes, and the ancestral bony fishes, mutations occurred in the MC2R gene in the bony fish lineage. These mutations resulted in the exclusive selectivity of MC2R for ACTH, but these changes also led to the dependence on MRAP1 for the functional expression of MC2R. The ramifications of these mutations will be discussed in a later section of this Discussion.

**Figure 29: Phylogeny of MC2R Ligand Selectivity and Interaction with MRAP**



*Tetrapod and Teleost ACTH(1-24) Analog Studies*

Prior studies have shown that ACTH(1-24) is the functional region of ACTH(1-39). These studies had also proposed that this functional region consists of two motifs that are essential for the activation of mammalian MC2R: the HFRW motif and KKRRP

motif (Eberle and Schwyzer, 1975; Schwyzer, 1977). Do these same requirements apply to other tetrapod MC2Rs such as *X. tropicalis* MC2R? Would these same requirements apply to a teleost MC2R? To address these questions this thesis analyzed the effect of introducing alanine substitutions into hACTH(1-24), and testing the effects of these analogs on the functional activation of xtMC2R. Another thesis project (Lisa Liang, 2013) conducted a parallel study on rainbow trout MC2R. The analogs that were tested were divided into three zone (A, B, C) as shown in the Methods (Table 1), which corresponded to the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif, the G<sup>10</sup>K<sup>11</sup>P<sup>12</sup>V<sup>13</sup>G<sup>14</sup> motif, and the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif, respectively.

Before discussing the results of the Zone A (HFRW) analog studies, it should be noted that all melanocortin peptides have the HFRW motif (Schwyzer, 1977), and conversely, the activation of all melanocortin receptors is dependent on the binding of this motif on the ligand to the receptor. Is there a common HFRW binding site on all melanocortin receptors? Pogosheva et al (2005) initially addressed this question by performing a modeling study and site directed mutagenesis study on the human melanocortin-4 receptor (hMC4R). That study identified critical amino acid positions in TM 2, 3, 6, and 7 that are required for interacting with the HFRW motif of  $\alpha$ -MSH. Those amino acid positions are shown in Figure 30A. Interestingly these same positions are nearly universally conserved in the MC1R, MC3R, MC4R, and MC5R paralogs of teleosts and tetrapods (Dores, 2009; Baron et al., 2009). Does xtMC2R have these critical positions? As shown in Figure 30B, nine of the twelve critical amino acid positions are

identical in xtMC2R and hMC4R. If the analysis is expanded to include human MC2R and rainbow trout MC2R, six of the positions are identical in all four sequences.

Figure 30

Comparison of the HFRW Binding site in hMC4R, xtMC2R, hMC2R, rMC2R

A. Human MC4R: Critical Residues in HFRW Binding Site

```

[-----TM2-----]      EC1      [-----TM3-----]
hMC4R FFICSLAVADMLVSVSNGSEETIVITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASICSLLS
          *                               * * * * *
[-----TM6-----]      EC3      [-----TM7-----]
hMC4R AITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPLIYAL
          * * * * *                               * * *
  
```

B. Comparison of hMC4R and xtMC2R: HFRW Binding Site

```

[-----TM2-----]      EC1      [-----TM3-----]
hMC4R FFICSLAVADMLVSVSNGSEETIVITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASICSLLS
xtMC2R FFICSLAVSDMLFSLYKILETIIIIILANIGFLDRNGPFEKKMDDVMDWIFVLSLLGSIFSISA
          *                               * * * * *
[-----TM6-----]      EC3      [-----TM7-----]
hMC4R AITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPLIYAL
xtMC2R AITLTILLGLFICCWSPFVLHLLLYVLCRYNPYCACYLSMLNVNGTLILFSSVIDPLIYAF
          * * * * *                               * * *
  
```

C. Comparison of hMC4R, xtMC2R, hMC2R, rMC2R: HFRW Binding Site

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[-----TM2-----]      EC1      [-----TM3-----]
hMC4R FFICSLAVADMLVSVSNGSEETIVITLLNSTD-TDAQSFTVNIDNVIDSVICSSLLASICSLLS
xtMC2R FFICSLAVSDMLFSLYKILETIIIIILANIGFLDRNGPFEKKMDDVMDWIFVLSLLGSIFSISA
hMC2R FFICSLAISDMLGSLYKILENILIILRNMGYLKPRGSFETTADDIIDSLFVLSLLGSIFSLSV
rMC2R FFICSLATFNTISSLSKTWETLMMEFSDVGQLDSRGDSVRRVDDIIDALLCMSFIGICFIFSILA
          *                               * * * * *
[-----TM6-----]      EC3      [-----TM7-----]
hMC4R AITLTILIGVFVVCWAPFFLHLIFYISCPQNPYCVCFMSHFNLYLILIMCNSIIDPLIYAL
xtMC2R AITLTILLGLFICCWSPFVLHLLLYVLCRYNPYCACYLSMLNVNGTLILFSSVIDPLIYAF
hMC2R AITLTILLGVFIFCWAPFVLHVLLMTFCPSNPYCACYMSLFQVNGMLIMCNAVIDPFIYAF
rMC2R ALTLTMLFGVFVVCCWAPFFLHLLLLMVCVENPYCECYRSLFQLNLVLLMSHAVVDPAIYAF
          * * * * *                               * * *
  
```

A) The critical residues present in HFRW binding site of human MC4R (hMC4R) are marked with a (\*) and colored red. B) A comparison of the hMC4R and *Xenopus tropicalis* MC2R (xtMC2R) in the HFRW binding site domain. Residues that are identical at the HFRW binding sites in hMC4R and xtMC2R are colored red. C) A comparison of hMC4R, xtMC2R, human MC2R (hMC2R), and rainbow trout MC2R (fMC2R) in the HFRW binding site domain. Residues that are colored red in the MC2R sequences are also found in hMC4R. Residues colored in blue are unique to some of the MC2R sequences.

*Analysis of Zone A Analogs of hACTH(1-24)*

As presented in the Results, there were five Zone A analog (HFRW analogs): A4 (AAAA), AFRW, HARW, HFAW, and HFRA. As expected, the A4 analog was unable

to stimulate activation at any concentration tested. These results were not surprising. However, it was unclear at the start of these experiments how single alanine substitutions would affect the activity of the Zone A analogs. Initially the analogs were tested on the hMC4R expressed in CHO cells (Liang et al., 2013). For hMC4R, the order of importance of the amino acid positions in the HFRW motif was  $W \gg R = F > H$ . Substitution at the  $W^9$  completely blocked stimulation of hMC4R, whereas substitution at either  $F^7$  or  $R^8$  shifted the  $EC_{50}$  value 15,000 fold relative to the positive control, and substitution at the  $H^6$  resulted in 100 fold shift in  $EC_{50}$  value relative to the control. When these same analogs were tested on human MC2R, the order of ligand importance was  $W \gg F > R > H$  (Liang et al., 2013). Once again substitution at  $W^9$  completely blocked activation of hMC2R. However, substitutions at  $F^7$ ,  $R^8$ , and  $H^6$  resulted in shifts in  $EC_{50}$  values of 8000 fold, 4000 fold, and 9 fold respective. Clearly, hMC4R and hMC2R did not respond to the Zone A analogs to the same degree. While the  $W^9$  position is clearly essential for both receptors, substitution at the other positions produced receptor-specific responses. These outcomes can be partially explained by the subtle differences in primary sequence at HFRW binding sites of the two receptors (Figure 29).

When these same single alanine Zone A analogs were tested on xtMC2R, there was no activation of the receptor following incubation with either the HARW analog or the HFRA analog (Table 2). In addition, only slight activation was observed at a concentration of  $10^{-6}$  M following incubation with the HFAW analog and AFRW analog ( $W = F \gg R = H$ ). Clearly primary sequence differences at the critical positions in the

HFRW binding site (Figure 30) appear to make xtMC2R much less tolerant of alanine substitutions to hACTH(1-24) than hMC2R. It would be reasonable to speculate that the 3-dimensional shape of the HFRW binding site in xtMC2R and hMC2R may be similar, but definitely not identical.

When these same single alanine Zone A analogs were used to stimulate the rainbow trout MC2R (rtMC2R), yet another pattern was observed with the respect the order amino acid position importance. For rtMC2R the order was W=R>F>H (Lisa Liang, Ph.D. thesis, 2013). For the rtMC2R, incubation with the HFRA analog actually resulted in a dose dependent stimulation at  $10^{-7}$ M and  $10^{-6}$ M. This level of stimulation was not observed for hMC2R, hMC4R, or xtMC2R. In addition, substitution at H<sup>6</sup> had no negative effect on activation; the EC<sub>50</sub> value for the AFRW analog was not statistically different from the positive control (Lisa Liang, Ph.D. thesis, 2013). Collectively, these observations lend support to the conclusion that while an HFRW binding site is a common feature of all melanocortin receptors, there appears to be differences in the 3-dimension structure of this site even between orthologs. Molecular modeling approaches are needed to resolve these shape differences.



*Analysis of Zone C Analogs of hACTH(1-24)*

The role of the KKRRP motif (Zone C) in ACTH(1-24) as the “address” motif required for initial interaction with the “ACTH” receptor (aka MC2R) was initially proposed by Schwyzer (1977). Analogs of this region included A5 (A<sup>15</sup>A<sup>16</sup>A<sup>17</sup>A<sup>18</sup>A<sup>19</sup>), A<sup>15</sup>A<sup>16</sup>RRP, and KKA<sup>17</sup>A<sup>18</sup>A<sup>19</sup>. As shown in Figure 15 there was no stimulation of xtMC2R following incubation with the A5 analog at any of the concentrations tested. The same outcome was observed when the A5 analog was tested on rtMC2R (Lisa Liang, Ph.D. Thesis, 2013). However, incubation of the hMC2R with the A5 analog resulted in a dose dependent increase in cAMP production at concentrations of 10<sup>-8</sup>M and 10<sup>-7</sup>M.

In addition, incubation of xtMC2R with the analogs, AARRP and KKAAA, resulted in diminished activation of xtMC2R, and the EC<sub>50</sub> values for these analogs were 5.0 x10<sup>-6</sup> M and 3.7 x 10<sup>-6</sup> M, respectively (Table 2). The rtMC2R show a similar response to these analogs (Lisa Liang, Ph.D. Thesis, 2013). For xtMC2R and rtMC2R it appears that the interaction with positions 15 and 16 in the KKRRP motif might be more important for activation than interaction at positions 17, 18, and 19. Just the opposite was observed for hMC2R (Liang et al., 2013).

**Figure 31: Amino Acid Sequences of ACTH in Multiple Species**

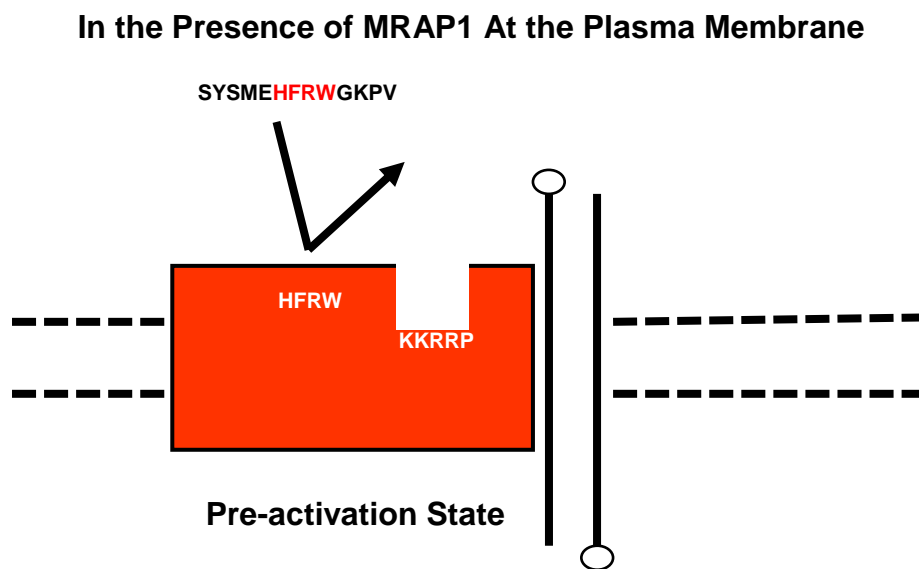
|         | [-----ACTH (1-39) -----]  |
|---------|---|
|         | [-----ACTH (1-24) -----]  |
|         | [ A ] [ B ] [ C ]   |
| Human   | SYSM <sup>A</sup> EHFRWGKPVGKRR <sup>B</sup> PKVYPNGA <sup>C</sup> EDSSA <sup>A</sup> AFLE <sup>L</sup> EP              |
| Chicken | SYSM <sup>A</sup> EHFRWGKPVGKRR <sup>B</sup> PIKVYPNGVDESSA <sup>A</sup> ESY <sup>P</sup> ME <sup>F</sup>               |
| Gecko   | SYAM <sup>A</sup> EHFRWGKPVGKRR <sup>B</sup> PVRVYPNGVEE <sup>E</sup> SS <sup>E</sup> SY <sup>P</sup> Q <sup>E</sup> F  |
| Xenopus | AYSM <sup>A</sup> EHFRWGKPVGKRR <sup>B</sup> PIKVYPNGVEE <sup>E</sup> SSA <sup>A</sup> ENY <sup>P</sup> ME <sup>L</sup> |
| Trout   | SYSM <sup>A</sup> EHFRWGKPVGKRR <sup>B</sup> PKVY <sup>T</sup> NGVEE <sup>E</sup> SSA <sup>A</sup> FP <sup>S</sup> EM   |
| Dogfish | SYSM <sup>A</sup> EHFRWGKPMGRKRR <sup>B</sup> PIKVYPNS <sup>F</sup> ED <sup>E</sup> SVEN <sup>M</sup> GP <sup>E</sup> L |

### *Analysis of Zone B Analogs of hACTH(1-24)*

It is surprising that since the studies by Schwyzer and colleagues on ACTH truncated analogs (Schwyzer, 1977), no analog studies have been done on the GKRVG (Zone B) motif of ACTH until recent thesis projects in our lab. The rationale for these experiments was that the GKRVG motif has been conserved in vertebrate evolution because this motif serves as “linker” region between the HFRW motif and KKRRP motif to properly position these motifs on the surface of MC2R so that the activation event can proceed (Liang et al., 2013). The results of the alanine substitution experiments on xtMC2R, hMC2R, and rtMC2R support this assumption. As shown for xtMC2R in Table 2, either single alanine substitution or double alanine substitution in this region of the ligand affected the EC<sub>50</sub> value in a negative manner. However, the most compelling argument for the importance of the GKPVG motif came from the truncation experiments. When the hACTH(1-24) sequence was shortened to the ACTH(1-21) or the ACTH(1-22) analog (Table 1) there was a complete lack of stimulation of, not only xtMC2R (Table 2), but also hMC2R and rtMC2R (Lisa Liang, Ph.D. thesis). Collectively, the results from the alanine substitution and the shortened Zone B ligand experiments underscore the importance of the secondary structure of Zone B (GKPVG), and suggest that this motif may play a crucial role in positioning the HFRW and KKRRP motifs, so that proper interaction of the ligand and receptor could occur to result in activation of the MC2Rs. These observations have led to the following hypothesis to account for the activation of tetrapod and teleost MC2Rs (Figure 32). This mechanism requires that tetrapod and

teleost MC2Rs interact with the MRAP1 homodimer at the rough endoplasmic reticulum. In the absence of MRAP1 the receptor will miss-fold and be degraded by the protein quality control mechanism in the endoplasmic reticulum (Hinkle and Sebag, 2010).

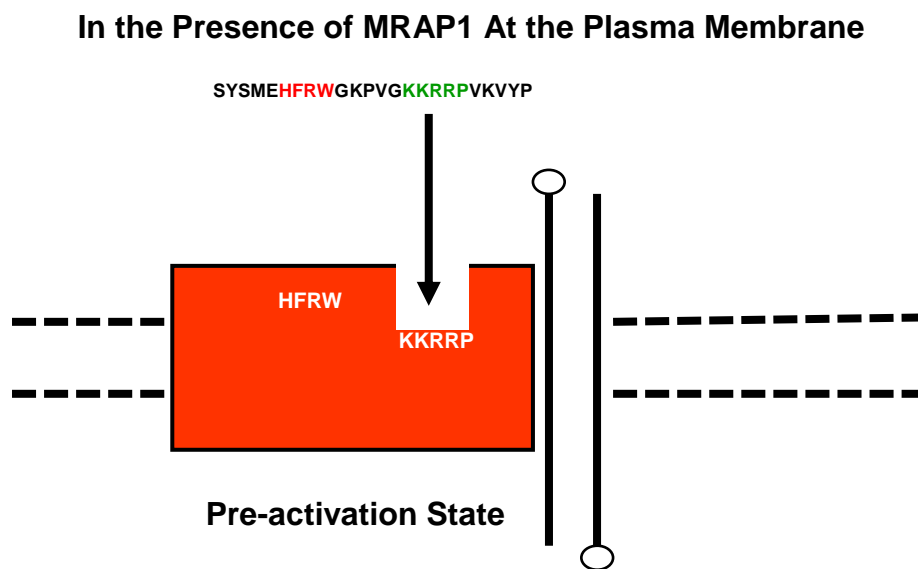
**Figure 32A: Proposed Model of MC2R Activation**



As shown in Figure 32A, MC2R is positioned on the plasma membrane, in contact with MRAP1, and in a “pre-activation” state. Based on the ligand and analog studies we assume that there are two binding sites on the receptor. The KKRRP binding site is exposed, but the HFRW binding site is not accessible in the pre-activation state. As a result in the pre-activation state,  $\alpha$ -MSH cannot activate the receptor. However, as shown in Figure 32B, when ACTH(1-24) makes contact with the receptor, the KKRRP motif on ACTH(1-24) can interact with the exposed KKRRP binding site on the receptor.

This event would be Step 1 of the activation process. The argument to support the Step 1 event comes from the observations by Lisa Liang (Ph.D thesis) that the analog ACTH(15-24) blocks the binding of ACTH(1-24) to the receptor and inhibits activation as a result.

**Figure 32B: Proposed Model of MC2R Activation**



**Activation Step 1 – bind to KKRRP binding site**

For this proposed mechanism, the prediction is that the docking at the KKRRP binding site results in a conformation change in the receptor (Figure 32C). This conformation change exposes the HFRW binding site. This is Step 2 of the activation process. Because of the “linker” role that the GKPVG motif plays, the HFRW motif in the ligand is properly positioned to interact with the exposed HFRW binding site on the receptor. The later interaction is predicted to induce another conformational change in the

receptor (Step 3) which results in the activation of the G protein and induces the subsequent biological response within the target cell.

**Figure 32C: Proposed Model of MC2R Activation**



**Activation Step 2 – exposure of HFRW binding site**

Based on this model, and given the slightly different responses to the Zone A, B, and C analogs observed for xtMC2R, hMC2R, and rtMC2R it would appear that difference in primary sequence (Figure 30) could contribute to subtle changes in the 3-dimensional shape of these receptors. Confirmation of this conclusion will depend on molecular 3-dimensional modeling of the tetrapod and teleost MC2Rs.

*Tetrapod and Teleost MC2Rs: Mutant Receptor Studies – the KKRRP site on MC2R*

As introduced in the preceding section, there is an extensive literature on the binding and signaling by  $\alpha$ -MSH for mammalian MC1R, MC3R, and MC4R (Yang et al., 2000; Chen et al., 2006). Furthermore, the study by Pogozeva et al. (2005) identified the HFRW binding site which is common to all melanocortin receptors including the MC2 receptor. Table 4 (Liang, 2013) summarized the comparison between hMC4R, hMC2R, xtMC2R and rtMC2R in support of the assumption that all melanocortin receptors have a common HFRW binding site.

**Table 4: Proposed HFRW binding sites in MC4R and the corresponding residues of MC2R**

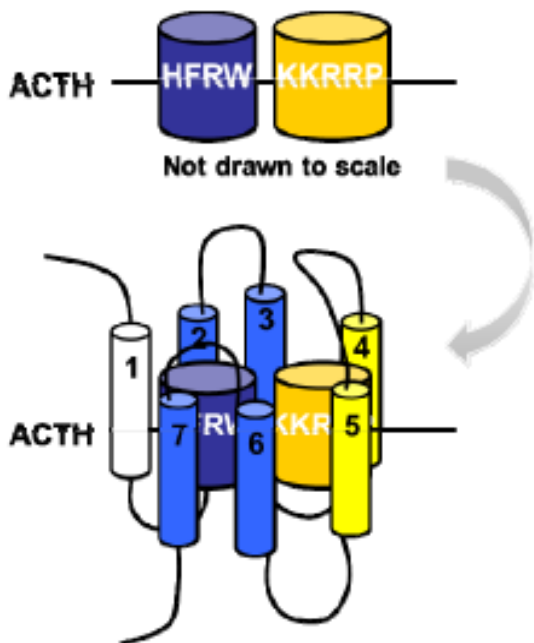
| AA   | Region | MC4R |   |    | MC2R |   |    |
|------|--------|------|---|----|------|---|----|
|      |        | H    | X | RT | H    | X | RT |
| E100 | TM2    | +    | + | +  | +    | + | +  |
| D122 | TM3    | +    | + | +  | +    | + | +  |
| D126 | TM3    | +    | + | +  | +    | + | +  |
| I129 | TM3    | +    | + | +  | -    | - | -  |
| L133 | TM3    | +    | + | +  | +    | + | -  |
| W258 | TM6    | +    | + | +  | +    | + | +  |
| F261 | TM6    | +    | + | +  | +    | + | +  |
| H264 | TM6    | +    | + | +  | +    | + | +  |
| N285 | TM7    | +    | + | +  | -    | + | -  |
| L288 | TM7    | +    | + | +  | -    | - | +  |

H: Human; X: *Xenopus tropicalis*; RT: Rainbow trout (*Onchorynchus mykiss*)

Although a high level of sequence similarity was found for the proposed HFRW binding sites on melanocortin receptors, there is still the question of where the docking site for the R/KKRRP is located within the melanocortin 2 receptor sequence. Working

from the model presented in Figure 33, this thesis focused on the TM 4, extracellular loop 2 (EL2), and the TM5 regions of xtMC2R.

**Figure 33: 3-Dimensional Diagram of the MC2R binding sites for the HFRW and R/KKRRP motifs**



The single alanine substitution experiments for xtMC2R are summarized in Figure 34A. There were nine residues in TM4, EL2, and TM5 that appear to be important, from a statistical perspective, for the activation of xtMC2R by hACTH(1-24) (see Table 3). However, as noted in the Results section, the alanine mutants that resulted in at least a 10 fold shift in  $EC_{50}$  values, relative to the positive control, were: A<sup>173</sup>, I<sup>175</sup>, F<sup>178</sup>, D<sup>180</sup>, A<sup>182</sup>, M<sup>183</sup>, and I<sup>184</sup>. Since the A<sup>173</sup> and A<sup>182</sup> sites are considered intrinsically inert in the wild-type receptor, we feel that the substitution of a glutamine residue at these two positions most likely disrupted natural interactions between the ligand and the

receptor. Hence, the critical sites for facilitating activation of xtMC2R are: I<sup>175</sup>, F<sup>178</sup>, D<sup>180</sup>, M<sup>183</sup>, and I<sup>184</sup>. Among these positions a 50 fold or greater shift in EC<sub>50</sub> values was observed for I<sup>175</sup>, F<sup>178</sup>, and I<sup>183</sup>, and a 100 fold or greater shift in EC<sub>50</sub> values was observed for the I/A<sup>175</sup> mutant and the I/A<sup>183</sup> mutant. It would appear then that positions I<sup>175</sup>, F<sup>178</sup>, and I<sup>183</sup> are the most important for activation of xtMC2R following the ligand binding event (Step1; Figure 33).

Recently, single alanine substitutions were performed on the TM4, EL2, and TM5 regions of hMC2R and rtMC2R (Lisa Liang, Ph.D. thesis, 2013). For hMC2R and rtMC2R a set of amino acid positions were also identified in these regions that affected EC<sub>50</sub> values by at least 10 fold (Figure 34C). While the model presented in Figure 33 targets TM4, EL2, and TM5 as the most likely location for the KKRRP docking site, a comparison of the primary sequences of xtMC2R, hMC2R, and rtMC2R resulted in a number of surprising observations (Figure 34B).

The underlined residues in Figure 34B were the amino acids positions that were targeted for single alanine or glutamine replacement. For TM4 and TM5 site directed mutagenesis was performed on the seven amino acid positions closest to the extracellular space. The rationale for selecting these positions was that Pogosheva et al. (2005) observed that the amino acid positions involved for the HFRW binding site are located within seven residues of the surface of the cell. Hence, it seemed reasonable to predict that the hydrophilic pocket for the KKRRP docking site would also be relatively close to the surface of the cell. The feature that is striking about Figure 34B is that the alignment



of the sequences of xtMC2R, hMC2R, and rtMC2R indicated that within the underlined zone only three positions are identical in the three receptors (residues in red). An additional eight positions (pink) have amino acids in the same group (i.e., hydrophobic, polar uncharged, or polar same charge). Hence, the striking feature is the lack of primary sequence identity in this region of the three receptors.

In terms of functionality, the critical positions in xtMC2R that have at least a 10 fold influence on  $EC_{50}$  value are located within TM4 and EL2 (Figure 34C), and the positions in this receptor that have a 100 fold or greater effect on  $EC_{50}$  value were located in TM4 and EL2. Note that positions in TM5 do not appear to be essential for the activation of xtMC2R. When the same comparison is done for hMC2R, the positions with at least a 10 fold influence on  $EC_{50}$  value are located in EL2 and TM5, the position in hMC2R that had a 100 fold or greater effect on  $EC_{50}$  value was restricted to a single position in TM5. Note the apparent absence of a role for TM4 in the activation of hMC2R. These observations are in contrast to rtMCR2 where the positions that had at least a 10 fold or 100 fold influence on  $EC_{50}$  value are located within TM4 and TM5. Note that for rtMC2R, the EL2 region does not appear to be important for the activation of the receptor. Collectively these observations indicate that while the TM4/EL2/TM5 region of these MC2Rs appears to be the target for the docking of the KKRRP motif of ACTH; the primary sequence of this zone in the three receptors do not provide a simple explanation for where the KKRRP docking event (Step 1) will occur. The prediction would be that the 3-dimensional shape of the TM4/EL2/TM5 region must be similar in all three

receptors. Another prediction is that the interaction between the KKRRP motif of ACTH and the corresponding docking site on the receptor relies upon hydrogen bonding and hydrophobic interactions between the R-groups rather than ionic interactions (note the absence of acidic amino acids in this region with the exception of the lone aspartic acid residue (D) in EL2 of xtMC2R. At this stage, it would be difficult to obtain further information from additional site-directed mutagenesis experiments. The next step should be to do molecular 3-dimensional modeling of these receptors.

**Figure 34: Tetrapod/Teleost Comparison of MC2R Single Alanine Substitutions**

**A. xMC2R: Summary of single alanine substitution**

[-----TM4-----] EL2 [-----TM5-----]  
**xMC2R** RASVILAVIWTFCGGSGIAIIMLFHDTAMIICLTVMFLLLLLVLIVCLYIHMF

**B. Sequence identity in TM4,EL2,and TM5 of hMC2R,xMC2R and rtMC2R**

[-----TM4-----] ELC2 [-----TM5-----]  
**xMC2R** RASVILAVIWTFCGGSGIAIIMLFHDTAMIICLTVMFLLLLLVLIVCLYIHMF  
**hMC2R** RTVVVLTVIWTFCTGTGITMVIFSHHVPTVITFTSLFPLMLVFILCLYVHMF  
**rMC2R** RAAAALAGIWALCGVAGAVMVAFCDATVIKIFFIVLFLISLLLLILFLYVHMF

**C. Mutations that resulted in a 10 fold or greater shift in EC<sub>50</sub>**

[-----TM4-----] EL2 [-----TM5-----]  
**xMC2R** RASVILAVIWTFCGGSGIAIIMLFHDTAMIICLTVMFLLLLLVLIVCLYIHMF  
**hMC2R** RTVVVLTVIWTFCTGTGITMVIFSHHVPTVITFTSLFPLMLVFILCLYVHMF  
**rMC2R** RAAAALAGIWALCGVAGAVMVAFCDATVIKIFFIVLFLISLLLLILFLYVHMF

**D. Mutations that resulted in a 100 fold or greater shift  
in EC<sub>50</sub>**

[-----TM4-----]    EC2    [-----TM5-----]  
**xMC2R** RASVILAVIWTFCGGSGIAI**I**MLFHDTAM**I**ICLTVMFLLLLLVLIIVCLYIHMF  
**hMC2R** RTVVVLTVIWTFCTGTGITMVIFSHHVPTVIT**F**TSLFPLMLVFILCLYVHMF  
**rMC2R** RAAAALAGIWALCGVAGAVM**V**AFCDATVIKIFFIVL**F**LISLLLILEFLYVHMF

*Final Observations*

Based on the observations made in this thesis, xtMC2R has several basic properties that are identical to mammalian MC2Rs. It appears that all of the tetrapod MC2Rs can only be activated by ACTH, but not by any MSH-sized melanocortin ligand. In addition, all of the tetrapod MC2Rs require interaction with MRAP1 to facilitate functional activation of the receptor at the plasma membrane following stimulation with ACTH (Gantz and Fong, 2003; Hinkle and Sebag, 2009; Webb and Clark, 2010). This thesis has examined the effects of alanine substitutions in the functional zones of hACTH(1-24) (Table1). The operating assumption was that there would be a differential response of amniote MC2Rs (e.g. human) to these analogs of hACTH(1-24) as compared to the response of an anamniote MC2R (i.e., frog) to the same analogs. However, the outcome of this analysis did not neatly follow this simple dichotomy of the tetrapod MC2R sequences.

The analysis of alanine-substituted analogs of the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif of hACTH(1-24) indicated that xtMC2R could not tolerate any single alanine substitution in this motif (Davis et al., 2013). By contrast, human MC2R could tolerate some alanine substitutions in the H<sup>6</sup>F<sup>7</sup>R<sup>8</sup>W<sup>9</sup> motif (Liang et al., 2013). These observations may indicate subtle differences in the 3-dimensional structure of the HFRW binding sites of tetrapod MC2Rs.

Similar results were observed when the alanine substituted analogs of the K<sup>15</sup>K<sup>16</sup>R<sup>17</sup>R<sup>18</sup>P<sup>19</sup> motif of hACTH(1-24) were analyzed. For xtMC2R it appears that positions 15 and 16 in the ligand are far more important for activation of the receptor (Table 2) than positions 17, 18, and 19 (Davis et al., 2013); whereas, for human MC2R, positions 17, 18, and 19 are clearly more essential for activation of the receptor (Liang et al., 2013). Figure 34 shows the proposed location of the putative KKRRP binding in MC2Rs. Given the lack of primary sequence identity in this region, secondary and tertiary structures at this proposed docking site would appear to be the critical factors for mediating the activation of these receptors.

The analysis of the G<sup>10</sup>K<sup>11</sup>R<sup>12</sup>V<sup>13</sup>G<sup>14</sup> motif in hACTH(1-24) provided further evidence for the importance of this region of the ligand for activation of the receptor. These observations also may explain the nearly universal conservation of this motif in gnathostome ACTH sequences (Dores and Lecaude, 2005). With respect to the alanine analogs of the GKPVG motif, the response of hMC2R (Liang et al., 2013) was distinct from the response of xtMC2R (Table 2). In addition, the analysis of the ACTH(1-21) and the ACTH(1-22) analogs do point to the importance of the spatial positioning of the

HFRW binding site and the putative KKRRP binding site in tetrapod MC2Rs with respect to the proposed 3 step mechanism for the activation of the receptor (Figure 32).

When these observations are viewed collectively, the response of xtMC2R to these ACTH(1-24) analogs was subtly different than the response of hMC2R to these same analogs. It is possible that mammalian MC2Rs have evolved structural features distinct from the non-mammalian MC2Rs. At present, the data set is small and the generalizations should be viewed conservatively until modeling of the 3-dimensional shape of these receptors has been done.

That said, the additional structure/function data provided in this thesis add to the growing literature on the distinctive features of melanocortin-2 receptor orthologs in teleosts and tetrapods (Agullerio et al., 201; Liang et al., 2011). In this regard, the exclusive selectivity for ACTH and the requirement for interaction with an MRAP1 ortholog are the two features which unite teleost and tetrapod MC2 receptor orthologs. The dependence of both teleost and tetrapod MC2Rs on MRAP1 for functional activation raises the question of whether there is an ortholog of MC2R that is MRAP independent. As noted in the Introduction, an MC2R ortholog detected in the genome of the cartilaginous fish, *Callorhinchus milii* [48], indicates that the *C. milii* MC2 receptor ortholog is MRAP independent. These observations would suggest that in the early evolution of the gnathostomes a MC2R-like receptor and a MRAP-like accessory protein functioned independent of each other. In this scenario following the divergence of the ancestral cartilaginous fish and the ancestral bony fish lineages, an interaction developed

between MC2R and MRAP1 that initially may have been neutral from a fitness perspective. However, as point mutations accumulated in the ancestral bony fish MC2R ortholog, the interaction with MRAP1 became essential for the functional activation of the receptor. The rescue of a misfolded MC2R by MRAP1 may have been a critical event during the early evolution of the bony fishes. Hence, the co-evolution of the MC2R gene and the MRAP1 gene appears to have been an important event in the evolution of the bony vertebrates (i.e., bony fishes, amphibians, reptiles, birds, and mammals).

## Works Cited

- Agulleiro, M.J., Roy, S., Sanchez, E., Puchol, S, Gallo-Payet, N., Cerda-Reverter, J.M., 2010, Role of melanocortin receptor accessory proteins in the function of zebrafish melanocortin receptor type 2. *Molecular and Cellular Endocrinology* 320, 145-152.
- Agulleiro, M.P., Sánchez, E., Leal, E., Cortés, R. Fernández-Durán, B., Guillot, R., Gallo-Payet, N., Dores, R.M., Davis, P., Cerdá-Reverter, J.M. (2013) Molecular Characterization and Functional Regulation of Melanocortin 2 Receptor (MC2R) in the Sea Bass. A Putative Role in the Adaptation to Stress. *PLOS ONE* 27;8(5):e65450.
- Aluru, K. and Vijayan, M.M. (2008), Molecular characterization, Tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. *Endocrinology* 149: 4577-4588.
- Barrett, K.E., Barman, S.M., Boitano, S., Brooks, H.L., 2010. *Ganong's Review of Medical Physiology*. The McGraw-Hill Companies, Inc., USA.
- Baron, A., Veo, K., Angleson, J., Dores, R.M. (2009), Modeling the evolution of the MC2R and MC5R genes: studies on the cartilaginous fish, *Heterodontus francisci*. *General and Comparative Endocrinology* 161, 13-19.
- Buckley, D.I., Ramachandran, J., 1981. Characterization of the corticotropin receptors on adrenocortical cells. *Proc. Natl Acad. Sci. USA* 78, 7435-7435.
- Chan LF, Clark AJ, Metherell LA. 2008. Familial glucocorticoid deficiency: advances in



- the molecular understanding of ACTH action. *Horm Res.* 69(2):75-82.
- Chen, M., Aprahamian, C. J., Celik, A., Georgeson, K. E., Garvey, W. T., Harmon, C. M., and Yang, Y. 2006. Molecular characterization of human melanocortin-3 receptor ligand receptor interaction, *Biochemistry* 45, 1128-1137.
- Chepurny, O.G., Holz, G.G., 2007. A novel cyclic adenosine monophosphate responsive luciferase reporter incorporating a nonpalindromic cyclic adenosine monophosphate response element provides optimal performance for use in G protein coupled receptor drug discovery efforts. *J. Biomol. Screen.* 12, 740–746.
- Cone, R.D. 2006. Studies on the physiological functions of the melanocortin system. *Endocr Rev* 27, 736–749.
- Cooray, S.N., Almiro Do Vale, I., Leung, K.Y., Webb, T.R., Chapple, J.P., Egertova, M., Cheetham, M.E., Elphick, M.R., Clark, A.J., 2008a. The melanocortin 2 receptor accessory protein exists as a homodimer and is essential for the function of the melanocortin 2 receptor in the mouse y1 cell line. *Endocrinology* 149, 1935–1941.
- Cooray, S.N., Clark, A.J.L., 2011. Melanocortin receptors and their accessory proteins. *Molecular and Cellular Endocrinology* 331, 215-221.
- Cooper, G.M., Hausman, R.E., 2009. *The Cell: A Molecular Approach*. The American Society of Microbiology, Washington D.C.

- Costa JL, Bui S, Reed P, Dores RM, Hochgeschwender U, Brennan MB. 2004. Mutational Analysis of Evolutionarily Conserved ACTH Residues. *Gen. Comp. Endocrinol.* 136 :12-16.
- Davis, P., Franquemont, S., Liang, L., Angleson, J.K., Dores, R.M. 2013. Evolution of the melanocortin-2 receptor in tetrapods: Studies on *Xenopus tropicalis* MC2R and *Anolis carolinensis* MC2R. *Gen Comp Endocrinol.* 118, 75-84.
- Dores, R.M., Lecaude, S., 2005. Trends in the evolution of the proopiomelanocortin gene. *Gen. Comp. Endocrinology* 142, 193-205.
- Dores, R.M., 2009. ACTH, the MSHS and melanocortin receptors: revisiting the works of Robert Schwyzer – a 30 year retrospective. *Ann. NY Acad. Sci.* 1163, 93-100.
- Dores, R.M., Baron, A.J., 2011. Evolution of POMC: origin, phylogeny, posttranslational processing, and the melanocortins. *Ann. NY Acad. Sci.* 1220, 34-48.
- Dores R.M., 2013. Observations on the evolution of the melanocortin receptor gene family: distinctive features of the melanocortin-2 receptor. *Front. Neurosci.* 7:28. doi: 10.3389/fnins.2013.00028
- Eberle, A., Schwyzer, R., 1975. Hormone-receptor interactions. Demonstration of the two message sequences (active sites) in  $\alpha$ -melanocortropin. *Helv. Chim. Acta.* 58, 1528-1535.
- Gantz, I., and Fong, T.M., The melanocortin system. *American Journal of Physiology – Endocrinology and Metabolism* 284 (2003) E468-E474.
- Haskell-Luevano, C., Sawyer, T. K., Trumpp-Kallmeyer, S., Bikker, J. A., Humblet, C., Gantz, I., and Hruby, V. J. 1996. Three-dimensional molecular models of the hMC1R

- melanocortin receptor: Complexes with melanotropin peptide agonists, *Drug Des. DiscoVery* 14, 197-211.
- Haitina, T., Klovins, J., Takahashi, A., Lowgren, M., Ringholm, A., Enberg, J., et al., 2007. Functional characterization of two melanocortin (MC) receptors in lamprey showing orthology to the MC1 and MC4 receptor subtypes. *BMC Evol. Biol.* 7:101. doi: 10.1186/1471-2148-7-101.
- Hinkle P.M., Sebag J.A., 2009. Structure and function of the melanocortin2 receptor accessory protein (MRAP). *Mol Cell Endocrinol.* 300(1-2):25-31.
- Holland, P.W., Garcia-Fernandez, J., Williams, N. A., Sidow, A., 1994. Gene duplications and origins of vertebrate development. *Dev. Suppl.* 125-133.
- Kageyama, K., Suda, T., 2009. Role and action in the pituitary corticotroph of corticotropin-releasing factor (CRF) in the hypothalamus. *Peptides* 30, 810-818.
- Klovins, J., Haitina, T., Fridmanis, D., Kilianova, Z., Kapa, K., Fredricksson, R., et al., 2004. The melanocortin system in fugu: determination of POMC/AGRP/MCR gene repertoire and synteny as well as pharmacology and anatomical distribution of the MCRs. *Mol. Biol. Evol.* 21, 563-579.
- Klovins, J., Haitina, T., Ringholm, A., Lowgren, M., Fridmanis, D., Slaidina, M., et al., 2004. Cloning of two melanocortin (MC) receptors in spiny dogfish. *Eur. J. Biochem.* 271, 4620-4331.
- Liang, L., 2013. Evolution of melanocortin-2 receptor activations: Studies on a mammal and a fish. Dissertation, University of Denver, Denver.

- Liang, L., Angleson J.K., Dores R.M., 2013. Review: using the human melanocortin-2 receptor as a model for analyzing hormone/receptor interactions between a mammalian MC2 receptor and ACTH(1-24). *Gen. Comp. Endocrinol.*, <http://dxdoi.org/10.11016/j.ygcen.2012.11.011>.
- Liang L, Sebag JA, Egelston L, Serasinghe MN, Veo K, Reinick C, Angleson J, Hinkle PM, Dores RM. 2011. Functional expression of frog and rainbow trout melanocortin 2 receptors using heterologous MRAP1s. *Gen Comp Endocrinol.* 174(1):5-14.
- Metherell, L.A., Chapple, J.P., Cooray, S., David, A., Becker, C., Ruschendorf, F., Naville, D., Begeot, M., Khoo, B., Nurnberg, P., Huebner, A., Cheetham, M.E., Clark, A.J. 2005. Mutations in MRAP, encoding a new interacting partner of the ACTH receptor cause familial glucocorticoid deficiency type 2. *Nat. Genet.* 37 (2), 166–170.
- Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Cone, R.D., 1992. The cloning of a family of genes that encode the melanocortin receptors. *Science* 257, 1248-1251.
- Nelson, J.S., *Fishes of the World*, 3<sup>rd</sup> edition, John Wiley and Sons, INC, New York, 1994.
- Pogozheva, I.D., Chai, B.X., Lomize, A.L., Fong, T.M., Weinberg, D.H., Nargund, R.P., Mulholland, M.W., Grants, I., and Mosberg, H.I. 2005. Interactions of human melanocortin 4 receptors with nonpeptide and peptide agonists. *Biochemistry* 44, 11329-11341.

- Rached, M., Mourabit, H.E., Buronfosse, A., Blondet, A., Naville, D., Begeot, M., Penhoat, A., 2005. Expression of the human melanocortin-2 receptor in different eukaryotic cells. *Peptides* 26, 1842-1847.
- Reinick, C.L., Liang L., Angleson, J.K., Dores, R.M., 2012. Identification of an MRAP-independent melanocortin-2 receptor: functional expression of the cartilaginous fish, *Callorhinchus milii*, melanocortin-2 receptor in CHO cells. *Endocrinology* 153, 4757-4765.
- Ringholm, A., Fredricksson, R., Roliakova, N., Tan, Y. L., Rostlethwait, J. H., Larhammer, D., et al., 2002. One melanocortin 4 and two melanocortin 5 receptors from zebrafish show remarkable conservation in structure and pharmacology. *J. Neurochem.* 82, 6-18.
- Roy, S., Rachel, M., Gallo-Payet, N., 2007. Differential regulation of the human adrenocorticotropin receptor [melanocortin-2 receptor (MC2R)] by human MC2R accessory protein isoforms  $\alpha$  and  $\beta$  in isogenic human embryonic kidney 293 cells. *Mol. Endocrinology* 21, 1656-1669.
- Schioth, H.B., Raudsepp, T., Ringholm, A., Fredricksson, R., Takeuchi, S., Larhammer, D., et al., 2003. Remarkable synteny conservation of melanocortin receptor in chicken, human and other vertebrates. *Genomics* 81, 504-509.
- Schwyzler R. 1977. ACTH: a short introductory review, *Ann. NY Acad. Sci.* 297: 3-26

- Sebag, J.A., Hinkle, P.M., 2008. Regions of melanocortin2 receptor accessory protein necessary for dual topology and MC2 receptor trafficking and signaling. *J. Biol. Chem.* 284, 610-618.
- Sebag, J.A., Hinkle, P.M., 2007. Melanocortin-2 receptor accessory protein MRAP forms antiparallel homodimers. *Proc. Natl. Acad. Sci USA* 104, 20244-20249.
- Vallarino, M., d'Amora, M., Dores, R.M., 2012. New insights into the neuroanatomical distribution and phylogeny of opioids and POMC-derived peptides in fish. *Gen. Comp. Endocrinology* 177, 338-347.
- Vastermark, A. and Schiöth, H.B., 2011, The early origin of melanocortin receptors, agouti-related peptide, agouti signaling peptide, and melanocortin receptor-accessory proteins, with emphasis on pufferfishes, elephant shark, lampreys, and amphioxus. *European Journal of Pharmacology*.  
Doi:10.1016/j.ejphar.2010.10.106.
- Viklund, H., Elofsson, A., 2004. Best alpha-helical transmembrane protein topology predictions are achieved using hidden Markov models and evolutionary information. *Protein Sci.* 13, 1908–1917.
- Webb, T.R., Clark, J.L., 2010. Minireview: the melanocortin-2 receptor accessory proteins. *Mol. Endocrinology* 24, 475-484.
- Webb, T.R., Chan, L., Cooray, S., Cheetham, J., Chapple, P., Clark, A.J.L., 2009. Distinct melanocortin 2 receptor accessory protein domains are required for

melanocortin 2 receptor interaction and promotion of receptor trafficking.

Endocrinology 150, 720-726.

Yang, Y. K., Fong, T. M., Dickinson, C. J., Mao, C., Li, J. Y., Tota, M. R., Mosley, R., van der Ploeg, L. H., and Gantz, I. 2000. Molecular determinants of ligand binding to the human melanocortin-4 receptor, *Biochemistry* 39, 14900-14911.