

## 60 YEARS OF POMC

# Melanocortin receptors: evolution of ligand selectivity for melanocortin peptides

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## Abstract

The evolution of the melanocortin receptors (MCRs) is linked to the evolution of adrenocorticotrophic hormone (ACTH), the melanocyte-stimulating hormones (MSHs), and their common precursor pro-opiomelanocortin (POMC). The origin of the MCRs and POMC appears to be grounded in the early radiation of the ancestral protochordates. During the genome duplications that have occurred during the evolution of the chordates, the organization plan for POMC was established, and features that have been retained include, the high conservation of the amino acid sequences of  $\alpha$ -MSH and ACTH, and the presence of the HFRW MCR activation motif in all of the melanocortin peptides (i.e. ACTH,  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH). For the MCRs, the chordate genome duplication events resulted in the proliferation of paralogous receptor genes, and a divergence in ligand selectivity. While most gnathostome MCRs can be activated by either ACTH or the MSHs, teleost and tetrapod MC2R orthologs can only be activated by ACTH. The appearance of the accessory protein, MRAP1, paralleled the emergence of teleost and tetrapods MC2R ligand selectivity, and the dependence of these orthologs on MRAP1 for trafficking to the plasma membrane. The accessory protein, MRAP2, does not affect MC2R ligand selectivity, but does influence the functionality of MC4R orthologs. In this regard, the roles that these accessory proteins may play in the physiology of the five MCRs (i.e. MC1R, MC2R, MC3R, MC4R, and MC5R) are discussed.

## Key Words

- ▶ MC1R
- ▶ MC2R
- ▶ MC3R
- ▶ MC4R
- ▶ MC5R
- ▶ ACTH
- ▶  $\alpha$ -MSH
- ▶ POMC
- ▶ evolution

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## Introduction

The determination of the amino acid sequences of mammalian adrenocorticotrophic hormone (ACTH) (Li *et al.* 1955) and  $\alpha$ -melanocyte-stimulating hormone (MSH) (Lee & Lerner 1956), an impressive accomplishment for the time, actually presented more questions than answers. While it was clear that the 13 amino acid sequence of  $\alpha$ -MSH were embedded in the 39 amino acid sequence of ACTH, the observation that

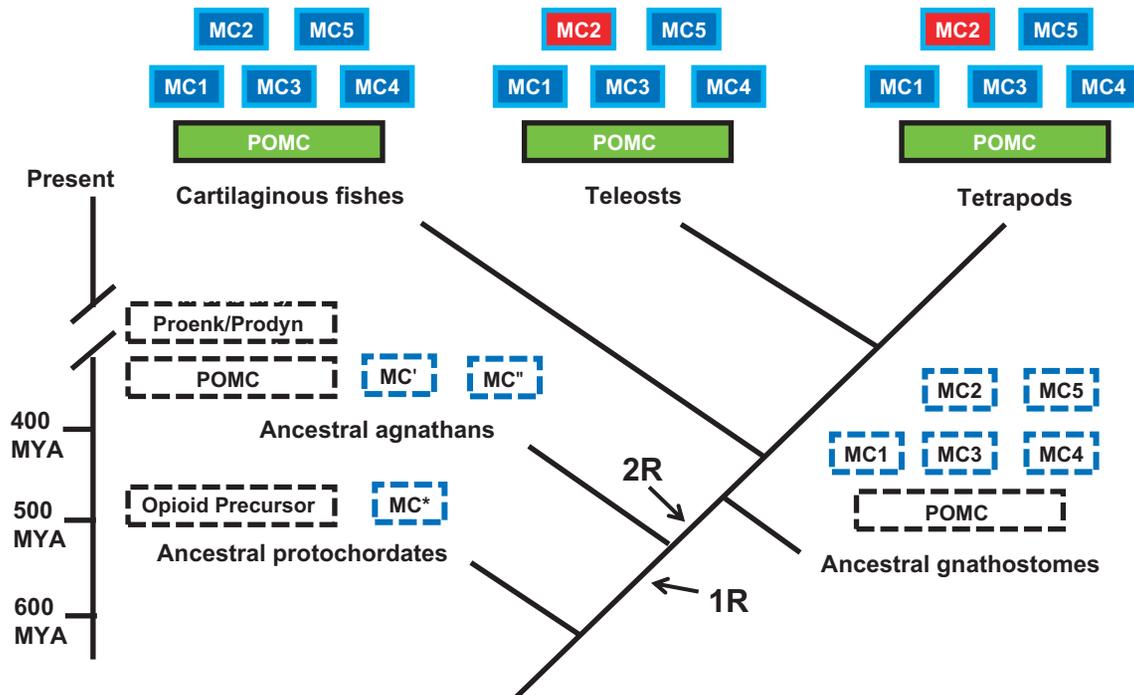
the former polypeptide appeared to be restricted to the cells of the intermediate pituitary, whereas the later polypeptide appeared to be restricted to a subset of cells in the anterior pituitary was perplexing (Baker 1979). In addition, several studies showed that while ACTH could bind to the 'ACTH' receptor on mammalian adrenal cortex cells and promote the synthesis of glucocorticoids,  $\alpha$ -MSH had no biological effect on these cells (Schwyzer 1977).

Conversely,  $\alpha$ -MSH could bind to the 'MSH' receptor on the melanophores of frogs and some reptiles to promote physiological color change, and ACTH had the same biological effect on these color-changing cells (Schwyzer 1977). Over the past 60 years, these puzzling observations have been reconciled as a result of the characterization of pro-opiomelanocortin (POMC), the common precursor for ACTH and MSH-related polypeptides (Nakanishi *et al.* 1979), and the realization that the melanocortin peptides (i.e. ACTH,  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH) selectively bind to a family of G protein-coupled receptors, which are now referred to as the melanocortin receptors (MCRs; Cone 2006). It should be noted that MCRs also interact with a family of antagonist peptides, Agouti-related protein (AgRP) and Agouti signaling protein (ASIP).

The evolution of these antagonists has been recently reviewed (Vastermark & Schiöth 2011).

### Co-evolution of POMC and the MCRs

The evolution of the melanocortin peptides and the MCRs are intertwined. There is reasonable agreement that the *pomc* gene and the ancestral *mcr* gene appeared during the early radiation of the chordates (Schiöth *et al.* 2003, 2005, Sundstrom *et al.* 2010, Dores *et al.* 2011), and the evolution of these ancestral genes has been influenced by the genome duplication events (Ohno *et al.* 1968, Lundin 1993, Holland *et al.* 1994) that have occurred during the radiation of the chordates. Phylum Chordata can be divided into three extant assemblages that have emerged



**Figure 1**

Proposed co-evolution of POMC and the MCR. This evolutionary tree for the chordates presents a working hypothesis for the origin of POMC and the MCRs. In this figure, proteins in white boxes etched with dashed lines are hypothetical, whereas proteins in colored boxes (green for POMC; blue or red for the MCRs) bordered by solid lines have been characterized in at least one species from the taxonomic group. For the MCRs, a blue box indicates a receptor that can be activated by ACTH or  $\alpha$ -MSH, and a red box indicates a receptor that can only be activated by ACTH. The operating assumptions for this proposed scheme are that (a) the genes that code for the ancestral opioid precursor and the ancestral MCR emerged in a lineage of ancestral protochordates; (b) the first genome duplication event (1R) resulted in a gene that coded for POMC and a gene that coded for proenkephalin/prodynorphin precursor, and two genes that coded for paralogous MCRs (MCR' and MCR''); (c) the second genome duplication event (2R) resulted in a gene that coded for POMC and four paralogous MCR genes; (d) a local gene duplication in the genome of the ancestral gnathostomes results in a fifth paralogous MCR gene. Among the extant cartilaginous fishes, all of the paralogous MCRs can be activated by ACTH or  $\alpha$ -MSH (solid blue). Among the extant teleosts and tetrapods, only the MC2R (solid red) has exclusive selectivity for ACTH. 1R, first chordate genome duplication event; 2R, second chordate genome duplication event; POMC, common precursor for ACTH and the MSHs; Proenk/Prodyn, a hypothetical protein that would have been ancestral to the opioid precursors proenkephalin and prodynorphin; MC\*, ancestral MCR; MC' and MC'', predicted hypothetical MCR paralogs after the 1R duplication event; MC1R, melanocortin-1 receptor; MC2R, melanocortin-2 receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; MC5R, melanocortin-5 receptor.

over the past 500 million years (Fig. 1): the protochordates (organisms lacking an internal cartilaginous or bony skeleton), the agnathans (bilateral organisms with an internal support system, but lacking a hinged jaw), and the gnathostomes (bilateral organisms that have an internal cartilaginous or bony skeleton and a hinged jaw). The *pomc* gene is present in the genome of some extant agnathans and all gnathostomes, and is a member of the opioid/orphanin gene family; a collection of precursor proteins genes that each encode at least one opioid (YGGF) or orphanin (FGGF) sequence (Dores *et al.* 2002). In the genomes of extant gnathostomes (i.e. cartilaginous fishes, bony fishes, and tetrapods), there are four genes in the opioid/orphanin family: *pomc*, *proenkephalin*, *prodynorphin*, and *proorphanin* or *pronociceptin*. In addition, there are usually five paralogous *mcr* genes in the genomes of extant gnathostomes.

The scenario to account for the presence of a *pomc* gene and five paralogous *mcr* genes in the genomes of extant gnathostomes goes as follows. During the early radiation of the chordates, an ancestral *opioid precursor* gene and an ancestral *mcr* gene are postulated to have emerged in the genome of a now extinct lineage of the protochordates (Fig. 1; Sundstrom *et al.* 2010, Dores & Baron 2011). During the early radiation of the jawless fishes (Agnatha), a genome duplication event (1R) occurred that resulted in paralogous *opioid precursor* (*proenkephalin/prodynorphin*) and *pomc* genes, and two paralogous *mcr* genes (Fig. 1). It is further postulated that a lineage of the jawless fishes underwent a second genome duplication event (2R). This event gave rise to 2R jawless fish lineages such as the extant lampreys (Smith *et al.* 2013), and to the ancestral gnathostomes (jawed vertebrates; Ohno *et al.* 1968). It is assumed that in the genome of the ancestral gnathostomes there were *proenkephalin*, *prodynorphin*, and *proorphanin* genes (not shown in Fig. 1) and a distinct *pomc* gene. In addition, initially as a result of the 2R event, four paralogous *mcr* genes were postulated, followed by a local gene duplication event to account for the fifth *mcr* gene (Fig. 1; Schiöth *et al.* 2003, 2005).

During the radiation of the gnathostomes, the ancestral cartilaginous fish lineage diverged from the ancestral bony fish lineage (Zhu *et al.* 2013). The ancestral bony fishes later diverged into the ancestral ray-finned bony fishes (ancestors to the modern bony fishes; the teleosts), and the ancestral lobe-finned fishes (represented today by the coelacanth and lungfishes; Rasmussen & Arnason 1999). In addition, at a later time the ancestral tetrapods evolved from lobe-finned fish ancestors (Sallan & Coates 2010). The ancestral tetrapods, in

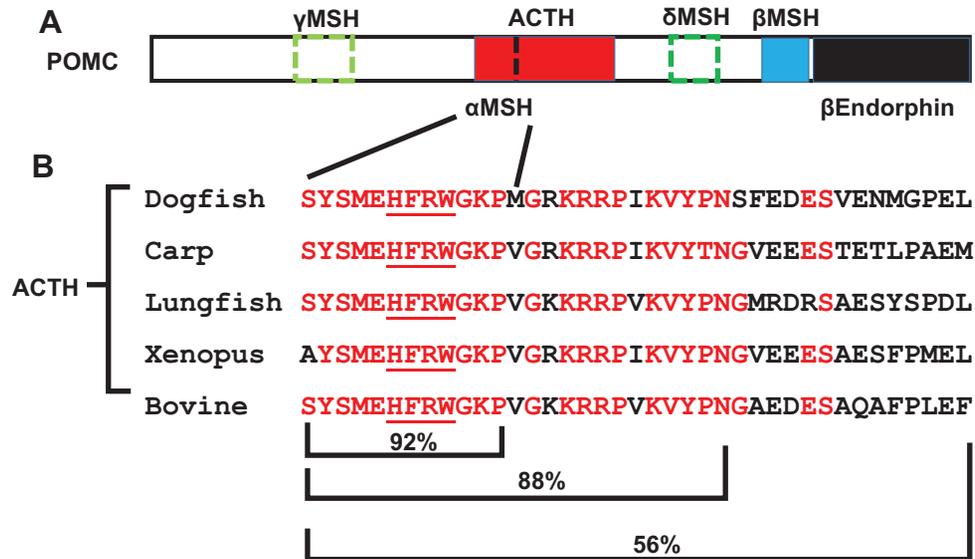
turn, would diverge into the contemporary amphibians, reptiles, birds, and mammals. During the radiation of the gnathostomes, the primary sequences of ACTH and  $\alpha$ -MSH, and the organization of POMC would be subject to selection pressures. In addition, during the radiation of the gnathostomes, selection pressure would play a role in the ligand selectivity properties of the five MCRs.

## Phylogeny of the organization of POMC

One of the striking features of POMC in extant gnathostomes is the adherence to a strict organizational plan (Fig. 2A). All gnathostome POMC sequences minimally have an ACTH sequence roughly in the middle of the precursor, with the  $\alpha$ -MSH sequence embedded in the ACTH sequence, and  $\beta$ -MSH and  $\beta$ -endorphin sequences at the C-terminal end of the precursor (Vallarino *et al.* 2012). Cartilaginous fish POMC sequences and tetrapod POMC sequences have an additional  $\gamma$ -MSH sequence. By contrast, the  $\gamma$ -MSH sequence and much of the N-terminal domain of the precursor have been deleted from teleost POMC sequences (for review see Dores & Baron 2011). In addition, cartilaginous POMC sequences have an additional  $\delta$ -MSH sequence (Takahashi *et al.* 2001). However, the most striking feature of gnathostome POMC sequences is the conservation of the ACTH and  $\alpha$ -MSH sequences (Fig. 2B).

The sequence identity for the first 13 amino acids at the N-terminal of ACTH (the  $\alpha$ -MSH sequence) is 91% for the set of sequences presented in Fig. 2B. By contrast, the  $\gamma$ -MSH and  $\beta$ -MSH sequences for these taxa have sequence identities in the 33–38% range, respectively (Dores & Baron 2011). Within the N-terminal sequence of ACTH, and in all of the MSH-related sequences, there is the HFRW motif (Schwyzer 1977). The presence of this motif in the melanocortin ligands is required for the activation of MCRs (Cone 2006). Finally, the sequence identity for the first 24 amino acids of ACTH is 88%, and ACTH(1–24) has the same biological activity as ACTH(1–39) (Schwyzer 1977). Thus, over a rather long period in the evolution of the gnathostomes, the first 24 amino acids of ACTH have been remarkably conserved.

As ACTH and  $\alpha$ -MSH are utilized in distinct neuroendocrine circuits, the mechanism for selectively producing these chemical signals is tissue-specific endoproteolytic posttranslational processing of POMC (Eipper & Mains 1980). In brief, through the action of distinct prohormone convertases (Seidah & Chretien 1999), the corticotrophic cells of the anterior pituitary will yield ACTH as the primary melanocortin end-product; whereas,

**Figure 2**

Organization of POMC and comparison of vertebrate ACTH sequences. (A) The diagram provides a schematic representation of the positioning of chemical signals within the precursor protein, POMC. ACTH (red box) is positioned in roughly the middle of POMC. The first 13 amino acids in ACTH (marked off by the dashed line) are the sequence of  $\alpha$ -MSH. The  $\beta$ -MSH sequence (blue box) is N-terminal to the sequence for the opioid,  $\beta$ -endorphin (black box). The  $\gamma$ -MSH sequence (white box, light green dash border) is present in cartilaginous fish and tetrapod POMC sequences (Dores & Baron 2011). The  $\delta$ -MSH sequence (white box, dark green dash border) is only found in the POMC sequences of cartilaginous fishes (Takahashi *et al.* 2001). (B) The amino acid sequences of the following ACTH sequences were aligned: dogfish (*Squalus acanthias*; accession number – AAS6720.1), Carp (*Cyprinus carpio*; accession number – CAA74968.1), lungfish (*Neoceratodus forsteri*; accession number – AAD37347.1), xenopus (*Xenopus laevis*; accession number – AAH54160.1), bovine (*Bos taurus*; accession number – NP\_776576.1). Amino acid positions that were identical in four or more of the sequences are in red.

the melanotropic cells of the intermediate pituitary and discrete neurons in the CNS will yield the MSHs (i.e.  $\alpha$ -MSH,  $\beta$ -MSH,  $\gamma$ -MSH, and  $\delta$ -MSH for cartilaginous fishes) as the major melanocortin end-products.

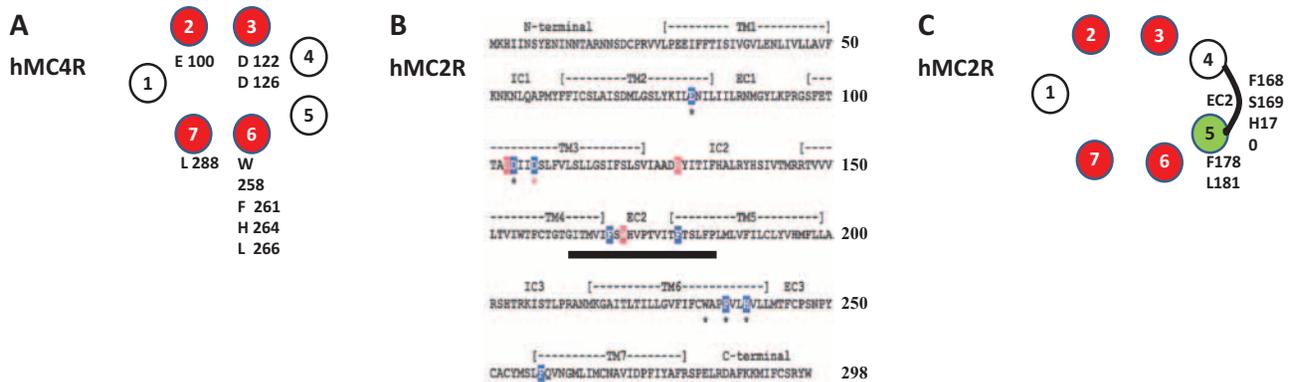
### Ligand selectivity of MCRs

Given the amino acid sequence overlap between ACTH and  $\alpha$ -MSH, it is probably not surprising that some MCRs can be activated by either chemical signal. In this regard, a number of studies have been done on the pharmacological properties of mammalian (Gantz & Fong 2003), avian (Ling *et al.* 2004), amphibian (Dores *et al.* 2014), and teleost (Klovins *et al.* 2004a) MC1R, MC3R, MC4R, and MC5R. Collectively, these studies all show that these paralogs can all be activated by either ACTH or  $\alpha$ -MSH with varying degrees of efficacy. As a result, in Fig. 1, these melanocortin receptor paralogs are represented by blue images. Given this lack of discrimination, the particular physiological function associated with these receptors is based on the anatomical source (i.e. region of the pituitary, selective neurons in the CNS, or paracrine

proximity) of the melanocortin peptides. The chemical communication circuits associated with MC1R, MC2R, MC3R, MC4R, and MC5R will be discussed in the next section on the physiology of MCRs.

Pharmacological studies also have been done on the MCRs from two species of cartilaginous fishes (Ringholm *et al.* 2003, Reinick *et al.* 2012a,b, Liang *et al.* 2013, Klovins *et al.* 2014b), and all of the cartilaginous fish MCRs analyzed, including the MC2R paralog (Reinick *et al.* 2012a), could be activated by either ACTH or  $\alpha$ -MSH. As a result, in Fig. 1, the cartilaginous fish receptor paralogs are also represented by blue images.

The preceding observations on the ligand selectivity can be explained by assuming that there is a common binding site for the HFRW motif of ACTH or  $\alpha$ -MSH on all of the receptors in Fig. 1 coded in 'blue'. Support for this conclusion comes from molecular modeling and site-directed mutagenesis studies on the human melanocortin-4 receptor (MC4R; Pogozeva *et al.* 2005). This study revealed critical amino acids located on TM2, TM3, TM6, and TM7 that facilitate the binding of  $\alpha$ -MSH to human MC4R (Fig. 3A). These residues are located in reasonably close proximity to the extracellular space.

**Figure 3**

Comparison of human MC4R and MC2R. (A) In this schematic representation, the transmembrane domains in the human hMC4R are represented by the spheres and are numbered 1 through 7. The red spheres form the HFRW binding site for  $\alpha$ -MSH (Pogozheva *et al.* 2005). The residues required for facilitating the binding of  $\alpha$ -MSH are listed (hMC4R accession number – NP-005903.2). (B) The amino acid sequence of the human hMC2R is presented (accession number – NP-001278840.1). Amino acid positions that are also found in the HFRW binding site for hMC4R (Pogozheva *et al.* 2005) are marked with asterisk (\*). Amino acid positions where a single alanine substitution decreased the activation of the mutant receptor expressed in OS3 adrenal cells are shaded in blue (Chen *et al.* 2007). Amino acid positions where amino acid substitutions as a result of a spontaneous point mutation that resulted in type 1 FGD and blocked activation of the mutant receptor (Chung *et al.* 2008) are shaded in red. The black bar demarcates the amino acid positions where single alanine mutations were made for the experiments presented in Figs 4 and 5. (C) A schematic representation of MC2R. The transmembrane domains are numbered as in Fig. 3A. The putative HFRW binding site for ACTH is represented by the transmembrane domains shaded in red. The putative KKRRP binding site for ACTH is represented by extracellular loop 2 (EC2) and transmembrane domain 5 shaded in green. The critical residues in this region that affect activation are listed based on the results from Figs 4 and 5 (data from Reinick *et al.* 2012b).

Molecular modeling studies predicted that TM2, TM3, TM6, and TM7 could create a hydrophilic binding pocket for  $\alpha$ -MSH (Pogozheva *et al.* 2005; Fig. 3A). In addition, nearly all of these ‘critical’ residues are present at the same positions in human MC1R, MC2R, MC3R, and MC5R (Pogozheva *et al.* 2005). Subsequent alignment of amino acid sequences of MCR paralogs from organisms as diverse as the marine lamprey and the chicken found that many of the key residues required for  $\alpha$ -MSH binding are conserved in TM2, TM3, TM6, and TM7 were conserved across the breath of vertebrate phylogeny (Baron *et al.* 2009, Dores 2009). These same amino acid positions are also conserved in all of the MCRs of the elephant shark, a cartilaginous fish. Given these observations, it would be reasonable to assume that the MCR paralogs of the ancestral gnathostomes (Fig. 1) also had a single ligand-binding site and could be activated by ACTH or  $\alpha$ -MSH.

By contrast, the ligand selectivity of tetrapod and teleost MC2R orthologs appears more straightforward, but in fact it is much more complicated. Before the characterization of mouse MC2R by Mountjoy *et al.* (1992), studies in the 1970s and 1980s on the activation of the ‘ACTH’ receptor on mammalian adrenal cortex cells found that these cells could be stimulated by ACTH, but not by  $\alpha$ -MSH (Schwyzer 1977). In fact,  $\alpha$ -MSH could not competitively inhibit the binding of ACTH to the ‘ACTH’ receptor (Buckley & Ramachandran 1981). The critical

difference between ACTH and  $\alpha$ -MSH is the presence of the tetrabasic amino acid motif (KKRRP; Fig. 2) in the sequence of ACTH. Removal of this motif renders the ACTH analog inactive (Schwyzer 1977). As a result, Schwyzer (1977) concluded that the ‘ACTH’ receptor had two binding domains; one site accommodated the KKRR motif of ACTH, and the other domain accommodated the HFRW motif of ACTH. The interaction between ACTH and its receptor may involve contact at the proposed KKRR binding site on the receptor, followed by ACTH contact at the HFRW binding site on the receptor. Note that several of the critical amino acid positions required for the HFRW binding site in human MC4R (Pogozheva *et al.* 2005) are present in the sequence of human MC2R (hMC2R; Fig. 3B, residues marked by a \*).

However, there is an unexpected property of tetrapod and teleost MC2R orthologs that confounded studies that attempted to analyze the activation of this receptor. While the gnathostome MC1R, MC3R, MC4R, and MC5R paralogs can be functionally expressed in mammalian cells lines such as Chinese hamster ovary cells (CHO) or human embryonic kidney cells (HEK-293) (Klovins *et al.* 2004a,b, Ling *et al.* 2004, Dores *et al.* 2014), mammalian MC2R orthologs (Noon *et al.* 2002, Rached *et al.* 2005) as well as teleost (Klovins *et al.* 2004a, Agulleiro *et al.* 2010), amphibian (Liang *et al.* 2011), reptilian (Davis *et al.* 2013), and avian (Ling *et al.* 2004, Barlock *et al.* 2014) MC2R

orthologs could not be functionally expressed in these cell lines.

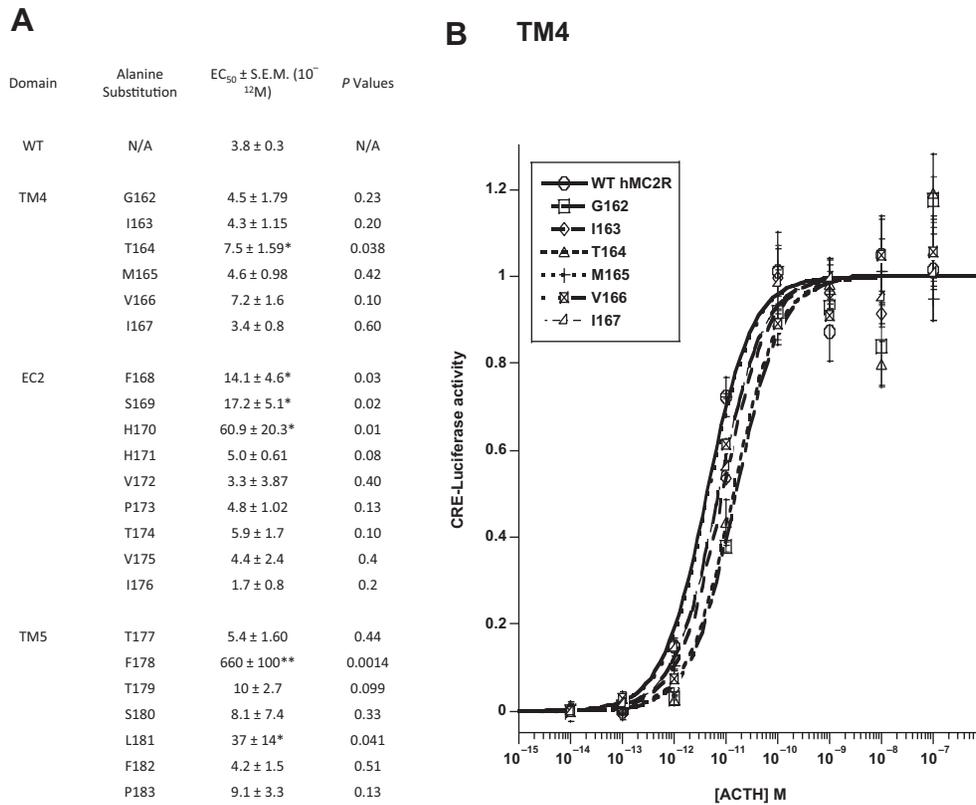
Subsequent studies revealed that the protein required for the functional activation of tetrapod and teleost MC2R orthologs is the melanocortin-2 receptor accessory protein (MRAP or MRAP1; Metherell *et al.* 2005). The MRAPs will be discussed in greater detail in the section on MCR physiology; however, it is now clear that the trafficking of tetrapod and teleost MC2R orthologs to the plasma membrane, and the activation of these orthologs by ACTH requires the formation of a MC2R/MRAP1 heterodimer (for reviews see, Hinkle & Sebag 2009, Webb & Clark 2010, Does & Garcia 2015). In addition, the *mrp1* gene is expressed in glucocorticoid producing cells (i.e. adrenal cortex cells and interrenal cells), adipose cells (Metherell *et al.* 2005), and apparently in several cell lines that express other MCRs such as Cloudman M3 cells (Mountjoy *et al.* 1992) and MIN6 cells (Al-Majed *et al.* 2004).

Once it was understood that functional expression of mammalian MC2R orthologs required either co-expression of the receptor construct with an *mrp1* construct in some heterologous mammalian cell line or expression of MC2R in a cell line derived from the adrenal cortex, Chen *et al.* (2007) did an alanine-substitution study on hMC2R, which revealed several domains in the receptor that participate in the activation of hMC2R by ACTH. The results of those experiments are summarized in Fig. 3B. As predicted from the Pogozheva *et al.* (2005) study, single alanine substitutions at E<sup>80</sup> (TM2), D<sup>104</sup> (TM3), D<sup>107</sup> (TM3), F<sup>235</sup>, and H<sup>238</sup> (TM6) all negatively affected the activation of the mutant receptor following ACTH stimulation. These observations are consistent with the assumption that MC2R orthologs have a HFRW binding site formed by the close interactions between TM2, TM3, and TM6. Chen *et al.* (2007) also hypothesized that phenylalanine residues may play a role in the interactions between the ligand and the receptor, and a single alanine substitution at F<sup>258</sup> is consistent with TM7 forming part of the HFRW binding site (Fig. 3B). However, the surprising finding was that substitutions at F<sup>168</sup> and F<sup>178</sup> blocked activation. F<sup>168</sup> is located on extracellular loop 2 (EC2), and F<sup>178</sup> is close to the surface on TM5 (Fig. 3B and C). EC2 and TM5 are rather far from the proposed HFRW binding site (i.e. TM2/TM3/TM6/TM7), hence, Chen *et al.* (2007) proposed that residues in the tetrabasic domain of ACTH might bind in this region.

Around this time Chung *et al.* (2008) compiled a list of mutations in the human *MC2R* gene that cause type 1 familial glucocorticoid deficiency (FGD). Several of these patients had point mutations that interfered with the

trafficking of MC2R to the plasma membrane. However, a few patients had single point mutations that interfered with the activation of the receptor (Fig. 3B). For example, replacement of D<sup>103</sup> with an asparagine residue or D<sup>107</sup> with an asparagine residue in TM3 decreased activation of the mutant hMC2R by apparently destabilizing the proposed HFRW binding site. Of particular interest is a mutated hMC2R in which H<sup>170</sup> in EC2 is replaced with a leucine residue. This substitution blocked the ACTH activation of the mutated hMC2R (Chung *et al.* 2008). Studies by Fridmanis *et al.* (2010, 2014) also point to the importance of extracellular loop 2 for the activation of hMC2R.

The results of the Chen *et al.* (2007) and Chung *et al.* (2008) studies warranted an in-depth analysis of TM4/EC2/TM5 region of hMC2R. To this end, we made single alanine mutants of hMC2R starting at G<sup>162</sup> in TM4 through P<sup>183</sup> in TM5 (Fig. 3B). The rationale was that residues in TM4 and TM5 that are close to the extracellular space could participate with residues on EC2 to form a docking site for ACTH. Each single-alanine hMC2R mutant construct was co-expressed with a mouse *mrp1* construct in CHO cells, and after 2 days in culture the transiently transfected CHO cells were stimulated with hACTH(1–24). The half maximal effective concentration (EC<sub>50</sub>) values for all the mutant constructs is presented in Fig. 4A, and the dose–response curves for TM4 mutants are presented in Fig. 4B. In addition, the dose–response curves for the EC2 mutants and the TM5 mutants are presented in Fig. 5A and B, respectively. Statistically significant decreases in activation, relative to the positive control, were observed for the following mutant constructs: T<sup>164</sup> (TM4), F<sup>168</sup>, S<sup>169</sup>, H<sup>170</sup> (EC2), F<sup>178</sup>, and L<sup>181</sup> (TM5). The observations for position F<sup>168</sup>, H<sup>170</sup>, and F<sup>178</sup> are consistent with the studies by Chen *et al.* (2007) and Chung *et al.* (2008). However, there is a hierarchy within this domain in terms of the importance of a given amino acid position for the ACTH activation event, and these results bring into focus the relationship between pharmacology and physiology. For a patient with a mutation at H<sup>170</sup>, MC2R is nonfunctional and the patient suffers from type 1 FGD. As shown in Fig. 4A, alanine substitution at this site results in a 16-fold shift in the sensitivity of the mutant receptor for ACTH. It would follow that the shift in EC<sub>50</sub> value at F<sup>178</sup> that is nearly 10-fold higher than the EC<sub>50</sub> value for H<sup>170</sup> would also have a negative effect from a physiological perspective. The alanine substitution at L<sup>181</sup> resulted in a 10-fold shift in the EC<sub>50</sub> value relative to the positive control; whether this mutation would completely block activation of adrenal cortex cells remains to be determined. By contrast,



**Figure 4**

Analysis of single alanine mutants of hMC2R – TM4. For these experiments, hMC2R was synthesized with a V-5 epitope tag, and the mouse MRAP1 (accession number – NM\_029844.3) was synthesized with a FLAG epitope tag by GenScript (Piscataway, NJ, USA). Both cDNA constructs were individually inserted in a pcDNA3.1+ vector for transfection into CHO cells. In addition, single alanine mutants of hMC2R were also made by GenScript, and the mutant cDNAs were individually co-transfected with mMRAP1 in CHO cells. The list of single alanine mutants is presented in panel A. CHO cells were transfected with the following plasmids: either human *MC2R* or human single alanine mutant *MC2R*, mouse *mrp1*, and the cAMP reporter plasmid *cre-luc* (Chepurny & Holz 2007) using a Cell Line Nucleofector Kit (Amaxa, Inc., Allendale, NJ, USA; [www.lonza.com](http://www.lonza.com)) as described by Liang *et al.* (2011). After 48 h, the transfected cells were stimulated with hACTH(1–24) (New England Peptide, Gardner, MA, USA) in serum-free CHO media for 4 h at 37°C at concentrations ranging from 10<sup>-7</sup> to 10<sup>-13</sup> M. After a 4-h incubation period, 100 µL of Bright-Glo Luciferase Assay System (Promega) was applied to each well and incubated at room temperature for 5 min. Luminescence was then measured using a BioTek Synergy HT plate reader. Average values and standard errors of the mean were graphed using KaleidaGraph software (v 4.1; [www.synergy.com](http://www.synergy.com)), and the EC<sub>50</sub> value for each ligand was determined (A). The EC<sub>50</sub> value for the wt *MC2R*/mMRAP1 transfection was compared with the EC<sub>50</sub> value for each mutant *MC2R*/mMRAP1 using the Student's *t*-test (*N*=3). (A) This figure has the EC<sub>50</sub> values for the 22 single alanine mutant *MC2R* constructed tested. Single asterisk (\*) indicates a drop in the EC<sub>50</sub> value of the mutant *MC2R* relative to the positive control with a *P* value between 0.04 and 0.01. Double asterisks (\*\*) indicate a drop in EC<sub>50</sub> values of the mutant *MC2R* relative to the positive control with a *P* value <0.01. (B) The dose-response curves for the transmembrane 4 (TM4) mutant constructs are presented.

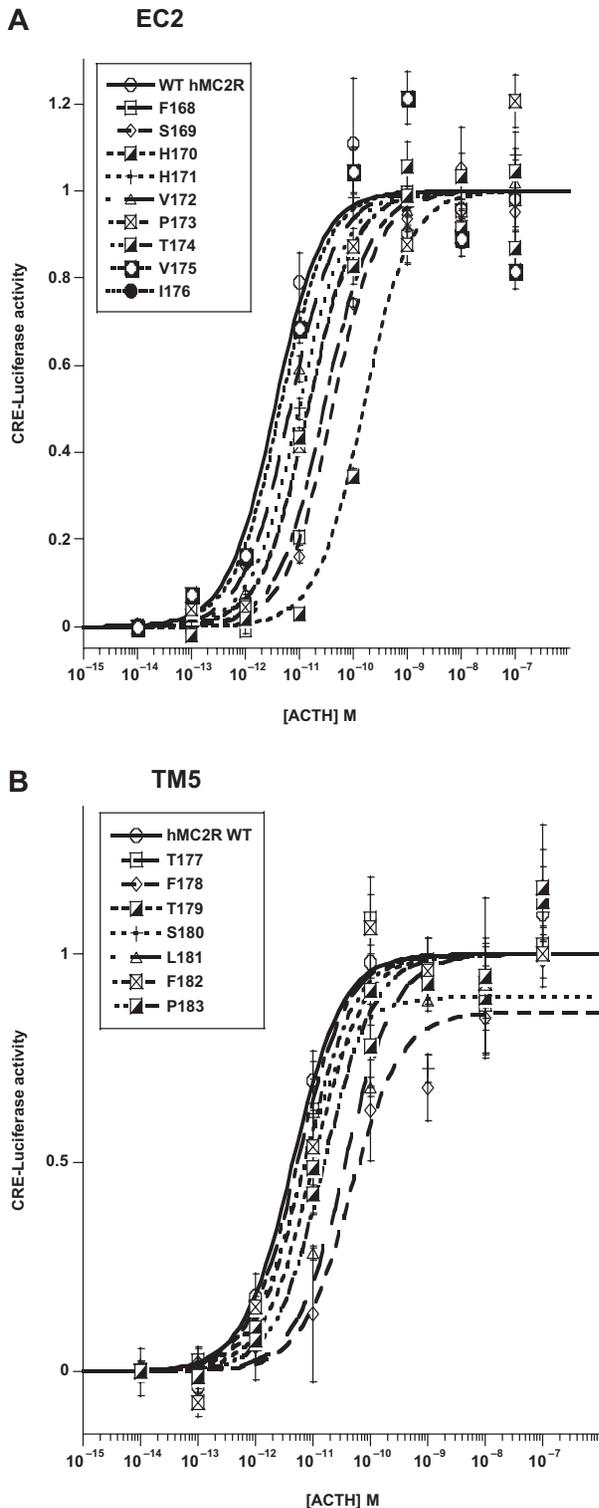
the two-fold shift observed for the T<sup>164</sup>/A<sup>164</sup> mutant may not be physiologically significant. Hence, the hierarchy at the proposed KKRR binding site on *MC2R* appears to be F<sup>178</sup>=H<sup>170</sup>>L<sup>181</sup>>S<sup>169</sup>=F<sup>168</sup>. Clearly, additional experiments are needed to verify that the EC2/TM5 region of *MC2R* is the location of the KKRR binding domain. However, it would appear, from a therapeutic perspective, that designing analogs of ACTH or drugs that selectively target the EC2/TM5 proposed binding site, and act as competitive inhibitors of ACTH, may be more efficacious than substances that target the HFRW binding site common to all MCRs.

## Physiology of MCRs

The MCRs mediate endocrine, neuronal, and paracrine chemical communication circuits (Cone 2006). MRAP1 (Metherell *et al.* 2005) and MRAP2 (Chan *et al.* 2009) add another layer of complexity to the regulation of some of these circuits.

## MC2R

*MC2R* plays a critical role in the hypothalamus/pituitary/adrenal (HPA) axis in mammals, birds, and reptiles



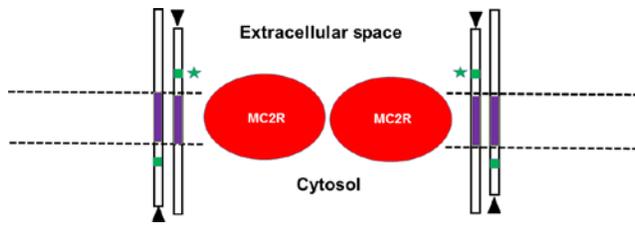
**Figure 5**  
Analysis of single alanine mutants of hMC2R – EC2 and TM5. The activation assays were done as described in the legend for Fig. 4. (A) Presentation of the dose–response curves for extracellular loop 2 (EC2) mutant constructs. (B) Presentation of the dose–response curves for the transmembrane 5 (TM5) mutant constructs.

(Vale *et al.* 1981), and the hypothalamus/pituitary/interrenal (HPI) axis in amphibians and teleosts (Dores & Garcia 2015). ACTH released from the anterior pituitary binds to MC2R on adrenal cortex cells (HPA axis) or to MC2R on interrenal cells (HPI axis), and leads to the synthesis and release of glucocorticoids from these steroid hormone-producing cells. The intracellular response to the binding of ACTH by mammalian adrenal cortex cells has recently been extensively reviewed (Gallo-Payet & Batista 2014), and a key feature of this signal transduction process is the interaction between MC2R and MRAP1.

As noted earlier, point mutations in hMC2R that interfere with the trafficking of the receptor to the plasma membrane or the activation of the receptor result in type 1 FGD (Chung *et al.* 2008), the inability of the adrenal cortex to respond to ACTH. However, there is a subpopulation of patients that have no point mutations in the MC2R gene, yet are incapable of responding to ACTH. This observation coupled with the realization that mammalian MC2R orthologs could not be functionally expressed in many nonadrenal mammalian cell lines (Noon *et al.* 2002, Rached *et al.* 2005) led to the discovery of the accessory protein MRAP1 (Metherell *et al.* 2005), and later to the discovery of its paralog, MRAP2 (Chan *et al.* 2009).

MRAP1 and MRAP2 are type 1/type 2 transmembrane proteins with a single transmembrane spanning domain. The type 1/type 2 designation comes from the observation that both MRAP1 and MRAP2 can form a homodimer at the rough endoplasmic reticulum (RER), and the homodimer has dual topology (Sebag & Hinkle 2007). Dual topology refers to the observation that at the plasma membrane, one monomer of the homodimer has the N-terminal domain oriented in the extracellular space (type 1), while the other monomer of the homodimer has the N-terminal domain oriented in the cytosol (type 2; Fig. 6A). MC2R and MRAP1 will come together to form a heterodimer complex at the RER as a result of contact between the transmembrane domain of MRAP1 and an unidentified transmembrane domain in MC2R (Cooray *et al.* 2008, Sebag & Hinkle 2009). Cooray *et al.* (2011) observed that the MC2R/MRAP1 heterodimer complex consisted of a MC2R homodimer and two MRAP1 homodimers as depicted in Fig. 6.

Within each MRAP1 N-terminal domain, there is a four amino acid activation motif (Fig. 6) that makes contact with MC2R. As a result of this interaction, at the plasma membrane ACTH can efficiently bind to the MC2R/MRAP1 heterodimer complex and initiate



**Figure 6**  
Interaction between MRAP1 and MC2R. This schematic presentation of the MC2R/MRAP1 heterodimer is based on data from the study by Cooray *et al.* (2011), with some modifications as a result of the study by Malik *et al.* (2015). MC2R (red sphere) will form a homodimer with two MRAP1 homodimers. In the MRAP1 homodimer, the N-terminal domain of each monomer is capped by a black triangle. The green box in the N-terminal domain is the activation motif. The purple zone on each monomer is the transmembrane domain. A star marks the activation motif that will make contact with an extracellular domain on MC2R.

activation of the receptor (Hinkle & Sebgag 2009, Webb & Clark 2010). For example, the activation motif in the N-terminal of mouse MRAP1 is L<sup>18</sup>D<sup>19</sup>Y<sup>20</sup>I<sup>21</sup> (Sebgag & Hinkle 2009). It is important to note that these residues are absent from the amino acid sequences of mammalian MRAP2 paralogs. As a result, the MC2R/MRAP2 heterodimer complex can move from the ER to the plasma membrane, but activation of this heterodimer by ACTH is extremely weak (Chan *et al.* 2009, Hinkle & Sebgag 2009, Webb & Clark 2010). Thus, the formation of the MC2R/MRAP1 heterodimer is critical for activation of the heterodimer complex by ACTH, and mutations to hMRAP1 that either disrupt the activation motif, disrupt the formation of the MRAP1 homodimer, or mutations in the transmembrane domain of MRAP1 can result in type 2 FGD (Metherell *et al.* 2005).

More recent studies have shown that avian, reptilian, amphibian, and teleost MC2R orthologs also require interaction with MRAP1 for functional activation by ACTH (Aguilleiro *et al.* 2010, Liang *et al.* 2011, Davis *et al.* 2013, Barlock *et al.* 2014). That said, given the dual topography of the MRAP1 homodimer (Fig. 6), which of the two activation motifs facilitates activation of the receptor or do both activation motifs make contact with MC2R? Recently, this question was addressed by Malik *et al.* (2015). In this study, a cDNA was designed in which two monomers of mouse MRAP1 were connected by a linker sequence, and the MRAP1 tandem sequence was linked to the N-terminal of hMC2R. The chimeric *mrp1/mc2r* cDNA construct was transiently expressed into HEK-293 cells, and then the transfected cells were stimulated with hACTH(1–24). In this experiment, the transfected cells responded in a dose-dependent manner to stimulation by ACTH

(Malik *et al.* 2015). In another experiment, a new chimeric MC2R/MRAP1 protein was made in which the activation motif on the MRAP1 monomer oriented toward the cytosol was inactivated, and an alanine was substituted for E<sup>80</sup> in hMC2R (Fig. 3B). HEK-293 cells were transfected with this mutant cDNA construct, and then stimulated with hACTH(1–24). No activation was observed in this experiment as a result of the E<sup>80</sup> to A<sup>80</sup> mutation in the MC2R region of the mutant chimeric protein (Malik *et al.* 2015). However, when this mutant chimeric *mc2r/mrp1* cDNA construct was co-expressed with a wt *hmc2r* cDNA construct, the transfected cells did respond to stimulation by hACTH(1–24) in a dose-dependent manner (Malik *et al.* 2015). The reciprocal experiment in which the activation motif on the MRAP1 monomer oriented toward the extracellular space was disrupted resulted in a mutant chimeric MC2R/MRAP1 protein that could not facilitate the activation of wt MC2R following stimulation of the co-transfected cells with hACTH(1–24). Collectively, these experiments indicate that the activation motif on the N-terminal domain of the MRAP1 monomer oriented on the extracellular space is interacting with some extracellular domain on hMC2R (Fig. 6). What remains to be determined is the exact contact site on MC2R. Presumably, the same extracellular domain on avian, reptilian, amphibian, and teleost MC2R orthologs is interacting with the activation motif on the extracellular N-terminal domain of the corresponding MRAP1.

The dependence of teleost and tetrapod MC2R orthologs on MRAP1 for trafficking and activation appears to parallel the proposed emergence of the *mrp1* gene in an ancestral bony fish lineage (Vastermark & Schiöth 2011). This lineage emerged after the divergence of the ancestral bony fishes and the ancestral cartilaginous fishes from a common ancestral gnathostome ancestor (Davis *et al.* 2012). In support of this conclusion, the MC2R ortholog in the genome of the elephant shark, a cartilaginous fish, does not apparently require MRAP1 for trafficking to the plasma or for activation (Reinick *et al.* 2012b). In addition, since this MC2R ortholog can be activated by either ACTH of  $\alpha$ -MSH, as can all of the cartilaginous fish MCR paralogs (Fig. 1), it is possible that cartilaginous fish have multiple HPI axes that are regulated by distinct hypothalamus/anterior pituitary and hypothalamus/intermediate pituitary circuits (Liang *et al.* 2013). Clearly, more detailed studies on the neuroanatomy, pharmacology, and physiology of the HPI axis in cartilaginous fishes are needed to resolve these issues.

## MC5R

There are several features that set MC5R apart from the other MCRs. For example in mice, MC5R has the broadest tissue expression distribution of all the MCRs (Chen *et al.* 1997), yet the corresponding physiological role(s) of this receptor in gnathostomes are not well understood. For mice, MC5R has been implicated in the regulation of secretion by some exocrine glands (Chen *et al.* 1997, van der Kraan *et al.* 1998, Zhang *et al.* 2011), and this receptor may also be involved in the regulation of thermoregulation in mice (Chen *et al.* 1997).

In the genomes of vertebrates as diverse as humans and pufferfish, the *mc5r* gene is found on the same chromosome as the *mc2r* gene (Klovins *et al.* 2004a), presumably as a result of a local gene duplication event. As might be predicted for two genes diverging independent of each other, the amino acid sequence identity for hMC2R and MC5R is only 39%, whereas the amino acid sequence identity for human MC5R and human MC4R is 60%. This observation has led to the alternative speculation that the *mc5r* gene might have been the result of a local gene duplication of the *mc4r* gene (Vastermark & Shioth 2011). As a *mc4r*-like gene might have been the ancestral *mcr* gene (Dores 2013), the amino acid sequence identity data would suggest that there are selection pressures to minimize the accumulation of mutations in the *mc5r* gene relative to the *mc2r* gene. However, this conclusion seems counterintuitive given the critical role that MC2R plays in the HPA/HPI axis, and the importance of HPA/HPI axis to the physiological fitness of vertebrates (Crespi *et al.* 2013).

Another peculiarity of MC5R is the co-expression of the *m5r* gene and the *mc2r* gene in the adrenal cortex cells of a bird (Takeuchi & Takahashi 1998), and the interrenal tissue of an amphibian (Dores & Garcia 2015) and several teleost fishes (Haitina *et al.* 2004, Metz *et al.* 2005, Aluru & Vijayan 2008, Kobayashi *et al.* 2011b). At present, it is unclear what role MC5R may be playing in the response to ACTH stimulation at these glucocorticoid-producing cells, and whether interactions between MRAP1 and MC5R in these cells play a role in the ligand selectivity of MC5R.

## MC4R and MC3R

MC4R and MC3R are expressed by distinct neurons in the CNS (Cone 2006) that synapse with neurons that express the *pomc* gene, and produce a  $\alpha$ -MSH-related end-product. The MC3R neuronal circuit is involved with the control of ingestive behaviors and regulation of metabolic expenditure/homeostasis (Renquist *et al.* 2011,

Begrache *et al.* 2013), whereas the MC4R circuit in the CNS regulates satiety (Cone 2006). The neuronal MC4R and MC3R circuits in the hypothalamus are of particular interest due to the fact that the *mrap2* gene is expressed in this region of the brain in organisms as diverse as mice (Asai *et al.* 2013) and zebrafish (Sebag *et al.* 2013).

The role of MC4R as a regulator of feeding behavior in mice was established not long after the characterization of the *mc4r* gene (Huszar *et al.* 1997). The binding of  $\alpha$ -MSH to neurons in the hypothalamus that express *mc4r* will decrease feeding behavior. Conversely, point mutations of the *mc4r* gene that render the receptor nonfunctional result in the onset of obesity (Santini *et al.* 2009). Taking advantage of the observation that the expressions levels of the *mrap2* gene are high in the hypothalamus of mice, Asai *et al.* (2013), observed that brain-specific deletion of the *mrap2* gene resulted in obese mice. Subsequent co-expression of mouse *mc4r* and *mrap2* in HEK-293 cells resulted in a higher sensitivity of *mc4r* to stimulation by  $\alpha$ -MSH. Hence, it appears that *mrap2* can interact with *mc4r* to enhance the neuronal circuit in the mouse brain to suppress feeding behavior.

The effects on the regulation of feeding behavior are not restricted to mice. The zebrafish genome has two MRAP2 paralogs, MRAP2a and MRAP2b (Agulleiro *et al.* 2010). Sebag *et al.* (2013) observed that during the larval stage of zebrafish development, a period when growth is a primary objective, MRAP2a will interact with MC4R to lower activation of this receptor, and as a result the suppression of feeding *via* the MC4R circuit is decrease. This mechanism would allow the larval zebrafish to grow faster. During the larval stage of development, the expression levels of the *mrap2b* gene are low. In the adult stage of the zebrafish life cycle, the expression levels of the *mrap2a* gene decline, and the expression levels of the *mrap2b* gene increase. Sebag *et al.* (2013) found that co-expression of zebrafish MRAP2b and zebrafish MC4R in HEK-293 cells increases the sensitivity of MC4R for  $\alpha$ -MSH; the same response was found in the study by Asai *et al.* (2013). Regulating food intake appears to improve the fitness of adult zebrafish (Sebag *et al.* 2013). These results are of interest not only because they present a function for MRAP2, but they also suggest that functional variants of MRAP2 may be present in other vertebrates besides the zebrafish. At present, the sites on MRAP2 and MC4R that make contact to effect ligand sensitivity have not been determined.

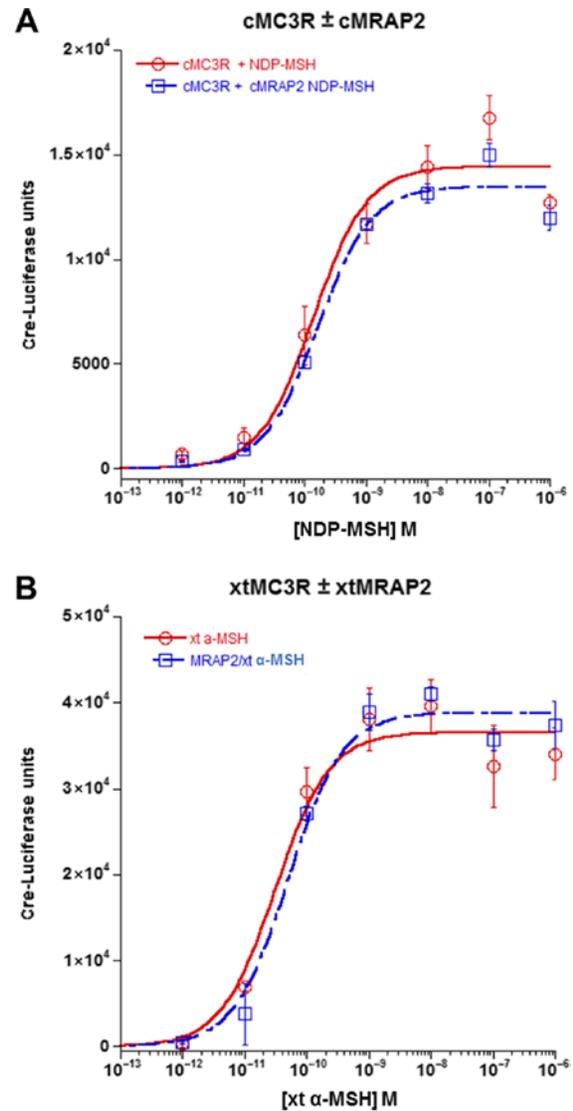
Given the observations on the interactions between MRAP2 and MC4R on neurons in the hypothalamus, the possibility that MRAP2 may interact with MC3R on neurons in the hypothalamus should be considered. Chan *et al.* (2009) found that co-expression of human MRAP2

and human MC3R in HEK-293 cells did not have an effect on the trafficking of MC3R to the plasma membrane, but there was a statistically significant drop in sensitivity to activation by  $\alpha$ -MSH. To determine whether other tetrapod MC3R orthologs are impacted by interaction with MRAP2, we co-expressed *Gallus gallus* (chicken; Fig. 7A) and *Xenopus tropicalis* (frog; Fig. 7B) MC3R orthologs separately in CHO cells in the presence or absence of their respective MRAP2 ortholog. The transfected cells were stimulated with  $\alpha$ -MSH, and in neither species was there an indication of a shift in ligand sensitivity as a result of co-expression with their species-specific MRAP2. It will be important to repeat these analyses for other vertebrate MC3R orthologs. It is possible that with the exception of human MC3R, other vertebrate MC3R orthologs may be resistant to interaction with MRAP2.

### MC1R

The role of the endocrine system in the regulation of color change in vertebrates was appreciated long before the characterization of  $\alpha$ -MSH or any of the MCRs (Waring 1942). However, the characterization of  $\alpha$ -MSH (Lee & Lerner 1956) established the link between the source of the color-changing hormone, the intermediate lobe of the pituitary, and the pigment-producing melanocytes and chromatophores in the integument. The mechanism for the translocation of pigment vesicles along with the microtubule networks in the transparent chromatophores of teleosts would be resolved before the characterization of MC1R (Mountjoy *et al.* 1992), and not long after the characterization of MC1R, long-acting analogs of  $\alpha$ -MSH would be discovered (Jiang *et al.* 1997). After the characterization of MC1R, studies on the regulation of coat color in mice led to the discovery of the agouti protein, an antagonist of MC1R and MC4R (Cone 2006). Recent studies on the melanophores and xanthophores in the integument of the teleosts, *Verasper moseri* and *Carassius auratus* have detected multiple MCRs in these pigmented cells (Kobayashi *et al.* 2010, 2011a,b), and have revealed that the acetylation state of  $\alpha$ -MSH has an effect on the activation of these pigmented cells (Kobayashi *et al.* 2012). The presence of multiple MCRs on the plasma membrane of teleost pigmented cells raises the possibility that receptor heterodimers may form and could have a role in the signal transduction process.

Although the activation of pigmented cells by pituitary-derived melanocortins has been a major focus of the research on the MC1R-mediated system in the integument, the relevance of these studies for humans has been obtuse



**Figure 7**

The effect of MRAP2 on the activation of chicken and frog MC3R. (A) A chicken (*Gallus gallus*) *mc3r* (cMC3R; accession number – BAA32555.1) cDNA was made by GenScript, and the cDNA was inserted into the pcDNA3.1+ expression vector. The *cmc3r* cDNA was expressed alone or co-expresses with a chicken *mrp2* cDNA (cMRAP2; accession number – XP\_004940463) in CHO cells as described in the legend for Fig. 4. All cells were transfected with the *cre-luc* cAMP reporter construct (Chepurny & Holz 2007). The transfected cells were stimulated with NDP-MSH (New England Peptide) at concentrations ranging from 10<sup>-12</sup> to 10<sup>-6</sup> M. Data are presented as mean ± s.e.m. (N=3). The EC<sub>50</sub> value for cMC3R was 1.4 × 10<sup>-10</sup> M ± 5.4 × 10<sup>-11</sup>, and the EC<sub>50</sub> value for cMC3R/cMRAP2 was 7.6 × 10<sup>-10</sup> M ± 4.7 × 10<sup>-11</sup>. (B) A frog (*Xenopus tropicalis*) *mc3r* cDNA (xtMC3R; accession number – XP\_002935936) was made by GenScript, and this cDNA was expressed either in the presence or absence of *X. tropicalis* *mrp2* cDNA (xtMRAP2; accession number – XP\_002933993) in CHO cells and stimulated with xt  $\alpha$ -MSH (New England Peptide) at concentrations ranging from 10<sup>-12</sup> to 10<sup>-6</sup> M. Data are presented as mean ± s.e.m. (N=3). The EC<sub>50</sub> value for xtMC3R was 1.1 × 10<sup>-11</sup> M ± 2.5 × 10<sup>-11</sup>, and the EC<sub>50</sub> value for xtMC3R/xtMRAP2 was 6.5 × 10<sup>-11</sup> M ± 1.3 × 10<sup>-11</sup>. Note that in (A) and (B), co-expression with the species-specific MRAP2 ortholog has no effect on the dose–response curves for either MC3R ortholog.

since the human pituitary lacks an intermediate lobe. However, the detection of POMC-derived end-products in human keratinocytes provides a mechanism for the paracrine activation of MC1R on melanocytes in response to UV exposure (Schauer *et al.* 1994, Slominski *et al.* 2000, Scott *et al.* 2002). In addition, chronic stressors will promote the release of ACTH from the anterior lobe of the pituitary that can also activate MC1R receptors on human melanocytes (Costin & Hearing 2007). This chronic stress response on integument color change by the anterior pituitary also appears to occur for an amphibian and a reptile (Greenberg 2002, Kindermann *et al.* 2013).

Whether MRAP2 plays a role in the physiology of melanocytes or melanophores remains to be determined. Pharmacological studies indicate that trafficking of human MC1R expressed in HEK-293 to the plasma membrane is not affected by co-expression with MRAP2, but sensitivity to  $\alpha$ -MSH was statistically lowered as a result of co-expression of human MC1R and human MRAP2 (Chan *et al.* 2009).

## Conclusions

The physiology of the melanocortin receptor-mediated networks is contingent upon the source of the melanocortin peptides (i.e. the pituitary, discrete neurons in the CNS, paracrine release) and the potential interactions with the accessory proteins, MRAP1 and MRAP2. MRAP1 is an essential component of the HPA/HPI axis of teleosts and tetrapods, and appears to dictate the strict ligand selectivity of teleost and tetrapod MC2R orthologs for ACTH. MRAP2 appears to be an important component of the hypothalamus MC4R circuit involved with the regulation of feeding. The roles that MRAP accessory proteins play in the physiology of cartilaginous fish MC2R-mediated processes and MC4R-mediated process remains to be determined. Similarly, the roles these accessory proteins may or may not play in the physiology of gnathostome MC5R-, MC3R-, and MC1R-mediated processes remains to be determined.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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