FUNCTIONAL CHARACTERIZATION AND PHARMACOLOGICAL RESCUE OF THE

PORCINE AND HUMAN NEURAL MELANOCORTIN RECEPTORS

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FUNCTIONAL CHARACTERIZATION AND PHARMACOLOGICAL RESCUE OF THE PORCINE AND HUMAN NEURAL MELANOCORTIN RECEPTORS

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DISSERTATION ABSTRACT

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During the past decade, numerous studies showed that both melanocortin-3 and -4 receptors (MC3R and MC4R) are critically involved in regulation of mammalian energy balance. The MC4R plays a vital role in regulation of food intake and energy expenditure, and the MC3R is critical in regulation of fat storage/adiposity.

In this study, porcine (p) MC3R was cloned, expressed and functionally characterized. Sequence analysis revealed that pMC3R was highly homologous (>80%) at nucleotide and amino acid sequences to human, rat, and mouse MC3Rs. Pharmacological analysis showed that pMC3R bound NDP-MSH with the highest

affinity followed by D-Trp^8-γ-MSH, γ-MSH and α-MSH. Both pMC3R and hMC3R bound Agouti-related protein (AgRP) with high affinity. D-Trp^8-γ-MSH was the most potent agonist to stimulate cAMP generation followed by NDP-MSH, γ-MSH, and α-MSH. These results suggest that D-Trp^8-γ-MSH is the best tool for *in vivo* studies.

Two naturally occurring pMC4R mutants, D298N and R236H, were functionally characterized. Both mutant pMC4Rs had similar binding capacities and affinities for the natural agonist α-MSH and the natural antagonist AgRP as wild-type (WT) pMC4R. In signaling assays, both mutants had normal EC_{50} and maximal signaling to α-MSH. Thus, both mutants do not have any overt functional defects. Therefore we urge caution using these variants as genetic markers for selection in breeding programs.

In addition, ten human (h) MC4R mutants were functionally characterized. The results showed that R7C had decreased cell surface expression but had similar binding capacity and signaling potency as WT MC4R. C84R, S127L, W174C and F261S were defective in either total or cell surface expression or both resulting in mutants with impaired function. A219V had normal total and cell surface expressions; however, its ability for binding and signaling were significantly reduced. P230L had decreased cell surface expression but had similar signaling potency as the WT hMC4R. S136F had normal total and cell surface expression and ligand binding but was defective in signaling. I317V and L325F might be polymorphisms of hMC4R. We also tested the effect of an MC4R antagonist, ML00253764, on the cell surface expression of C84R and W174C. The results showed that the cell surface expression and signaling of the mutants were rescued. These results suggested a novel way to treat obese patients carrying intracellularly retained MC4R mutations.

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CHAPTER I

INTRODUCTION

1.1. GENERAL INTRODUCTION

The melanocortin receptors (MCRs) consist of five members and are named MC1R to MC5R according to the sequence of their cloning (Gantz and Fong, 2003). Tissue distribution analysis demonstrated that MC1R is specifically expressed in the skin and is the classical melanocyte-stimulating hormone (MSH) receptor to regulate pigmentation. The MC2R, previously called ACTH receptor (ACTHR), is expressed in the adrenal gland and plays a critical role in regulating adrenal steroidogenesis and growth. MC5R is mainly expressed in exocrine glands and regulates exocrine gland secretions (Chen, et al. 1997). In contrast to the other MCRs, both MC3R and MC4R are expressed primarily in the central nervous system. Thus, MC3R and MC4R are neuronal MCRs.

Sequence comparison and functional analysis showed that the MCRs are members of the rhodopsin-like G protein-coupled receptors (GPCRs) (also called family A GPCRs). As is typical of GPCRs, MC3R and MC4R share the common heptahelical structure of GPCRs that are connected by alternating extracellular and intracellular loops with an extracellular NH_2 terminus and an intracellular COOH terminus. Both receptors couple to the stimulatory heterotrimeric G protein (Gs) upon stimulation with agonists and subsequently activate the adenylyl cyclase to raise the intracellular cAMP levels (Gantz,

et al. 2003). The downstream signaling pathway of MC3R and MC4R is cAMP-dependent. The activities of MC3R and MC4R are regulated by a balance finely maintained between MSHs and Agouti-related protein (AgRP). MSHs, including α-, β-, and γ-MSH, are short peptide derivatives of a precursor polypeptide called pro-opiomelanocortin (POMC) and serve as endogenous agonists of either MC3R (α- and γ-MSH) or MC4R (α- and b-MSH). POMC can be produced in several tissues and processed posttranslationally in a tissue-specific manner. The processing is catalyzed by either prohormone convertase 1 (PC1) alone for generation of ACTH or PC1 and PC2 for production of MSHs (Smith, et al. 1988). he physiological activities of MC3R and MC4R are inhibited by AgRP acting as an endogenous antagonist (Ollmann, et al. 1997). AgRP are synthesized and secreted from a subset of neurons, called AgRP neurons that are, like POMC neurons, located in the arcuate nucleus of hypothalamus.

MC3R and MC4R are downstream mediators of the leptin-regulated pathway that has previously been shown critical in controlling food intake and body weight. In this pathway, leptin plays a critical role in regulating the downstream signal transduction. Leptin (LEP), a small polypeptide molecule, is released into the bloodstream once synthesized by the adipocyte. Leptin can cross the blood-brain barrier and subsequently bind to the leptin receptors (LEPR) expressed on the cell surface of the AgRP and POMC neurons in the arcuate nucleus of the hypothalamus. The binding of leptin to leptin receptor upregulates the POMC expression and thus increases the release of α-, β- and γ-MSH. On the other side, this binding also inhibits AgRP expression and release from the AgRP neurons. The overall outcome of this event is the activation of MC4R thus resulting in decreased food intake and increased energy expenditure.

Considering the importance of the leptin-regulated pathway in controlling food intake and body weight, it is reasonable to expect that functional disruptions of any genes involved in this pathway will disturb the energy balance. So far, mutations in six genes including leptin (*Lep*), leptin receptor (*Lepr)*, prohormone convertase 1 (*PC1*), pro-opiomelanocortin (*Pomc*), melanocortin-4 receptor (*Mc4r*), and single-minded homolog 1 (*Sim1*) are found to cause monogenic early-onset obesity in both rodent and human (Synder, et al. 2004). Among those genes, *Mc4r* has been identified as a key switch in the leptin-regulated pathway (Huszar, et al. 1997; Graham, et al. 1997; Ollmann, et al. 1997; Fan, et al. 1997; Thiele, et al. 1998). Mutations occurred in the coding region of the *MC4R* gene represent the most common monogenic form of obesity. In some population groups, it was estimated that ~6% of early-onset obesity was caused by mutated MC4Rs (Farooqi, et al. 2003a). Currently, mutations in *MC4R* serve as the best available genetic model to investigate human obesity (Spiegelman, et al. 2001; Cone, 2005; Tao, 2005). The possible application of naturally occurring loss-of-function *MC4R* mutations has been investigated in domestic pigs to improve pork production.

Unlike MC4R, the evidences from rodent genetic and pharmacological studies did not support a role of MC3R in regulating food intake and energy expenditure. However, accumulating data from rodent studies showed that this receptor might play an important role in fat storage/feeding efficiency. In addition, screening efforts of the *MC3R* mutations from obese patients are not very successful. So far, a total of eight *MC3R* mutations have been identified and functionally characterized (Tao, 2007). The causative relationship of the *MC3R* mutations to human adiposity remains to be established.

Over the past few years, detailed functional analysis of naturally occurring *MC3R* and *MC4R* mutations revealed multiple receptor defects such as intracellular retention, inability to bind ligand or responding to ligand stimulation (Tao, 2005). Currently, these mutant receptors are grouped into five classes according to the classification scheme proposed by Tao and Segaloff in 2003 (Tao and Segaloff, 2003). Insights from these analyses not only add significant understanding of the structure and function of these receptors but also elucidate the molecular mechanism of how the mutations affect receptor functions, highlighting potential therapeutic options.

Functional analysis of the naturally occurring MC4Rs mutations revealed that the majority of the MC4R mutants are defective in receptor maturation/trafficking. It was noticed that several loss-of-function MC4R mutants such as N62S and P78L (Tao and Segaloff, 2003) have residual activities in terms of hormone-stimulated cAMP generation indicating that these mutations might not disrupt ligand binding and signaling. Thus, any approach that leads to increased expression of the mutant receptors on the cell surface could potentially be of therapeutic value. Small molecule pharmacological chaperones might be reagents to reach this goal (Tao, 2005; 2006). Currently, pharmacological chaperones such as SR49059 have been employed in preclinical trials to treat nephrogenic diabetes insipidus caused by transportation-defective vassopressin V2 receptor (V2R) (Bernier, et al. 2006; also see review Conn et al., 2007).

1.2. LITERATURE REVIEW

1.2.1. Gene structures and tissue distributions of the neuronal MC3/4R

The human *MC4R* was first cloned by Gantz, et al. (1993a) using a adegenerate PCR method. The human *MC4R* gene consists of a 5′-nontranscriptional region, a coding region harboring a single exon and a 3′-nontranscriptional region. The open reading frame (ORF) of the human *MC4R* is 999 bp in length encoding a small protein of 332 amino acids (Gantz, et al. 1993a). Genomic localization assay showed that human *MC4R* gene is mapped to chromosome 18q21.3 (Magenis, et al. 1994). Over the past decades, *MC4R*s from other species have been cloned from mammals including mouse (Magenis, et al. 1994), rat (Mountjoy, et al. 1994), pig (Kim, et al. 2000; Fan, et al. 2008a), cattle (Haegeman, et al. 2001) as well as nonmammalian species such as trout (Haitina, et al. 2004), flounder (Kabayashi, et al. 2008) and goldfish (Cerdá-Reverter, et al. 2003). MC4Rs from mammals all share the same gene structure and have the same coding size as that of the human *MC4R*. However, *MC4R*s from non-mammalian species, although all containing a single exon like the human *MC4R*, might have a coding region with different sizes. For example, the ORF of flounder *MC4R* is only 978 bp in size and thus encodes a protein of 325 amino acid residues, 7 amino acids shorter than that of the mammalian MC4Rs. The *MC4R* gene of trout encodes a 339 amino acid protein with 7 more amino acids compared to human *MC4R*. Tissue distribution analysis in rodents demonstrated the expression of the MC4R in the paraventricular nuclei (PVN) of the hypothalamus in the brain. In addition, high level expression of the MC4R is also detected in the brainstem (Mountjoy, et al. 1994).

The human *MC3R* was first cloned by Gantz and coworkers (1993b). The chromosome location of human *MC3R* is 20q13.2 (Gantz, et al. 1993b; Magenis, et al. 1994). Like human *MC4R* gene, the human *MC3R* consists of a 5′-nontranscriptional region, a coding region and a 3′-nontranscriptional region. The coding region of human *MC3R* contains a single exon of 1083 bp encoding a protein of 360 amino acid residues. After the cloning of the human *MC3R*, *MC3R*s from other mammals including mouse (Desarnaud, et al. 1994), rat (Roselli-Rehfuss, et al. 1993), pig (Fan, et al., 2008b) and elephant (Rompler, et al. 2006) have been cloned. In lower vertebrates, the *MC3R*s have been cloned from the chicken (Takeuchi and Takahashi, 1999), zebrafish (Logan, et al. 2003) and dogfish (Klovins, et al. 2004). *MC3R*s from different species share the same gene structure but might have a coding size different from human *MC3R*. For example, the ORFs of mouse and dogfish MC3Rs are 972 bp and 981 bp in size, respectively, and encode a protein of 323 and 326 amino acid residues, respectively.

Tissue distribution analysis in rodents revealed that MC3R is expressed centrally in several brain regions including the hypothalamus, cortex, thalamus, and hippocampus (Gantz, et al. 1993a; Roselli-Rehfuss, et al. 1993). MC3R is also expressed peripherally in the placenta, heart and the gut (Gantz, et al. 1993a) as well as in immune cells such as macrophages (Getting, et al. 2003, 2004). Recently, several studies have supported an important role of the MC3R in regulation of the cardiovascular system (Humphreys, 2004; Mioni, et al. 2003; Ni, et al. 2003; Versteeg, et al. 1998) and controling of the inflammation process (Manna and Aggarwal, 1998).

1.2.2. Role of the neuronal MC3/4R in energy homeostasis

Mouse genetic studies showed that targeted disruption of either *Mc4r* or *Pomc* result in increased food intake associated with hyperphagia, hyperinsulinemia and hyperglycemia (Huszar, et al. 1997; Yaswen, et al. 1999). The *Mc4r* heterozygotes have an intermediate phenotype implying that the *Mc4r* gene in mice has a dosage-dependent effect. Significant elevation of the AgRP expression is observed in arcuate nucleus of the hypothalamus of *obese* and *diabetic* mice (Ollmann, et al. 1997; Shutter, et al. 1997) and over-expression of agouti (Ay mice) or AgRP also results in obesity (Lu, et al. 1994; Graham, et al. 1997; Ollmann, et al. 1997). These results demonstrated that functional disruption of either MC4R itself or its endogenous agonists (POMC-derived MSH) or upregulated expression of its antagonist AgRP all result in the same obese phenotype, thus supporting a critical role of MC4R in regulation of food intake and body weight of rodents. Recently, mice containing mutagen-induced mutations in the coding region of the *MC4R* gene showed the same obese phenotype as that of *Mc4r*-knockout (KO) mice (Meehan, et al. 2006). Functional analysis of these MC4R mutants demonstrated that they are defective in either receptor signaling (I194T) or ligand binding (L300P) *in vitro* (Meehan, et al. 2006) further confirmed the critical role of MC4R in regulating food intake and energy expenditure of rodents. The experiments described above established a causative relationship of the MC4R with obesity in rodents.

Involvement of the MC4R in food intake and body weight was also supported by human genetic studies. For example, mutations in human *POMC* (Krude, et al. 1998) and *PC1* (Jackson, et al. 1997) result in severe early onset obesity, similar to the phenotypes showed by *Pomc*- and *Mc4r*-KO mice. Because endogenous agonists of hMC4R

including α- and β-MSH are derived from POMC and PC is involved in POMC processing, it is reasonable to assume that hMC4R also is critical in regulation of food intake and energy expenditure in human being. However, direct evidences came from the naturally occurring mutations in human MC4R identified from obese patients (see review, Tao, 2005). Functional analysis of the hMC4R mutants showed multiple defects such as truncated protein, intracellular retention, no ligand binding and/or no response to ligand stimulation. Thus, a causal relationship between the receptor mutations and obesity has been established. Just like the *Mc4r*$^{+/-}$ mice showing an intermediate obese phenotype between that of WT and *Mc4r*$^{-/-}$ mice, genetics study clearly showed that human MC4R mutations caused obesity by haploinsufficiency (Cody, et al. 1999).

Pharmacological studies also support a critical role of MC4R in regulation of rodent feeding. It was clear that intracerebroventricular (i.c.v.) administration of the MC4R synthetic agonist melanotan II into PVN of the hypothalamus resulted in suppressed food intake and decreased body weight of the rodent (Fan, et al. 1997; Thiele, et al. 1998). In contrast, the MC4R synthetic antagonist SHU 9119, once delivered into the PVN of the hypothalamus by i.c.v. administration, not only significantly stimulated feeding of the rodent but also reversed the suppressive effects of melanotan II on food intake (Fan, et al. 1997). In addition to the PVN as the possible sites of action for these melanocortin analogs, the brainstem may also be involved in the control of feeding (Grill, et al. 1998).

Studies on the involvement of pig MC4R in feed intake and energy balance have resulted in conflicting results. Barb and colleagues showed that i.c.v. administration of a synthetic analog of α-MSH, [Nle^4-D-Phe^7]-a-MSH (NDP-MSH), decreased feed intake, but treatments with the synthetic antagonist SHU9119 or AgRP, the natural antagonist of

the MC4R, failed to stimulate feed intake (Barb, et al. 2004), which differs from the results obtained in rodents (Fan, et al. 1997; Seeley, et al. 1997; Rossi, et al. 1998) and sheep (Wagner, et al. 2004). *In vitro*, these ligands did act as antagonists (Barb, et al. 2004; Fan et al. 2008a). Thus, these data cannot provide an explanation for the lack of effect of AgRP on feed intake in pigs as observed by Barb and colleagues (Barb, et al. 2004).

Rodent genetic studies demonstrated that *Mc3r*-KO mice did not show hyperphagia and obesity (Butler, et al. 2000; Chen, et al. 2000). These mice had similar or even decreased levels of food intake and normal energy expenditure as compared to their WT littermates (Butler, et al. 2000; Chen, et al. 2000). This observation showed that MC3R is not involved in regulation of food intake and energy expenditure. Interestingly, *Mc3r*-KO mice presented increased fat mass and decreased lean mass indicating that MC3R might affect feed efficiency rather than food intake and body weight (Butler, et al. 2000; Chen, et al. 2000). Therefore the MC3R probably play a critical role in regulation of feeding efficiency and fat storage. This conclusion was also supported by another experiment. In 2000, Van der Ploeg and coworkers showed that double KO mice lacking both *Mc3r* and *Mc4r* had exacerbated obesity as compared to *Mc3r* or *Mc4r* single gene KO mice. All of these data, taken together, suggested that the two neuronal MCRs regulate different aspects of energy homeostasis. Whether MC3R is a critical factor in regulation of the adiposity of human or other mammals remains unknown and needs to be investigated.

1.2.3. Naturally occurring MC3/4R mutations

Since the establishment of causative relationship between mutations in human *MC4R*

and morbid early onset obesity (Vaisse, et al. 1998; Yeo, et al. 1998), large-scale screening efforts have been made to detect the occurrence and frequency of human *MC4R* mutations in obese subjects with different ethnic backgrounds. Currently, a total of 110 naturally occurring human *MC4R* mutations have been identified and the majority of them have been functionally characterized. These mutations including frameshift, inframe deletion, nonsense and missense mutations, scattered throughout the coding region of human *MC4R* gene (Vaisse, et al. 1998; Yeo, et al. 1998; Biebermann, et al. 2003; Donohoue, et al. 2003; Dubern, et al. 2001; Farooqi, et al. 2000, 2003; Gu, et al. 1999; Hinney, et al. 1999, 2003, 2006; Jacobson, et al. 2002; Kobayashi, et al. 2002; Larsen, et al. 2005; Lubrano-Berthelier, et al. 2003b, 2004; Marti, et al. 2003; Mergen, et al. 2001; Miraglia Del Giudice, et al. 2002; Santini, et al. 2004; Tarnow, et al. 2003; Vaisse, et al. 2000; Valli-Jaakola, et al. 2004; Yeo, et al. 2003; Rettenbacher, et al. 2007; Buono, et al. 2005; Shao, et al. 2005). Functional analyses showed that the majority of human *MC4R* mutations result in the production of mutant receptors defective either in receptor biosynthesis, cell surface expression, ligand binding or signaling (Tao, 2005, Tao, 2006). Currently, the frequency of human *MC4R* mutations caused early onset severe obesity is estimated at ~6% in some population group (Farooqi, et al. 2003a).

Rodent and human genetics studies showed that the majority of obesity caused by MC4R mutations results from haploinsufficiency. That means the *MC4R* gene has a dose-dependent effect. It is well known that dimerization of GPCRs occur to almost all GPCRs (Bulenger, et al. 2005), including MCRs (Mandrika, et al. 2005). However, molecular characterization showed that most of human *MC4R* mutations identified from obese patients are heterozygous and do not have dominant negative activity (Farooqi, et

al. 2000; Ho and MacKenzie, 1999; Yeo, et al. 2003). These data thus support the clinical observation at a molecular level and clearly indicate that the expression level of the *MC4R* gene might be critical in regulation of food intake and body weight. The exceptions are D90N and S136F. Each of these mutants showed dominant negative activity (Biebermann, et al. 2003; Rettenbacher, et al. 2007). This might be another mechanism of how *MC4R* mutations caused obesity.

As mentioned above, *MC4R* gene has a dose-dependent effect and decreased MC4R expression level might cause obesity. Thus, mutations affecting the MC4R expression level might also cause the obese phenotype. Several research groups have tried to identify mutations or variants in the regulatory sequences of the *MC4R* gene, especially promoter region, in obese subjects. Although several variants were identified, none of them segregates with obesity phenotype (Lubrano-Berthelier, et al. 2003a; Ma, et al. 2004) therefore their relevance to the pathogenesis of obesity is unknown at present. It is likely that defect in gene transcription of *MC4R* is not a major cause of severe early-onset obesity.

Several large-scale screening studies have been carried out to identify the naturally occurring human *MC3R* mutations. These studies have been focusing on the obese subjects and patients with type 2 diabetes as well as normal subjects. Compared to the numerous human *MC4R* mutations, only a total of eight human *MC3R* mutations were identified so far including K6T, A70T, I81V, M134I, I183N, A293T, I335S, X361S (Boucher, et al. 2002; Feng, et al. 2004; Hani, et al. 2001; Li, et al. 2000; Mencarelli, et al. 2004; Schalin-Jantti, et al. 2003; Wong, et al. 2002; Lee, et al. 2002; Lee, et al. 2007; Tao, 2007). Among them, K6T and I181V were also found in control subjects at similar

frequencies and thus likely represent polymorphisms (Boucher, et al. 2002; Hani, et al. 2001; Li, et al. 2000; Schalin-Jantti, et al. 2003; Wong, et al. 2002). Functional analysis showed that K6T and I181V are normal functionally (Tao and Segoloff, 2004) thus agreeing with the clinical studies that both receptor mutants represent polymorphrism of the MC3R. The mutant receptor, I183N, however completely lacks signaling in response to hormone stimulation, thus representing a loss-of-function mutant (Tao and Segoloff, 2005). Further studies showed that I183N, A293T, I335S and X361S do not exert dominant negative activity on the WT allele suggesting they display haploinsufficiency rather than dominant negative activity (Tao and Segaloff, 2004; Tao, 2007).

1.2.4. Molecular classification of neuronal MCR mutations

As described above, the mutant MC4Rs might be defective either in biosynthesis, cell surface expression, ligand binding or signaling. To classify the human *MC3/4R* mutations into groups with different defects, Tao and Segaloff (2003) proposed a molecular classification system, modified after the classification of mutations in the low density lipoprotein receptor and cystic fibrosis transmembrane conductance regulator (Hobbs, et al. 1990; Welsh and Smith, 1993).

Class I mutants contain mutations that lead to the production of a premature stop codon in encoding region of the *MC3/4R* genes or a frameshift that caused the generation of a premature receptor protein. This group of mutant receptors is defective in receptor biosynthesis. Human MC4R mutants W16X (Marti, et al. 2003), Y35X (Hebebrand, et al. 2002; Hinney, et al. 1999), L64X (Jacobson, et al. 2002), ΔCTCT at codon 211 (Ho and MacKenzie, 1999), the TGAT insertion at codon 244 (Ho and MacKenzie, 1999), and a

Δ750-751GA (Lubrano-Berthelier, et al. 2004) likely belong to this class.

Class II mutants are defective in intracellular trafficking. This group of mutant receptors can be synthesized but are trapped intracellularly, most likely in the ER. This class comprises the largest set of *MC3/4R* mutations reported to date, including hMC4R mutants S58C (Lubrano-Berthelier, et al. 2003b; Tao and Segaloff, 2003), N62S (Tao and Segaloff, 2003; Yeo, et al. 2003), P78L (Lubrano-Berthelier, et al. 2003b; Nijenhuis, et al. 2003; Tao and Segaloff, 2003), N97D (Yeo, et al. 2003), G98R (Tao and Segaloff, 2003), I102S (Lubrano-Berthelier, et al. 2003b), L106P (Yeo, et al. 2003), I125K (Yeo, et al. 2003), R165Q (Nijenhuis, et al., 2003), R165W (Lubrano-Berthelier, et al. 2003b; Nijenhuis, et al. 2003), N240S (Tao and Segaloff, 2005), L250Q (Lubrano-Berthelier, et al. 2003b), Y287X (Yeo, et al. 2003), C271R (Tarnow, et al. 2003), C271Y (Tao and Segaloff, 2003; Yeo, et al. 2003), P299H (Lubrano-Berthelier, et al. 2003b), I316S (Yeo, et al. 2003), I317T (Lubrano-Berthelier, et al. 2003b; VanLeeuwen, et al. 2003).

Class III mutants include hMC4R mutants N97D, L106P, I125K (Yeo, et al. 2003), I137T (Gu, et al. 1999), I316S (Yeo, et al. 2003), Δ88-92 (Donohoue, et al. 2003), I102S and I102T (Tao and Segaloff, 2005) and hMC3R mutants A70T and V83I (Lee, et al. 2007). This group of mutant receptors is normal in both biosynthesis and cell surface expression but defective in ligand binding with either decreased binding capacity and/or affinity, resulting in impairments in hormone-stimulated signaling. Different from other GPCRs, the activities of the neuronal MC3/4Rs are inhibited by their endogenous antagonist AgRP. If the mutants are more sensitive to inhibition by AgRP, the mutants are also functionally defective. Therefore, these mutants can be grouped as a subclass within this class. However, most of the functional studies to date did not include AgRP in

ligand binding studies. It is not known how prevalent this kind of defect is. The only example for this subclass is human *MC4R* mutation I316S (Yeo, et al., 2003). This mutant receptor showed altered relative affinities for its endogenous agonist (α-MSH) and antagonist (AgRP).

Class IV mutants include hMC4R mutants D90N (Biebermann, et al. 2003), I137T (Gu, et al. 1999), A175T and V253I (Yeo, et al. 2003) and hMC3R mutants I183N (Tao and Segoloff, 2004; Rached, et al. 2004; Lee, et al. 2007). This group of the mutant receptors is normal in biosynthesis and cell surface expression. They also show ligand binding with normal affinity. However, mutants belonging to this group are defective in signaling with decreased efficacy and/or potency.

Class V mutants have apparently normal function. These variants, similar to the WT MC3/4R, are normal in receptor biosynthesis, cell surface expression, ligand binding and signaling in heterologous expression systems. Therefore, these variant simply represent polymorphism of WT MC3/4R. The mutant receptors in this group include hMC4R mutants T11A, D37V, P48S, V50M, F51L, V103I, T112M, 1251L, A154D, I170V, M200V, N274S, and S295P (Farooqi, et al. 2000; Gu, et al. 1999; Hinney, et al. 1999; Tao and Segaloff, 2003) and hMC3R mutants K6T, I181V, A293P and X361S (Boucher, et al. 2002; Hani, et al. 2001; Li, et al. 2000; Schalin-Jantti, et al. 2003; Tao, 2007; Wong, et al. 2002). Whether and how these variants cause energy imbalance and therefore obesity is unclear.

1.2.5. Therapeutic implications

Functional analysis showed that the majority of the mutant hMC4Rs belongs to the

class II mutants according to the classification system proposed by Tao and Segaloff (2003). That means most of the identified mutant hMC4Rs are defective in intracellular trafficking. It was already well known that, of the naturally occurring mutations in GPCRs that cause human diseases, intracellular retention is the most common defect for loss-of-function phenotype. The examples include retinitis pigmentosa (rhodopsin mutants) (Sung, et al. 1991, 1993), nephrogenic diabetes insipidus (V2 vasopressin receptor mutants) (reviewed in Morello and Bichet (2001)), Hirschsprung's disease (endothelin B receptor mutants) (Tanaka, et al. 1998), familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism (calcium-sensing receptor mutants) (Bai, et al. 1996), idiopathic hypogonadotropic hypogonadism (gonadotropin-releasing hormone receptor mutants) (Leanos-Miranda, et al. 2002), infertility (lutropin and follitropin receptors mutants) (Themmen and Huhtaniemi, 2000) (reviewed in Tao, 2006). Thus, any approach that can rescue the cell surface expression of the intracellularly retained mutant receptors could potentially be of therapeutic value.

In vitro studies showed that cell surface expression of wild type (WT) and mutant GPCRs can be significantly elevated when a low culture temperature was used (Fan, et al. 1997; Jaquette & Segaloff, 1997; Jeyaraj, et al. 2001; Robben, et al. 2006) or treatment of cells with high concentration of so-called chemical chaperones such as glycerol and DMSO were performed (Sato, et al. 1996; Robben, et al. 2006). Due to the observation that high concentration of chemical chaperones is toxic to cells and their effect is nonspecific to a certain receptor, it was obvious that these approaches are not practical options for a clinical treatment.

In 2000, Bouvier and coworkers (Morello, et al. 2000) reported an interesting finding

that small molecule antagonists of V2R can specifically increase the cell surface expression of the WT and mutant receptors by working as a pharmacological chaperone. Similar results were achieved recently in μ- and δ-opioid receptor (Petaja-Repo, et al. 2002), melanin-concentrating hormone receptor 1 (Robert, et al. 2005), V1a and V1b vasopressin receptors (Morello and Bichet, 2001; Chaipatikul, et al. 2003; Hawtin, 2006), gonadotropin-releasing hormone receptor (Janovick, et al. 2002; 2003) and the prototypical GPCR, rhodopsin (Noorwez, et al. 2003; 2004). Pharmacological chaperones are defined as small molecules, generally synthetic antagonists, that can across the cell membrane and bind to specific receptors to help them fold into the correct conformation and traffick onto the cell surface (see reviews Castro-Fernandez, et al. 2005; Conn, et al. 2007). The rescuing effect of the pharmacological chaperones does not depend on their specific chemical structure. For example, three chemicals with different structures, indoles, quinolines, and etythromycinmacrolides, can rescue the majority of the GnRHR mutants (Janovick, et al. 2003). Recently, treatment of nephrogenic diabetes insipidus patients carrying V2R mutants with the nonpeptide antagonist SR49059 decreased urine volume and water intake in patients (Bernier, et al. 2006), proving the clinical utility of pharmacological chaperones.

Since several hMC4R mutants with intracellular retention showed residual activities in terms of hormone-stimulated cAMP generation, approaches that result in increased expression of the mutant receptor on the cell surface could potentially be of therapeutic value. It has been reported that the C-terminal peptide of AgRP can increase the cell surface expression of MC4R (Shinyama, et al. 2003). It is possible that of the numerous antagonists developed for MC4R, some might act as pharmacological chaperones, which

can potentially be of therapeutic value. The fact that some mutants, such as N62S, I102S, Y157S and C271Y, can respond to NDP-MSH stimulation with increased cAMP production in spite of minimal binding capacity (less than 5% of weight) (Tao and Segaloff, 2003, 2005) suggests that these mutants are competent in G protein coupling and effector activation. Increasing their expression could be beneficial for treating obesity in the subjects carrying these mutant *MC4R* genes.

1.3. CONCLUSION

Obesity has become one of the most significant public health problems all over the world, especially in developed countries. Human obesity is strongly associated with diseases such as hypertension, type-II diabetes mellitus, stroke, coronary artery diseases and certain cancers and thus causes an increased risk of mortality and morbidity (Hill and Peters, 1998; Comuzzie and Allison, 1998).

During the past decade, rodent and human genetic studies have revealed that the leptin-regulated melanocortin circuit plays a critical role in controlling body weight. These studies demonstrated that genetic factors are also critical in regulation of energy balance. Currently, mutations in six genes including *LEP*, *LEPR*, *PC1*, *POMC*, *MC4R*, and *SIM1* cause monogenic early-onset obesity (Synder, et al. 2004). Among them, *MC4R* has been identified as the most frequent monogenetic form causing human early-onset obesity (Tao, 2005). It was estimated that up to 6% of the early-onset obesity were caused by the mutations in human *MC4R* gene (Farooqi, et al. 2003a). MC3R might not be an important factor in regulation of food intake and body weight but is critical in

fat storage/adiposity for both rodent and human. The exact mechanism of the increased feed efficiency in the Mc3r-KO mice and obese patients with increased fat mass is not clear.

Currently, the number of the naturally occurring mutations identified in the coding region of *MC4R* gene has reached 110 (Spiegelman, et al. 2001; Cone, 2005; Tao, 2005). A total of 8 mutations in human *MC3R* gene have also been identified. These mutations have been identified from obese as well as normal weight subjects. About 30% of the residues of the hMC4R have been found to be mutated naturally and new mutations continue to be identified. Detailed functional studies are necessary to bridge the clinical studies identifying an association of the mutation with obesity or other phenotype (such as binge eating disorder) to a causative relationship. In addition, it is imperative to identify the exact defect if we are ever going to use the results of these studies for personalized treatment for mutation harboring obese patients.

CHAPTER II

MOLECULAR CLONING AND PHARMACOLOGICAL CHARACTERIZATION OF PORCINE MELANOCORTIN-3 RECEPTOR

2.1. Introduction

Melanocortins including α–, β- and γ-melanocyte stimulating hormones (α-MSH, β-MSH and γ-MSH) and adrenocorticotropic hormone (ACTH) are short peptide hormones derived from pro-opiomelanocortin (POMC) through tissue-specific posttranslational processing (Smith and Funder, 1988). The melanocortin receptors (MCRs) are members of rhodopsin-like (family A) G protein-coupled receptors (GPCRs). They are expressed on the cell surface, predicted to have seven transmembrane domains (TMDs) connected by alternating extracellular and intracellular loops, with the N terminus lying on the outside of the cell and the C terminus locating on the inside of the cell. Once bound to MCRs, melanocortins induce conformational changes in MCRs facilitating their coupling to and activation of the stimulatory G protein, Gs. Activated Gs enhances adenylyl cyclase activity resulting in increased production of the intracellular second messenger cyclic AMP (cAMP) triggering downstream signal transduction pathways (Gantz and Fong, 2003). Agouti and agouti-related protein (AgRP) are the endogenous antagonists of the MCRs. So far, five MCRs have been cloned, named

MC1R to MC5R according to the sequence of their cloning (Gantz and Fong, 2003). Of the MCRs, MC3R and MC4R are the main subtypes expressed in the brain. They are expressed in the hypothalamic paraventricular nucleus (PVN) and arcuate nucleus as well as in other brain regions including the cortex, thalamus, and hippocampus (Gantz, et al. 1993a; Gantz, et al. 1993b; Mountjoy, et al. 1994; Roselli-Rehfuss, et al. 1993). The MC3R is also expressed in the placenta and gut (Gantz, et al. 1993a) and immune cells such as macrophages (Getting, et al. 1999).

Although both the MC3R and the MC4R are involved in the central control of energy homeostasis, it is clear that the MC4R primarily regulates food intake and energy expenditure (Cone, 2005). On the other hand, the MC3R does not regulate food intake. MC3R knockout mice did not have hyperphagia and obesity; they had similar or even decreased levels of food intake and normal energy expenditure as compared to their wild-type littermates (Butler, et al. 2000; Chen, et al. 2000). Of particular interest was the observation that these mice have increased fat mass and decreased lean mass. Therefore the MC3R is involved in regulation of feeding efficiency and fat storage. Compared with MC3R or MC4R single gene knockout mice, mice lacking both MC3R and MC4R developed exacerbated obesity, suggesting that the two central MCRs regulate different aspects of energy homeostasis (Chen, et al. 2000). However, a recent gene knockout study performed with an obesity-resistant mice strain (Black Swiss 129) indicated that MC3R single gene knockout mice developed a comparable degree of increased adiposity as the MC4R knockout mice (Zhang, et al. 2005) suggesting that the MC3R might also be involved in regulating food intake and energy expenditure. A potential pathway that the

MC3R might regulate food intake is by releasing the inhibitory effect of the MC3R on POMC neurons (Marks, et al. 2006).

To begin to understand what role(s) the MC3R might play in regulating fat storage and energy homeostasis in the pig, we report herein the molecular cloning and pharmacological characterization of the porcine MC3R (pMC3R). For the pharmacological characterizations, we tested several ligands including the endogenous agonists α- and γ-MSH, a superpotent agonist specific for the MC3R, D-Trp^{8}-γ-MSH (Grieco, et al. 2000), and a superpotent analogue of α-MSH specific for all MCRs except MC2R (the ACTH receptor), [Nle^{4}-D-Phe^{7}]-MSH (NDP-MSH) (Sawyer, et al. 1980). We also tested the binding of the cloned receptor to the natural antagonist of the MC3/4-R, AgRP.

2.2. Materials and Methods

2.2.1. Plasmid and peptides

Human MC3R cDNA inserted in a mammalian expression vector, pcDNA3.1, was kindly provided by Dr Ira Gantz (University of Michigan, Ann Arbor, MI, USA). α-, γ-, and NDP-MSH were purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). D-Trp^{8}-γ-MSH and AgRP(86–132) were obtained from Peptides International Inc. (Louisville, KY, USA). ^{125}I-iodinated NDP-MSH was purchased from Peptide Radioiodination Service Center, The University of Mississippi (University, MS, USA). Molecular cloning of porcine MC3R Sequence analysis performed with DNAman program (Lynnon Corp., Quebec, Canada) suggested that the putative coding region of

the pMC3R gene consists of a single exon of 960 bp. Thus, the pMC3R coding region was amplified directly from porcine genomic DNA (Novagen, SanDiego, CA, USA) using a primer pair (sense primer, 5'-AAGAATTCATGAATGCTTCGTGCTGC-3' and antisense primer, 5'-CCTCTAGAGCCTCCTACCCCAGGTTC-3') designed based on the published pig genomic DNA sequence from clone CH242-163M14 on chromosome 17 (GenBank accession no. CR956393). To facilitate the cloning, EcoRI and XbaI sites (underlined) were incorporated in sense and antisense primers respectively. The PCR was performed in a 50 µl mixture containing 100 ng porcine genomic DNA, 0.25 mM dNTPs, 0.4 mM of each of the primers, 5 µl pfu Turbo DNA polymerase buffer, 1.5 mM MgCl2, and 2.5 U pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) with the following parameters: 2 min at 95°C for one cycle and 1 min at 95°C, 45 s at 56°C, and 90 s at 72°C for 45 cycles followed by a final cycle at 72°C for 10 min. Subsequently, the 960 bp PCR fragment was visualized after electrophoresis with ethidium bromide using a 1% agarose gel, purified with Qiagen PCR purification kit (Qiagen), double digested with EcoRI and XbaI (New England Biolabs, Beverly, MA, USA). The EcoRI-XbaI DNA fragment was purified again with Qiagen PCR purification kit and then cloned into the expression vector pcDNA3.1(+) using T4 DNA ligase (Roche) according to the manufacturer's protocol. The nucleotide sequence of the cloned pMC3R gene was determined by sequencing three independent plasmids and deposited in NCBI GenBank with an accession number EU091085. Plasmids were prepared with Qiagen Plasmid Maxi kit for transfection of human embryonic kidney (HEK) 293T cells as described below.

2.2.2. Homology and phylogenetic analysis of pMC3R

Homology and phylogenetic analyses at nucleotide and amino acid levels were performed between different species including porcine, human, mouse, and rat using DNAman program and MacVector 8.0 (Accelrys Software Inc., San Diego, CA, USA) respectively, according to the manufacturer's protocols.

2.2.3. Transient expression of plasmids

Transient expression of plasmids was performed in HEK293T cells (American Type Culture Collection, Manassas, VA, USA). HEK293T cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 10 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin (all fromInvitrogen). Cells were plated on gelatin-coated 35 mm six-well plates (Corning, Corning, NY, USA) or on gelatin-coated 100 mm plates. Transient transfection was performed using the calcium precipitation method (Chen and Okayama, 1987). For each 35 mm well of the six-well plates or 100 mm plates, 2 ml media containing 4 mg plasmid or 10 ml media containing 20 mg plasmid were added respectively. Cells were used for measuring ligand binding and agonist-stimulated cAMP generation 48 hrs post-transfection as described below.

2.2.4. Radioligand-binding assay with agonists

The method for binding assay has been described in detail before (Tao and Segaloff, 2003). Briefly, 48 hrs after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich) modified to contain 1 mg/ml BSA (referred herein as

Waymouth/BSA). One milliliter of fresh Waymouth/BSA with or without different concentrations of unlabeled agonists including α-, γ-, NDP-, and D-Trp^{8}-γ-MSH and 100, 000 c.p.m. of ^{125}I-NDP-MSH (50 µl) was added to each well. The final concentration of unlabeled ligands ranged from 10^{-10} to 10^{-5} M (for α-, γ-, and D-Trp^{8}-γ-MSH) or from 10^{-11} to 10^{-6} M (for NDP-MSH). After incubation at 37°C for 1 hr, cells were washed twice with cold Hanks' balanced salt solution (Sigma-Aldrich) modified to contain 1 mg/ml BSA (referred herein as HBSS/BSA). Then, 100 µl of 0.5 M NaOH were added to each well. Lysed cells were collected from each well using cotton swabs to plastic tubes, and ligand binding was counted in a γ-counter. All determinations were performed in duplicates.

2.2.5. Radioligand-binding assay with AgRP

For AgRP-binding assay, transient transfection of plasmids was performed in 100 mm dishes as described above. Twenty-four hrs after transfection, cells were trypsinized, split into 24-well plates, and incubated for an additional 24 hrs. Cells were washed twice with warm Waymouth/BSA. Fresh Waymouth/BSA containing 20 µl of ^{125}I-NDP-MSH (40,000 c.p.m.) and 20 µl different concentrations (including zero) of unlabeled antagonist AgRP were added to each well to a total volume of 200 µl per well. The final concentration of the unlabeled AgRP ranged from 10^{-11} to 10^{-6} M. After incubation at 37°C for 1 hr, cells were washed twice with cold HBSS/BSA. Then, 50 µl of 0.5 M NaOH were added to each well. Cell lysate collection and ligand binding were performed as described above. All determinations were performed in triplicates.

2.2.6. Intracellular cAMP generation

HEK293T cells were plated and transfected as described above. Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then, 1 ml fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well. After incubation at 37°C for 15 min, either buffer alone or different concentrations of ligands (final concentrations of 10^{-11}-10^{-5} M for α- and γ-MSH, and 10^{-12}-10^{-6} M for NDP-MSH and D-Trp^{8}-γ-MSH) were added, and the incubation was continued for an additional 1 h. Cells were then placed on ice, media were aspirated, and intracellular cAMPs were extracted by the addition of 1.5 ml fresh 0.5 M perchloric acid containing 180 mg/ml theophylline, and measured using RIA. All determinations were performed in triplicate.

2.2.7. Statistical analysis

Maximal binding (Bmax), IC_{50}, maximal responses (Rmax), and EC_{50} were calculated using Prism 4.0 (GraphPad, San Diego, CA, USA). Data were presented as the mean and SEM. All statistical analyses were done using Prism 4.0.

2.3. Results

2.3.1. Nucleotide and deduced amino acid sequences of the putative pMC3R

Civanova and colleagues reported the partial sequence of the pMC3R and mapped the *pMC3R* gene to porcine chromosome 17 (Civanova, et al. 2004). Recently, the complete nucleotide sequence of porcine chromosome 17 was deposited in GenBank. We

performed nucleotide sequence alignment among the partial nucleotide sequence of *pMC3R* gene (GenBank accession no. AJ744762), nucleotide sequence of the clone CH242-163M14 on porcine chromosome 17 (GenBank accession no. CR956393) and nucleotide sequences of human, rat, and mouse MC3Rs (GenBank accession nos. NM_019888, NM_001025270 and NM_008561, respectively). We found that the putative *pMC3R* gene was intronless and contained an open reading frame of 960 bp encoding a putative protein of 319 amino acids (Fig. II-1-A). The TATA box, characteristic of the mammalian promoter region, was identified 382 bp upstream of the translation initiation site of the putative pMC3R; a potential poly(A)+ signal was recognized 1381 bp downstream from the translation stop site of the putative pMC3R (highlighted in bold in Fig. II-1-A). Comparison of the putative pMC3R sequence with that of the MC3Rs from other species showed that pMC3R is 88.2%, 85.3%, and 86.0% homologous at nucleotide level (Fig. II-1-B) and 87.5%, 83.1%, and 83.7% identical at amino acid level (Fig. II-2-A and 2-B) to human, mouse, and rat MC3Rs, respectively. Further comparison indicated that pMC3R is significantly conserved in amino acid sequence to other species in the transmembrane domains (TMDs), extracellular and intracellular loops. But lower conservation is observed at the amino- and carboxyl termini. In addition, the putative pMC3R is 4 amino acids shorter at the carboxyl terminus than human, rat and mouse MC3Rs and 37 amino acids shorter at the N-terminus than human MC3R (but the same as that of rat and mouse MC3Rs). Phylogenetic analysis indicates that pMC3R clusters together with MC3Rs from other species at nucleotide level (Fig. II-1-C) but is significantly divergent from human MC3R and to a less degree from mouse and rat MC3Rs at amino acid level (Fig. II-2-C).

2.3.2. Expression and functional analysis of the cloned pMC3R in HEK293T cells

Based on the sequence analysis described above, primers were designed and the 960 bp of putative pMC3R was directly amplified from porcine genomic DNA by PCR and cloned into mammalian expression vector pcDNA3.1 for functional analysis. To demonstrate if the cloned pMC3R is capable of producing a biologically active receptor protein, we transfected the pMC3R construct obtained into HEK293T cells and analyzed its ligand binding and signaling properties using the natural agonist for MC3R, γ-MSH, and ligand binding property with the natural antagonist for MC3R, AgRP. Human MC3R was used for comparison. As shown in Fig. II-3 and Table 1, γ-MSH bound the cloned pMC3R with an IC_{50} of 129.07 nM and hMC3R with an IC_{50} of 293.00 nM. Both the cloned pMC3R and hMC3R bound AgRP with similar high affinity: the cloned pMC3R with an IC_{50} of 0.67 nM and hMC3R with an IC_{50} of 2.35 nM. Furthermore, γ-MSH also induced dose-dependent accumulations of intracellular cAMP with EC_{50} of 79.16 nM and 111.13 nM for the cloned pMC3R and hMC3R, respectively. These results suggested that the cloned pMC3R encoded a functional MC3R protein.

2.3.3. Binding and signaling of pMC3R to three other MC3R agonists

For *in vivo* investigation of the role that pMC3R plays in pigs, the most potent and selective agonist for pMC3R must be selected from the pool of known MC3R agonists. Herein, we tested the pharmacological properties of three MC3R agonists on the cloned pMC3R. These agonists include the natural agonist of MC3R, α-MSH, a superpotent analogue of α-MSH for MC3R and MC4R, NDP-MSH, and a superpotent analogue specific for MC3R, D-Trp^8-γ-MSH. As shown in Fig. II-4, ligand binding assays revealed

that pMC3R bound NDP-MSH with the highest affinity (IC_{50} of 4.36 nM) followed by D-Trp^8-γ-MSH (IC_{50} of 10.65 nM) and α-MSH (IC_{50} of 674.90 nM). The same ranking was also observed for hMC3R with an IC_{50} of 21.71 nM for NDP-MSH, 90.74 nM for D-Trp^8-γ-MSH and 1151.33 nM for α-MSH. It is interesting to note that pMC3R always had 2-9 fold higher affinities for these ligands than the hMC3R (Table 1). The data from the signaling assays demonstrated that D-Trp^8-γ-MSH was the most potent agonist to stimulate does-dependent cAMP generation with an EC_{50} of 1.24 nM, followed by NDP-MSH (EC_{50} of 2.24 nM) and α-MSH (EC_{50} of 59.03 nM) (Table 1). Human MC3R has the same ranking with an EC_{50} of 1.95 nM for D-Trp^8-γ-MSH, 5.01 nM for NDP-MSH and 70.27 nM for α-MSH (Table 1). Taken together, these data demonstrated that D-Trp^8-γ-MSH was the most potent agonist in eliciting pMC3R signaling. As can be seen from Table 1, the maximal responses to α-MSH, γ-MSH, and D-Trp^8-γ-MSH are similar between the pMC3R and hMC3R. However, the hMC3R has significantly larger maximal response to NDP-MSH than pMC3R. One reason might be that hMC3R is expressed at slightly higher level (148 + 8% of pMC3R, n=12). However, the maximal responses to the other three agonists are similar between the two MC3Rs. We suggest that the NDP-MSH binding to hMC3R induce a conformation with higher coupling efficiency than the pMC3R (Ballesteros, et al. 1998).

2.4. Discussion

Previous pharmacological and genetic studies in rodents suggested that the MC3R was not involved in food intake regulation. Administration of MC3R-specific agonist

such as γ-MSH did not inhibit food intake (Kask, et al. 2000). In fact, a recent study showed that peripheral administration of D-Trp^{8}-γ-MSH stimulated food intake in rats by releasing the inhibitory effect of the MC3R on POMC neurons (Marks, et al. 2006). Changes in energy balance such as food restriction or diet-induced obesity did not change MC3R expression level in the brain whereas the MC4R density was modulated (Harrold, et al. 1999). Finally, MC3R knockout mice did not have increased food intake as did the MC4R knockout mice (Butler, et al. 2000; Chen, et al. 2000; Huszar, et al. 1997). Mice lacking the MC3R have increased fat contents but are normal in terms of food intake and body weight (Butler, et al. 2000; Chen, et al. 2000), suggesting that the MC3R pathway modulates feeding efficiency and fat storage. The recent gene targeting study by Zhang and colleagues showed that Black Swiss; 129 mice lacking MC3R gene had comparable level of adiposity as mice lacking the MC4R gene (Zhang, et al. 2005). In humans, only a few naturally occurring mutations in the MC3R gene have been identified (Lee, et al. 2007; Lee, et al. 2002; Tao, 2007). These mutations were found to cause defects in signaling (Lee, et al. 2007; Rached, et al. 2004; Tao, 2007; Tao and Segaloff, 2004). In contrast, in the MC4R, numerous mutations have been identified from various patient populations (Tao, 2005; 2006). Therefore the roles of the MC3R in energy homeostasis are much less understood.

Pork is an important protein source in our diet. About 60% of the saturated fat comes from animal products in typical American diet. Leaner meat will decrease intake of saturated fat and decrease blood cholesterol levels. To increase the economic return for hog farmers and reduce the obesity epidemic, it is of great interest to decrease the fat content in pork. Therefore a better understanding of fat storage in pigs is of great

economic interest. In the present study, as the first step towards elucidating the role(s) of the MC3R in pig energy homeostasis, we describe the molecular cloning and pharmacological characterization of the porcine MC3R. Through bioinformatics analysis and progress in pig genome sequencing, we were able to identify the putative full-length coding region of pMC3R. Primers designed based on this sequence were used to amplify the coding region from pig genomic DNA by taking advantage of the fact that the mammalian MCRs, including the putative pMC3R, are intronless. This amplified fragment was inserted into pcDNA3.1 and the sequence verified. This construct was used for detailed pharmacological analysis using a series of ligands. From ligand binding studies, we showed that of the four agonists tested, the rank order in terms of affinity is: NDP-MSH>D-Trp^{8}-γ-MSH>γ-MSH>α-MSH. Human MC3R has the same rank order for these ligands. From signaling experiments, we showed that the rank order in terms of potency for these four ligands are: D-Trp^{8}-γ-MSH>NDP-MSH >γ-MSH>α-MSH. Again, hMC3R has the same rank order for these ligands. Both pMC3R and hMC3R bind to AgRP with high affinity, with IC_{50}s of 0.67 and 2.35 nM, respectively.

Previously, the MC3Rs have been cloned from a number of species, including human (Gantz, et al. 1993a), rat (Roselli-Rehfuss, et al. 1993), mouse (Desarnaud, et al. 1994), and elephant (Rompler, et al. 2006). In lower vertebrates, the MC3Rs have been cloned from the chicken (Takeuchi and Takahashi, 1999) and zebrafish (Logan, et al. 2003) but was found to be absent in some other fishes such as the pufferfishes (Klovins, et al. 2004a). The numbers we reported herein for pMC3R are roughly in line with previous studies. For example, with NDP-MSH, the most commonly used analog for MCR studies, the IC_{50} reported here for pMC3R is 4.36 nM. Previously, the Kd for rat

MC3R was reported to be 3.78 nM (Kim, et al. 2002), and the Ki for human MC3R was 5.6 nM (Chen, et al. 2006), and 0.93 nM for the dogfish MC3R (Klovins, et al. 2004b). In the original publication describing the discovery of D-Trp^8-γ-MSH as a potent and selective MC3R agonist (Grieco, et al. 2000), an IC_{50} of 6.7 nM and EC_{50} of 0.33 nM were reported for hMC3R. Our data for hMC3R are somewhat higher than those reported before (IC_{50} of 90.75 nM and EC_{50} of 1.95 nM). These differences might be due to the use of different cell lines and different methods in the two studies. For example, for binding studies, Grieco and colleagues used membranes prepared from L cells stably expressing hMC3R, whereas we used live HEK293T cells transiently expressing hMC3R (only cell surface receptor binding are detected). However, our data for pMC3R (IC_{50} of 10.65 nM and EC_{50} of 1.24 nM) are very close to the numbers reported for hMC3R by Grieco and colleagues (Grieco, et al. 2000).

It should be pointed out that the ranking in terms of affinity is not the same as that in terms of potency. For example, for pMC3R, NDP-MSH has higher binding affinity than D-Trp^8-γ-MSH. However, it is not as potent in eliciting activation of signaling pathways as the latter. This may be due the difference in their relative efficacies. Both affinity and efficacy can independently affect the potency of a particular ligand (Besse and Furchgott, 1976). The data presented herein suggest that D-Trp^8-γ-MSH is a superpotent agonist for the pMC3R. Since it does not activate the MC4R efficiently (Grieco, et al. 2000), it is an excellent tool for *in vivo* studies directed towards dissecting the role(s) of the MC3R in regulating pig energy balance. However, the *in vitro* data described herein should only be used as the starting point for further *in vivo* studies. Previous studies showed that pMC4R respond to the natural (AgRP) and artificial (SHU9119) antagonists normally *in vitro*.

However, *in vivo*, these antagonists do not increase food intake as observed in rodents (Barb, et al. 2004). It was suggested that the MC4R might not play a critical role in regulating feed intake in the pig (Barb, et al. 2004). Similarly, two naturally occurring mutations (R236H and D298N) in the pMC4R were identified in different strains of pigs that are associated with differences in performance characteristics such as average daily gain and average daily intake as well as carcass fat (Kim, et al. 2000; Meidtner, et al. 2006). In the case of D298N, not all studies could confirm the original associations (Park, et al. 2002; Stachowiak, et al. 2006). Similarly, our experiments *in vitro* could not replicate the original functional studies (Fan, et al. 2007; Kim, et al. 2004). Therefore the data on the naturally occurring mutations in the pMC4R are also much less convincing than those on human MC4R (Tao, 2005; 2006). All these point to the importance of using caution when extrapolating *in vitro* data as well as the potential difference of the relevance of the melanocortin system in regulating energy homeostasis in the pig.

In summary, we have cloned the pMC3R. Expression of the cloned pMC3R in HEK293 cells revealed that indeed it was functional. Pharmacological characterizations using a series of ligands revealed that the pMC3R and hMC3R have the same rank orders for these ligands. We showed that D-Trp^{8}-γ-MSH was the most potent ligand (and selective for the MC3R), therefore best suited for further *in vivo* studies to elucidate the functional implication.

Table 1. Ligand binding and agonist-stimulated cAMP response of pMC3R and hMC3R

Ligands	MC3R	Assay no.	Ligand binding	Assay no.	Ligand-stimulated cAMP response	
			IC_{50} (nM)		EC_{50} (nM)	Rmax (pmol/10^6 cells)
α-MSH	P	3	674.90 ± 83.76	4	59.03 ± 7.51	2854 ± 96
	H	3	1151.33 ± 85.54	4	70.27 ± 11.97	2765 ± 238
NDP-MSH	P	5	4.36 ± 0.43	4	2.24 ± 0.24	1286 ± 160
	H	5	21.71 ± 2.64	4	5.01 ± 0.87	2438 ± 333
γ-MSH	P	3	129.07 ± 3.28	4	79.16 ± 6.05	2354 ± 147
	H	3	293.00 ± 21.64	4	111.13 ± 11.78	2623 ± 185
D-Trp^8-γ-MSH	P	3	10.65 ± 3.31	4	1.24 ± 0.58	1894 ± 256
	H	3	90.74 ± 12.46	4	1.95 ± 0.64	1972 ± 258
AgRP	P	3	0.67 ± 0.06	N/A	N/A	N/A
	H	3	2.35 ± 0.42	N/A	N/A	N/A

Data shown are the mean ± SEM of the indicated number of experiments.

IC_{50} is the concentration of ligand that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of agonist that results in 50% stimulation of the maximal response.

N/A, not applicable.

P, porcine.

H, human.

Figure II-1. Deduced nucleotide and amino acid sequences of pMC3R and comparison with MC3Rs from other mammalians. (A) Sequence of the full-length pMC3R cDNA with *in silico* translation is shown. Arrows indicate the position and direction of the primers used for amplification of the cDNA by PCR. (B) Homology and (C) phylogenetic analysis of MC3R nucleotide sequences from several species.

A

```
-430                                  GGCTGCACTGAAGATTAAGTAGGAGTTGTCTGCCTCCAGCGATGTATAAACCAG
-375  TGTTCACCCCTCACTCCCAACGACTCCTACCACAGCCCTCTCTTGCTATGCAAAGAGCCGTAACTGTAGCAACCG
-300  GGGGCTGGTGTGTTCCATTCCCAGGTGGGGAGGTGAGGAGAGGAAGGTAAGACAGGAGAGAGCTAAGAGCATCTA
-225  GAAAACTGCTTGCCCTGGAGAAGCAAAGTTCTCTGCATGTCTCGGAGCTGACTCTCTCTCTCTCCCTCTTCTC
-150  TCCATCTCGGGCAGAAGAGAATCAAAGTCCAGACTGGACTAGCATCCAAAACAAGCACCTGCAGGGAGATTTTTC
-75   TCTTTCCTGGAAGCAGCAGCCACAGCAGCCTCTGCACCCTGCTGGAGCCCGGGCTCCGATCCCCGGCCGTGAGCA
+1    ATGAATGCTTCGTGCTGCCTGCTCTCTGCTCCACCGGCGCTGCCTAACAGCTCAGAGCACCTCCCCGCCCCTTCC
+1     M  N  A  S  C  C  L  L  S  A  P  P  A  L  P  N  S  S  E  H  L  P  A  P  S
+76   TTCAGCAACCAGAGCAGCAGCGGCTTCTGCGAGCAGGTCGTCATCCAGCCCGAGGTCTTCCTGGCTCTGGGCATC
+26    F  S  N  Q  S  S  S  G  F  C  E  Q  V  V  I  Q  P  E  V  F  L  A  L  G  I
+151  CTCAGCCTGCTGGAGAACGTGCTGGTCATCCTGGCCGTGGCCAGGAACGGCAACCTGCACTCGCCCATGTACCTC
+51    L  S  L  L  E  N  V  L  V  I  L  A  V  A  R  N  G  N  L  H  S  P  M  Y  L
+226  TTCCTCTGCAGCCTGGCCGTGGCCGACCTGCTGGTGAGCGTGTCCAACGCCCTGGAGACCATCATGATCGCCGTG
+76    F  L  C  S  L  A  V  A  D  L  L  V  S  V  S  N  A  L  E  T  I  M  I  A  V
+301  GTCAACAGCGACGCCCTGACCTTCGAGGACCAGTTCGTCCAGCACATGGACAACGTCTTCGACTCCATGATCTGC
+101   V  N  S  D  A  L  T  F  E  D  Q  F  V  Q  H  M  D  N  V  F  D  S  M  I  C
+376  ATCTCGCTGGTGGCCTCCATCTGCAACCTCTTGGCCATCGCCGTGGACAGGTACGTCACCATCTTCTACGCGCTG
+126   I  S  L  V  A  S  I  C  N  L  L  A  I  A  V  D  R  Y  V  T  I  F  Y  A  L
+451  CGCTACCACAGCATCATGACCGTGCGGAAGGCGGGGGCCCTGATCGCGGCCATCTGGGTGTGCTGCGGCGTCTGC
+151   R  Y  H  S  I  M  T  V  R  K  A  G  A  L  I  A  A  I  W  V  C  C  G  V  C
+526  GGCGTGGTCTTCATCGTCTACTCCGAGAGCAAGATGGTCATCGTGTGCCTTGTCGTCATCTTCTTCGCCATGCTG
+176   G  V  V  F  I  V  Y  S  E  S  K  M  V  I  V  C  L  V  V  I  F  F  A  M  L
+601  CTCCTCATGGGCACCCTCTACGTGCACATGTTTCTCTTCGCCCGGCTGCACGTCCAGCGCATCGCCGCGCTGCCG
+201   L  L  M  G  T  L  Y  V  H  M  F  L  F  A  R  L  H  V  Q  R  I  A  A  L  P
+676  CCCGCCGACGGGGGGCCCCCCCCGCAGCGCTCGTGCCTGAAGGGGGCCGTGACCATCTCCCTCCTGCTGGGGGTC
+226   P  A  D  G  G  P  P  P  Q  R  S  C  L  K  G  A  V  T  I  S  L  L  L  G  V
+751  TTCATCTTCTGCTGGGCCCCCTTCTTTCTCCACCTGGTTCTCATCATCACCTGCCCCACCCACCCCTACTGCATC
+251   F  I  F  C  W  A  P  F  F  L  H  L  V  L  I  I  T  C  P  T  H  P  Y  C  I
+826  TGCTACACCGCCCACTTCAACACCTACCTGGTCCTCATCATGTGCAACTCGGTCATCGACCCCCTGATCTACGCC
+276   C  Y  T  A  H  F  N  T  Y  L  V  L  I  M  C  N  S  V  I  D  P  L  I  Y  A
+901  TTCCGGAGCCTGGAACTGCGCAACACCTTCAAGGAGATCCTGTGCAGCTGCAACGCCTCAACCTGGGGTAGGAGG
+301   F  R  S  L  E  L  R  N  T  F  K  E  I  L  C  S  C  N  A  *
+976  CAGGGCCCCGCCGGAGCGGTCCTCAGCCTGGTCGCGTTTCACCTGCCGGCCAGCCAAGGTGTTTAGGAAGCGGGG
+1051 AGAAACGCACTCAAAAGATGGAAAGATGTGTTCACGGTCATGATCATACAGGTCCTTTTGTTTTTAAGCTTACAA
+1126 AACGTTGAGAAAGAAGGGGGGGGGGCTTTCTGAAAGGACGCCAACTTGGGTAAGTCCCCAACTCTGCTTTCCCAA
+1201 AGAGTCGCGGGGGCAAATCCGTGGAGACGGCTCTGGGTGCTGTCTGCGTTCGTTTCCATGCACCCGGGATCCGTG
+1276 ATGCCCCGCCTGCTCCCCGGCTTCCCACCCACGCCTCCGTGTTTCAAGTTCAAACCTCTCCCAAGCAAACCTGTT
+1351 CCAGCTATTGACCTTCAGCTACCAGCTGCTCGGGTGGCTTTGCCCTCATTTTGTGAGACCCTGAGTCTCTATTAC
+1426 TCTCTTTACAAGAAAACGGTTTCCTAAAAACAATGACCTCTTTGTGGAAGAAGTGATGCTATTTGCCCCTCCTCT
+1501 TGACCCCAGAGCTCCCCTGCAAAAATAGGGTGGGAATGTAGCCCACTTCTAGTCTGTTGTTATTGTTATTGTTGC
+1576 TGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGTTAGGCTTTTATGCAGGCAACATAGGGCAGCTT
+1651 TCAAAACTCTTCTGAAAGAGAAAGTGCTTTGGAAAATCAGAACATACCCATCTACAAAGTATTTGGAGACCTTGC
+1726 TACTTTGAGGTGTACATTTTCCAATTTTGCAAAAGTCATGTTTTCCTAATATGCCTTTTTTCGTCTTTAAAGCAA
+1801 GGAAGCAACTCCTGGGAGGGAGGGCATCTGTCTCAAGAAATTGCTGGACTGTAGTTTCCGAGAGTTTTAGTGCCA
+1876 GGGAGCTGCCTGCGGCTTTCGTGGAAGCTGTTTTTGCTCAGAGAAACTGGGTCTCTACCACGTGTGGTCTATGGG
+1951 ATGTGGGCGATGAAGGAGAATGGGGTTTTGGTTCACTGTTCCTTTTGCTTGACATGATGCTAAAGCTGGGTGACC
+2026 CTCCTGTCGAGGTCTACACTGCATCAAGTAAAGCGATCCATTCCTGCGAGCGAGCGCAGGGTGAGATTCGCTGAG
+2101 CAGTGTTGTACACCAGTGCGCTGCAAGCATCCATCAGACCCCTTTCTGCCTCAGATGACTTCCGGCTGTGCTCTC
+2176 CAGCTGTCCTCACCCCCCCCCCCCCATGAATCCTTATATTACGCTGTGTCTTTGGTATACGACTGTGTCTTTTTC
+2251 TTGTGCTGGAATATATTGGGGGTTTTTTGTACTTGATATGTGCTCGTGTGCATACTATTGTTTCTTGCTTTGATT
+2326 GTGATGTTTGAAAATAATAAACATCTTCTGTGGCTGATAAACATGTGTCACTTTAAACTACCCCACCTGGACGCA
+2401 CCCTAGTGACGTCTGCACGTGGCCATCATATGTGACACTG
```

B

	Pig	Human	Mouse	Rat
Pig	100.0	88.2	85.3	86.0
human		100.0	87.6	87.6
Mouse			100.0	93.0
Rat				100.0

C

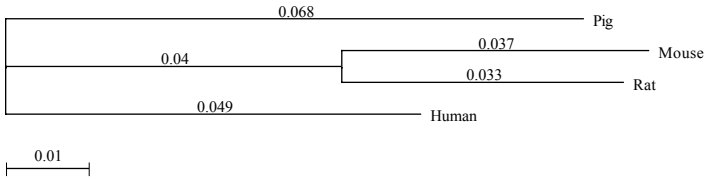


Figure II-2. Comparison of amino acid sequences between pMC3R and MC3Rs from other species. Alignment of the amino acid sequences between pMC3R and human, mouse and rat MC3Rs. Positions of different regions of the receptor are indicated above the sequences and labeled as follow: transmembrane domains as TMD1-7, extracellular loops as EL1-3, intracellular loops as IL1-3, amino- and carboxyl- termini as extracellular amino terminus and cytoplasmic tail, respectively. The conserved residues are shaded in gray. The transmembrane residues are in bold. (B) Homology and (C) phylogenetic analysis of MC3R amino acid sequences from several species.

A

```
                                  Extracellular Amino Terminus
pMC3R:  ------------------------------------MNASCCLLSAPPALPNSSEHLPA  23
hMC3R:  MSIQKTYLEGDFVFPVSSSSFLRTLLEPQLGSALLTAMNASCCLPSVQPTLPNLSQHPAA  60
mMC3R:  ------------------------------------MNSSCCLSSVSPMLPNLSEHPAA  23
rMC3R:  ------------------------------------MNSSCCPSSSYPTLPNGSEHLQA  23

                                     TMD1              IL1       TMD2
pMC3R:  PSFSNQSSSGFCEQVVIQPEVFLALGILSLLENVLVILAVARNGNLHSPMYLFLCSLAVA  83
hMC3R:  PFFSNQSSSAFCEQVFIKPEVFLSLGIVSLLENILVILAVVRNGNLHSPMYFFLCSLAVA  120
mMC3R:  PPASNRSGSGFCEQVFIKPEVFLALGIVSLMENILVILAVVRNGNLHSPMYFFLCSLAAA  83
rMC3R:  PSASNRSGSGFCEQVFIKPEVFLALGIVSLMENILVILAVVRNGNLHSPMYFFLLSLLQA  83

                                  EL1                 TMD3
pMC3R:  DLLVSVSNALETIMIAVVNSDALTFEDQFVQHMDNVFDSMICISLVASICNLLAIAVDRY  143
hMC3R:  DMLVSVSNALETIMIAIVHSDYLTFEDQFIQHMDNIFDSMICISLVASICNLLAIAVDRY  180
mMC3R:  DMLVSLSNSLETIMIAVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAIDRY  143
rMC3R:  DMLVSLSNSLETIMIVVINSDSLTLEDQFIQHMDNIFDSMICISLVASICNLLAIAVDRY  143

             IL2               TMD4          EL2          TMD5
pMC3R:  VTIFYALRYHSIMTVRKAGALIAAIWVCCGVCGVVFIVYSESKMVIVCLVVIFFAMLLLM  203
hMC3R:  VTIFYALRYHSIMTVRKALTLIVAIWVCCGVCGVVFIVYSESKMVIVCLITMFFAMMLLM  240
mMC3R:  VTIFYALRYHSIMTVRKALTLIGVIWVCCGICGVMFIIYSESKMVIVCLITMFFAMVLLM  203
rMC3R:  VTIFYALRYHSIMTVRKALSLIVAIWVCCGICGVMFIVYSESKMVIVCLITMFFAMVLLM  203

                          IL3                              TMD6
pMC3R:  GTLYVHMFLFARLHVQRIAALPPADGGPPPQRSCLKGAVTISLLLGVFIFCWAPFFLHLV  263
hMC3R:  GTLYVHMFLFARLHVKRIAALPPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  300
mMC3R:  GTLYIHMFLFARLHVQRIAVLPPAGVVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  263
rMC3R:  GTLYIHMFLFARLHVQRIAALPPADGVAPQQHSCMKGAVTITILLGVFIFCWAPFFLHLV  263

             EL3               TMD7            Cytoplasmic Tail
pMC3R:  LIITCPTHPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCSCNA----  319
hMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFREILCGCNGMNLG  360
mMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCGCNSMNLG  323
rMC3R:  LIITCPTNPYCICYTAHFNTYLVLIMCNSVIDPLIYAFRSLELRNTFKEILCGCNGMNVG  323
```

B

	Pig	Human	Mouse	Rat
Pig	100.0	87.5	83.1	83.7
human		100.0	88.7	89.3
Mouse			100.0	94.0
Rat				100.0

C

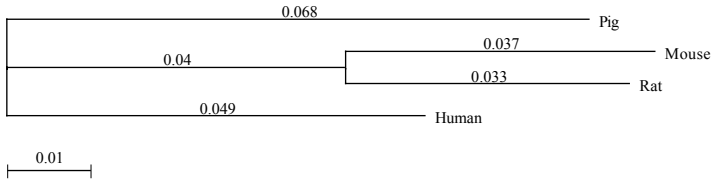
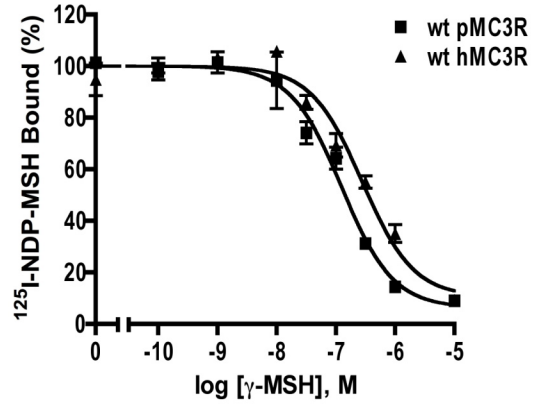
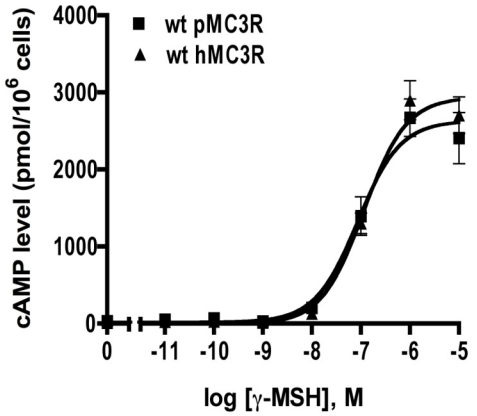


Figure II-3. Expression and functional analysis of the cloned pMC3R in HEK293T cells. HEK293T cells were transiently transfected with pMC3R and hMC3R and binding and signaling assays were performed as described in *Materials and Methods*. For ligand binding, different concentrations of unlabeled γ-MSH (A) or AgRP (B) were used to displace the binding of ^{125}I-NDP-MSH to pMC3R or hMC3R on intact cells. Results shown are expressed as the transfected cells were stimulated with various concentrations of γ-MSH and intracellular Camp levels were measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment and all experiments were performed at least three times.

A


wt pMC3R
wt hMC3R
^{125}I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-10
-9
-8
-7
-6
-5
log [γ-MSH], M


wt pMC3R
wt hMC3R
cAMP level (pmol/10^6 cells)
4000
3000
2000
1000
0
0
-11
-10
-9
-8
-7
-6
-5
log [γ-MSH], M

B

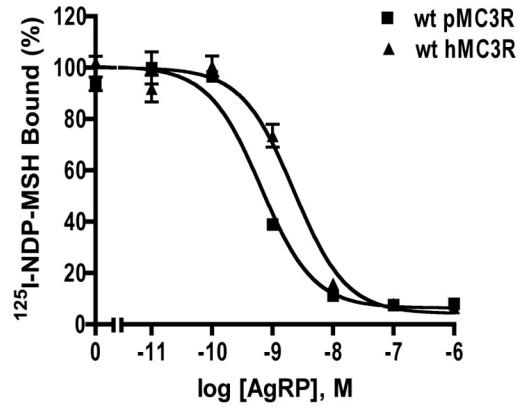
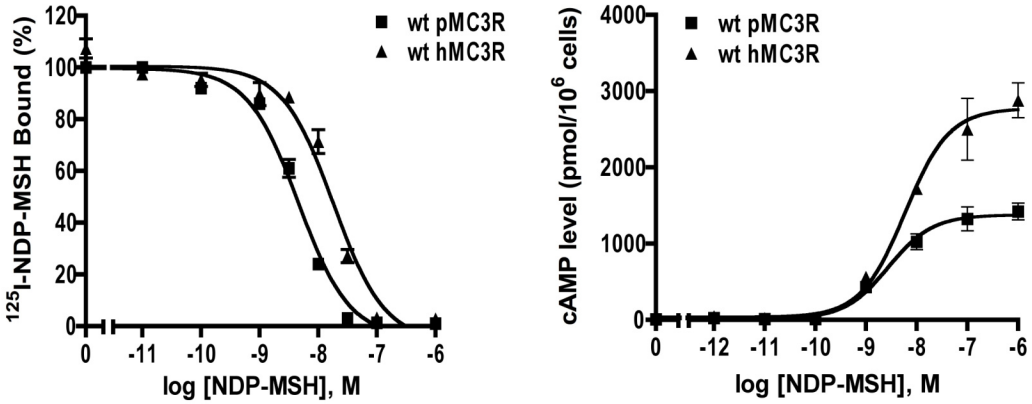
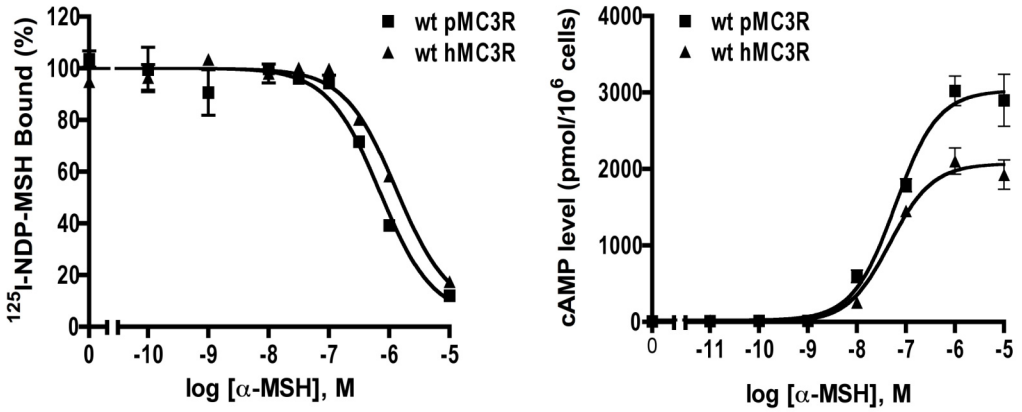

wt pMC3R
wt hMC3R
^{125}I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M

Figure II-4. HEK293T cells were transiently transfected with pMC3R or hMC3R. Binding assays were performed as described in *Materials and Methods*. Different concentrations of unlabeled NDP-MSH (A), α-MSH (B) or D-Trp^{8}-γ-MSH (C) were used to displace the binding of ^{125}I-NDP-MSH to pMC3R or hMC3R on intact cells. To measure accumulation of intracellular cAMP, the transfected cells were stimulated with various concentrations NDP-MSH (A), α-MSH (B) or D-Trp^{8}-γ-MSH (C) and intracellular cAMP levels were measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment and all experiments were performed at least three times.

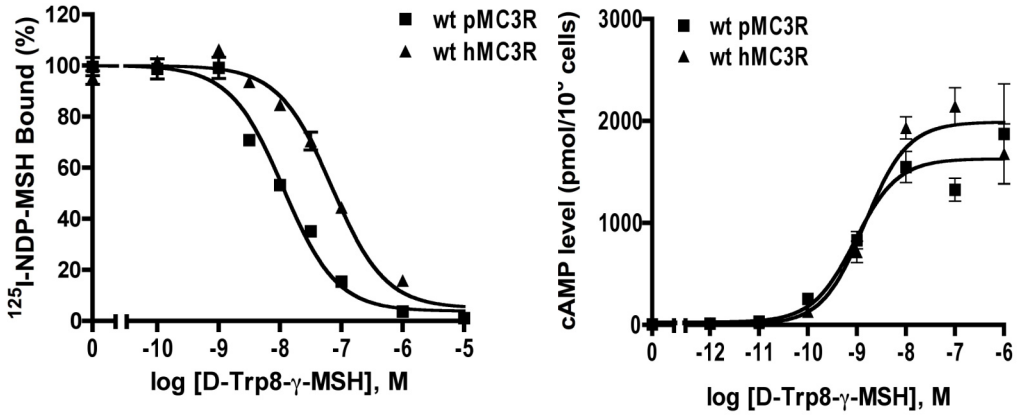
A


wt pMC3R
wt hMC3R
125I-NDP-MSH Bound (%)
log [NDP-MSH], M
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M

B


wt pMC3R
wt hMC3R
125I-NDP-MSH Bound (%)
log [α-MSH], M
cAMP level (pmol/10^6 cells)
log [α-MSH], M

C


wt pMC3R
wt hMC3R
125I-NDP-MSH Bound (%)
log [D-Trp8-γ-MSH], M
cAMP level (pmol/10^6 cells)
log [D-Trp8-γ-MSH], M

CHAPTER III

PHARMACOLOGICAL ANALYSIS OF TWO NATURALLY OCCURRING PORCINE MELANOCORTIN-4 RECEPTOR MUTATIONS IN DOMESTIC PIGS

3.1. Introduction

The melanocortin-4 receptor (MC4R) is a member of the superfamily of G protein-coupled receptors (GPCRs) consisting of the hallmark seven transmembrane domains (TMs) connected by alternating extracellular and intracellular loops, with an extracellular NH2 terminus and intracellular COOH terminus. Upon hormone stimulation, MC4R couples to the stimulatory heterotrimeric G protein (Gs) and then activates adenylyl cyclase to promote the intracellular accumulation of cAMP. The endogenous agonist of MC4R, α-melanocyte stimulating hormone (α-MSH), is derived from proopiomelanocortin (POMC) via tissue-specific posttranslational processing. Agouti-related protein (AgRP) is the endogenous antagonist of the MC4R.

During the past decade, ample evidences demonstrated the central role of the MC4R in regulating energy homeostasis of rodents (Cone, 2005). MC4R or POMC knockout mice have increased food intake and obesity (Huszar, et al. 1997; Yaswen, et al. 1999). Mice over-expressing AgRP are hyperphagic, obese and hyperinsulinemic (Ollmann, 1997; Graham, 1997) whereas mice administered with α-MSH or melanotan II, an

analogue of α-MSH, had decreased food intake (Fan, 1997; Thiele, 1998). Furthermore, mice co-administrated melanotan II and the MC4R antagonist SHU9119 showed a normal food intake, suggesting that SHU9119 inhibits the effect of melanotan II on food intake (Fan, 1997; Thiele, 1998). MC4R activation also increases energy expenditure (Ste Marie, 2000; Balthasar, 2005).

Human genetic studies also showed that the MC4R plays a critical role in controlling food intake and body weight in humans. So far more than 110 distinct human MC4R (hMC4R) mutations have been identified from obese patients of various ethnic populations (Tao, 2006). These mutants are caused by frameshift, in-frame deletion, nonsense and missense mutations. Although some mutants do not have obvious functional defects as determined by *in vitro* assays and might represent rare polymorphic variants, others are defective in cell surface expression, ligand binding or G protein coupling/activation (Tao, 2005). Up to 6% of patients with severe early-onset obesity are caused by mutations in *MC4R* gene in some cohorts, thus representing the most common monogenic form of obesity (Farooqi, et al. 2003a).

In 2000, Rothschild and colleagues identified a missense mutation in the porcine MC4R (pMC4R) gene that change a highly conserved Asp in TM7 in melanocortin receptors (MCRs) to Asn (Kim, et al. 2000). This Asp, designated 7.49 according to the numbering scheme of Ballesteros and Weinstein (Ballesteros, et al. 1995), lies in the highly conserved N/DPxxY motif. The majority of Family A (rhodopsin-like) GPCRs has an Asn at this position. MCRs, together with gonadotropin-releasing hormone receptor and prostanoid receptors, are some of the few receptors that have an Asp at position 7.49. Within the MCR subfamily, however, this Asp is fully conserved in all the MCRs cloned

to date, pointing to its potential functional importance. In our structure-function studies on the hMC4R, we generated D298N hMC4R and found that the mutant receptor has relatively normal functions. These results are dramatically different with the results reported for the pMC4R mutant, where a total loss of signaling was reported (Kim, et al. 2004). Subsequent genotypephenotype association studies on this locus resulted in divergent conclusions, with some studies supporting the original association (Liu, et al. 2002; Hernandez-Sanchez, et al. 2003; Houston, et al. 2004; Meidtner, et al. 2006) while other studies failed to find an association (Park, et al. 2002; Andersson, 2003; Stachowiak, et al. 2006). Based on extensive studies on other GPCRs, we hypothesized that D298N pMC4R has normal function. We therefore generated the mutant pMC4R and analyzed its function. A novel pMC4R mutant, R236H, was recently identified (Meidtner, et al. 2006). This mutant was identified in a Piétrain founder pig, a relatively slow-growing pig. It was not found in the Mangalitsa pigs in the same breeding program. R236 is located in the third intracellular loop that could be important for G protein coupling. However, the functional properties of this mutant were not investigated in the original study. Detailed functional studies are necessary to establish a causal relationship between the variant identified from genetic screening and any potential contribution of the variant to growth traits (Tao, 2005). We therefore studied its functional properties.

3.2. Materials and methods

3.2.1. Peptides and supplies

[Nle^4, D-Phe^7]-α-MSH (NDP-MSH), a superpotent analogue of α-MSH, was obtained from Phoenix Pharmaceuticals (Belmont, CA). β-MSH was purchased from Bachem (King of Prussia, PA). Human AgRP(86-132)was obtained from Peptides International (Louisville, KY). ^{125}I-iodinated NDP-MSH was purchased from the Peptide Radioiodination Service Center at The University of Mississippi (University, MS) with a specific activity of 2176 Ci/mmol. Tissue culture plastic wares were purchased from Corning (Corning, NY). Dulbecco's modified Eagles medium (DMEM) and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

3.2.2. Molecular cloning of pMC4R

The pMC4R coding region was amplified directly from porcine genomic DNA (Novagen, San Diego, CA) using a primer pair (sense primer 5'-AAGAATTC**ATG**AACTCAACCCATCAC-3' and anti-sense primer 5'-CCTCTAGATTAATATCTGCTAGACAAATC-3') designed based on the published nucleotide sequence of pMC4R (GenBank access no. AB021664) incorporating EcoRI and XbaI restriction sites in sense and anti-sense primers, respectively (underlined). PCR amplification was performed in a 50 μl mixture containing 100 ng of the porcine genomic DNA, 0.25 mM dNTPs, 0.4 μM of each primer, 1×pfu DNA polymerase buffer, 1.5 mM of $MgCl_2$, and 2.5 U pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with the following cycling parameters: 2 min at 95°C for one cycle and 1 min at 95°C, 45 s at

56°C, and 90 s at 72°C for 35 cycles followed by a final cycle of extension at 72°C for 10 min. The PCR products of expected size as visualized by agarose gel electrophoresis were purified with Qiagen PCR purification kit (Qiagen, Valencia, CA) and double digested with EcoRI and XbaI (New England Biolabs, Beverly, MA). The PCR fragment was further purified, ligated into the expression vector pcDNA3.1, and transformed into JM109 competent cells. Cells were grown overnight on LB agar plates containing ampicillin and 8 clones were selected for growing in LB medium. Plasmid DNA was extracted with Qiaprep spin miniprep kit (Qiagen) to screen clones with the insert of expected size after digestion with EcoRI and XbaI. The nucleotide sequence of the cloned pMC4R was determined by sequencing three independent plasmids performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center. Plasmid DNA containing the pMC4R of correct sequence was prepared with Qiagen plasmid maxi kit (Qiagen) for site-directed mutagenesis or transfection as described below.

3.2.3. Site-directed mutagenesis

Nucleotide sequencing of the cloned pMC4R showed that it is D298N pMC4R. Wild-type (WT) pMC4R was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol described previously (Tao and Segaloff, 2003). R236H pMC4R was made using the WT pMC4R as the template by the same method. Wild-type hMC4R attached with a myc epitope at its N terminus (after the initiating Met) has been described previously (Tao and Segaloff, 2003). The hMC4R mutant D298N was generated using this construct as template by the same mutagenesis method.

3.2.4. Cells and transfection

Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas,VA) were grown at 5% CO_2 in DMEM supplemented with 10% newborn calf serum, 10 mM HEPES, and 100 units/ml of penicillin and 100 ng/ml streptomycin. For transient expression of the MC4Rs, cells were plated on gelatincoated 35-mm 6-well plates and transfected using the calcium precipitation method (Chen, et al. 1987). Four-microgram plasmid in 2 ml media was used per 35-mm well. The transfection cocktail included 86 µl water, 10 µl 2.5M $CaCl_2$, 4 µl of plasmid DNA, and 100 µl of 2×BSS (consisting of 280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM *N*,*N*-bis[2-hydroxyl]-2-aminoethane sulfonic acid, pH 6.95). After 15 min incubation in the hood at room temperature, 1.8 ml of growth media was combined with the cocktail and put into a well in 6-well clusters. Forty-eight hours after transfection, cells were used for measuring ligand binding and hormone stimulation of cAMP generation.

3.2.5. Radioligand binding assay

Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) modified to contain 1 mg/ml BSA (referred to as Waymouth/BSA). One milliliter of fresh Waymouth/BSA was added to each well, and then 100,000 cpm of ^{125}I-NDP-MSH (50 µl) was added to each well, with or without different concentrations of unlabeled α-MSH or NDP-MSH. The final concentration of unlabeled ligands ranged from 10^{-10} to 10^{-5} M (for α-MSH) or 10^{-11} to 10^{-6} M (for NDP-MSH). For the AgRP binding assay, cells were plated in 100 mm dishes and transfected when the cells reached 50-70% confluency. After an overnight

incubation in transfection cocktail, cells were trypsinized and replated into 24-well plates. The total volume for binding assay in the 24-well plate was 200 µl. (The reason for performing the AgRP binding assays in 24-well plate is to save the amount of AgRP used.) The final concentration of unlabeled AgRP ranged from 10^{-11} to 10^{-6} M. After incubation at 37°C for 1 hr, cells were placed on ice, washed twice with cold Hanks' balanced salt solution (Sigma-Aldrich) modified to contain 1 mg/ml BSA (referred as HBSS/BSA). Then 100 µl of 0.5 N NaOH was added to each well. Lysed cells were collected using cotton swabs, and ligand binding was counted in a gamma counter. Binding capacity and IC_{50} were calculated using Prism software version 4 (GraphPad Software, San Diego, CA).

3.2.6. Ligand stimulation of intracellular cAMP generation

Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then 1ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well. After incubation at 37°C for 15 min, either buffer alone or different concentrations of α-MSH or NDP-MSH were added. The final concentrations ranged from 10^{-12} to 10^{-6} M (for NDP-MSH) or 10^{-11} to 10^{-5} M (for α-MSH). After incubation at 37°C for 1 hr, cells were then placed on ice, media aspirated, and intracellular cAMPextracted by the addition of 0.5 N percholoric acid containing 180 ng/ml theophylline, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramines T method. Polyclonal antibody against cAMP was obtained from Strategic Biosolutions (Newark, DE). The radioimmunoassay was performed as

described before (Steiner, et al. 1969) except that polyethylene glycol 8000 was used to precipitate the antibody-bound fraction of cAMP instead of a second antibody in the original publication. Maximal responses (Rmax) and EC_{50} values were calculated using Prism software version 4 (GraphPad Software).

3.2.7. Statistic analysis

Statistical calculations were performed using Prism 4 (Motulsky, 2003). For comparisons on maximal binding and signaling, one sample *t*-test was used. For comparisons on IC_{50} and EC_{50}, an unpaired *t*-test was used.

3.3. Results

3.3.1. Ligand binding and signaling properties of the MC4R mutants using NDP-MSH as the ligand

HEK293T cells have been used extensively as an *in vitro* system for evaluation of functional properties of MC4Rs from various species. Herein, WT and mutant pMC4Rs were transiently transfected into HEK293T cells and their ligand binding and signaling properties were analyzed. Wild type and D298N hMC4Rs were included in these experiments for comparison. The superpotent analogue of α-MSH widely used in MCR studies, NDP-MSH, was used in these studies. As shown in Fig. III-1-A and Table 2, WT, D298N and R236H pMC4Rs bound NDP-MSH with an IC_{50} of 2.46, 6.70 and 14.00 nM, respectively. Maximal binding (Bmax) of the two mutants were similar to that of WT pMC4R (Table 2). Similar results were obtained for hMC4Rs: WT and D298N hMC4Rs

bound NDP-MSH with IC_{50} of 7.70 and 14.07 nM, respectively, and the Bmax of hMC4R D298N was at the same level as that of WT hMC4R (Fig. III-1-A and Table 2). The signaling properties of the WT and mutant MC4Rs were analyzed upon stimulation with NDP-MSH in HEK293T cells transiently transfected with these receptor constructs. The results showed that NDP-MSH induced dose-dependent increases of intracellular cAMP in all groups (Fig. III-1-B). As shown in Table 2, NDP-MSH stimulated cAMP accumulation with EC_{50} of 2.80, 27.62 and 15.43 nM for pMC4R WT, D298N and R236H, respectively. The maximal responses of pMC4R D298N and R236H were reduced to 76% and 65% of that of WT pMC4R, respectively. NDP-MSH stimulated cAMP generation with EC_{50s} of 1.63 and 12.96 nM for hMC4R WT and D298N, respectively. The maximal responses were similar for hMC4R WT and D298N (Table 2). We also measured the basal (constitutive) activities of the expressed receptors. The results showed that pMC4R D298N, but not R236H, had decreased basal signaling compared with WT pMC4R whereas hMC4R D298N has normal constitutive activity (Fig. III-2).

3.3.2. Ligand binding and signaling properties of the MC4R mutants using α-MSH as the ligand

Although NDP-MSH is widely used in MCR studies, it is a superpotent long-lasting analog of the natural agonist derived from POMC, α-MSH. Different pharmacological properties of the mutant receptors might be observed using NDP-MSH *vs.* α-MSH. Therefore we also measured ligand binding and signaling properties of the mutant pMC4Rs using α-MSH as the ligand. These results showed that there is no difference

between the WT pMC4R and either of the pMC4R mutants, R236H and D298N, in any of the parameters measured (Fig. III-3 and Table 3).

3.3.3. Ligand binding properties of the MC4R mutants using the antagonist AgRP

Since the balance between the agonist α-MSH and the antagonist AgRP determines the function of the MC4R, it is important to determine whether the mutant receptors have increased affinity for AgRP. Increased affinity for AgRP, even in the face of normal affinity for and response to α-MSH, would result in decreased function of the MC4R. Therefore we also determined the binding properties of the mutant receptors to AgRP. As shown in Fig. III-4, both WT and mutant pMC4Rs bound AgRP with similar high affinity (Table 2). Similarly, WT and D298N hMC4Rs bound to AgRP with the same affinities (Fig. III-4 and Table 2)

3.4. Discussion

More than 110 distinct mutations have been identified from obese humans in the *MC4R* gene. Functional studies are essential for establishing a causal relationship between the mutations identified and the obesity phenotype (Tao, 2005; Farooqi, et al. 2003b). This is highlighted by several recent studies where functional studies provided strong support for the genetic studies (Farooqi, et al. 2003a; Tao and Segaloff, 2005; Rong, et al. 2006).

Studies on the involvement of pig MC4R in feed intake and energy balance have resulted in conflicting results. Barb and colleagues showed that intracerebroventricular

administration of NDP-MSH decreased feed intake, but treatments with the synthetic antagonist SHU9119 or AgRP, the natural antagonist of the MC4R, failed to stimulate feed intake (Barb, et al. 2004), which differs from the results obtained in rodents (Fan, et al. 1997; Seeley, et al. 1997; Rossi, et al. 1998) and sheep (Wagner, et al. 2004). *In vitro*, these ligands did act as antagonists (Barb, et al. 2004). We showed herein that WT and D298N pMC4Rs have similar binding affinities for AgRP (Fig. III-4 and Table 2). Therefore these data, while conforming the *in vitro* data of Barb and colleagues, cannot provide an explanation for the lack of effect of AgRP on feed intake in pigs as observed by Barb and colleagues (Barb, et al. 2004).

Rothschild and colleagues identified a missense mutation in pMC4R, D298N, which mutated the conserved Asp in N/DPxxY motif in TM7. They showed that this mutation was associated with fatness, growth, and feed intake traits (Kim, et al. 2000). Subsequent functional studies on this mutant revealed that the mutation did not affect ligand binding but totally abolished ligand-stimulated signaling (Kim, et al. 2004) therefore belonging to the Class IV category according to our classification scheme (Tao, 2005; Tao and Segaloff, 2003): mutants defective in G protein coupling/activation. From a structure-function point of view, this mutation is interesting in that a fully conserved Asp in MCRs is mutated to Asn, the most commonly found amino acid in Family A GPCRs. Since Asn is found in most of the Family A GPCRs, it can be reasoned that Asn should confer normal functions for the receptor. Our preliminary results with hMC4R showed that indeed D298N hMC4R is fully functional in terms of both ligand binding and signaling. Therefore we were interested in identifying the molecular determinants dictating the dramatic differences between our own data on hMC4R and the data on the

pMC4R. We cloned the pMC4R from genomic DNA purchased from a commercial vendor and determined that the cDNA we cloned was in fact the mutant D298N. We then generated the WT cDNA using site-directed mutagenesis. As reported herein, our results differ from those of Kim et al. (2004). The D298N pMC4R, similar to the D298N hMC4R, can bind radiolabeled NDP-MSH with normal affinity and generate cAMP with similar maximal signaling, although the EC_{50}s are increased (Figs. III-1 and Table 2). The reason for this discrepancy is not clear. We noticed that the maximal stimulation in the study by Kim and colleagues, about 16 pmoles per million cells, is extremely low in this transient transfection system using HEK293 cells. In our radioimmunoassay for cAMP, this is about the lowest level we can detect. Recently, Patten and colleagues also showed that D298N hMC4R retained cAMP formation in response to NDP-MSH stimulation (Patten, et al. 2007).

Another mutation in pMC4R, R236H, was recently identified in another screening effort (Meidtner, et al. 2006). We showed here that the mutant had normal ligand binding and signaling properties compared to WT pMC4R when the natural ligand α-MSH was used for these assays (Fig. III-3 and Table 3), suggesting that it might not cause significant changes in feed intake and growth in pigs. The corresponding mutation was also identified in hMC4R from a patient and found to have normal functions, including cell surface expression as well as basal and stimulated activities (Hinney, et al. 2006). This arginine is not conserved in the cloned MCRs. In other MCRs, histidine, tryptophan, or glutamine was observed. It should be pointed out that in functional studies of hMC4R mutations identified from obese patients; several groups have reported a number of mutations that result in no overt functional defects (Tao, 2005). We classify these

mutants as Class V: mutants with no known defects (Tao, 2005; Tao and Segaloff, 2003). Similarly, in other GPCRs that result in diseases, mutations identified from patients that have normal functions were observed not infrequently (Tao, 2006), suggesting that these mutations were not the cause of the disease. One drawback of these experiments is that the MC4R is expressed in neurons. Our experiments in HEK293T cells may not reveal any neuron-specific aspects of the MC4R function.

Another interesting aspect that is worth mentioning is that from all the screening efforts in different strains of pigs, only two mutations in pMC4R were identified. In humans, numerous mutations were identified from both obese patients and the general population. The reason for this lower occurrence of pMC4R mutation is unknown at this time.

In summary, we reported here that the two mutations in pMC4R, R236H and D298N, do not result in overt defects in receptor functions, suggesting that they might not cause significant increases in feed intake and growth. We therefore suggest caution using these mutations as selection markers in breeding programs.

TABLE 2. **Ligand binding and agonist-stimulated cAMP generation of wt and mutant pMC4Rs and hMC4Rs**

	n	NDP-MSH binding		NDP-MSH-stimulated cAMP		AgRP binding
		IC_{50} (nM)	Bmax (% wt)	EC_{50} (nM)	Rmax (% wt)	IC_{50} (nM)
pMC4R wt	3	2.46 ± 0.27	100	2.80 ± 0.41	100	3.87 ± 1.00
pMC4R D298N	3	6.70 ± 1.53	114 ± 7	27.62 ±3.16[b]	76 ± 10	3.42 ± 1.22
pMC4R R236H	3	14.00 ±1.90[b]	89 ± 10	15.43 ±1.02[c]	65 ± 3 [b]	2.08 ± 0.45
hMC4R wt	3	7.70 ± 1.56	100	1.63 ± 0.53	100	5.43 ± 1.52
hMC4R D298N	3	14.07 ±1.16[a]	88 ± 8	12.96 ±2.77 [a]	116 ± 4	4.02 ± 1.41

[a] Significantly different from corresponding wt receptor, $p<0.05$
[b] Significantly different from corresponding wt receptor, $p<0.01$
[c] Significantly different from corresponding wt receptor, $p<0.001$

The data are expressed as the mean ± SEM of three independent experiments. The maximal binding (Bmax) of intact HEK293T cells expressing wt pMC4R or hMC4R were 15601 ± 1568 and 15646 ± 1214 cpm bound/10^6 cells, respectively. The maximal response (Rmax) was 4520 ± 359 and 1513 ± 271 pmol cAMP/10^6 cells for wt pMC4R and hMC4R, respectively. IC_{50} is the concentration of NDP-MSH that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of NDP-MSH that results in 50% stimulation of the maximal response.

TABLE 3. **Ligand binding and agonist-stimulated cAMP generation of wt and mutant pMC4Rs**

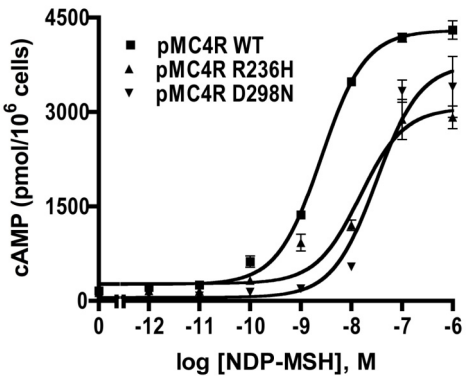
	n	α-MSH binding		α-MSH-stimulated cAMP	
		IC_{50} (nM)	Bmax (% wt)	EC_{50} (nM)	Rmax (% wt)
pMC4R wt	3	10.10 ± 1.67	100	11.02 ± 0.85	100
pMC4R D298N	3	13.02 ± 2.58	103 ± 7	12.03 ± 0.45	100 ± 2
pMC4R R236H	3	13.08 ±3.60	103 ± 3	11.43 ± 0.75	104 ± 4

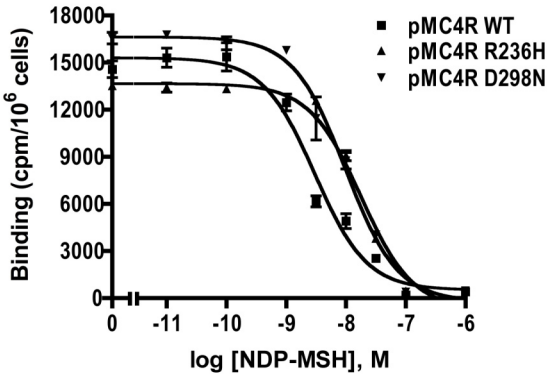
[a] Significantly different from corresponding wt receptor, $p<0.05$
[b] Significantly different from corresponding wt receptor, $p<0.01$
[c] Significantly different from corresponding wt receptor, $p<0.001$

The data are expressed as the mean ± SEM of three independent experiments. The maximal binding (Bmax) of intact HEK293T cells expressing wt pMC4R was 17102 ± 510 bound/10^6 cells for α-MSH. The maximal response (Rmax) under α-MSH stimulation was 1668 ± 221 pmol cAMP/10^6 cells for wt pMC4R. IC_{50} is the concentration of α-MSH that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of α-MSH that results in 50% stimulation of the maximal response.

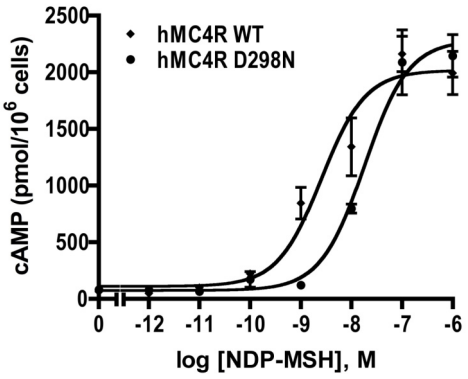
Figure III-1. Ligand binding and signaling properties of the WT and mutant MC4Rs using NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated pMC4R and hMC4R constructs and binding and signaling assays were performed as described in *Materials and Methods*. In A, different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I-NDP-MSH to pMC4Rs or hMC4Rs on intact cells. Results shown are expressed as the mean ± SEM from duplicate determinations within one experiment. In B, HEK293T cells transiently transfected with the indicated pMC4R or hMC4R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using radioimmunoassay. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed three times.

A


4500
3000
1500
0
cAMP (pmol/10^6 cells)
pMC4R WT
pMC4R R236H
pMC4R D298N
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M


18000
15000
12000
9000
6000
3000
0
Binding (cpm/10^6 cells)
pMC4R WT
pMC4R R236H
pMC4R D298N
0
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

B


2500
2000
1500
1000
500
0
cAMP (pmol/10^6 cells)
hMC4R WT
hMC4R D298N
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

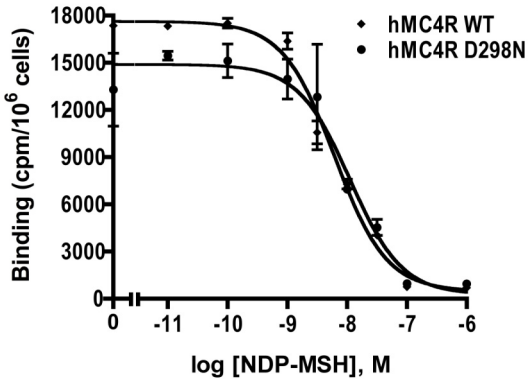

18000
15000
12000
9000
6000
3000
0
Binding (cpm/10^6 cells)
hMC4R WT
hMC4R D298N
0
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

Figure III-2. Basal activities of WT and mutant pMC4Rs and hMC4Rs. The basal activities of WT and mutant MC4Rs were measured by assaying intracellular cAMP levels in the absence of NDP-MSH. The results for the mutants were normalized to the corresponding WT MC4R (designated as 100%) and shown herein as mean percentage ± SEM from three independent experiments. The basal cAMP levels for WT pMC4R and hMC4R were 194 ± 48 pmol/10^6 cells and 155 ± 23 pmol/10^6 cells, respectively. Star (*) indicates significantly different from the wt hMC4R.

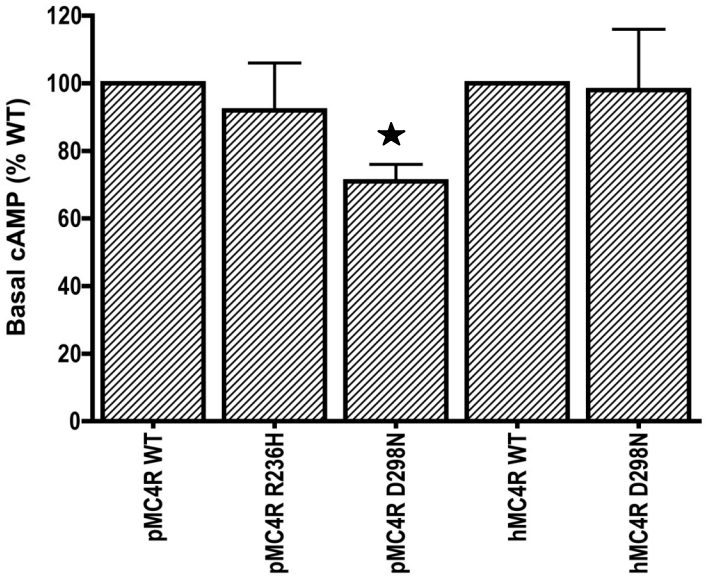
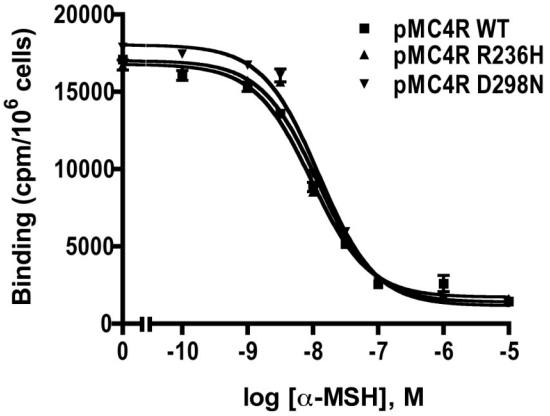

Basal cAMP (% WT)
120
100
80
60
40
20
0
pMC4R WT
pMC4R R236H
pMC4R D298N
hMC4R WT
hMC4R D298N

Figure III-3. Ligand binding and signaling properties of the WT and mutant pMC4Rs using α-MSH as the ligand. HEK293T cells were transiently transfected with the indicated pMC4R constructs and binding and signaling assays were performed as described in *Materials and Methods*. In A, different concentrations of unlabeled α-MSH were used to displace the binding of ^{125}I-NDP-MSH to pMC4Rs on intact cells. Results shown are expressed as the mean ± SEM from duplicate determinations within one experiment. In B, HEK293T cells transiently transfected with the indicated pMC4R constructs were stimulated with different concentrations of α-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed three times.

A


20000
15000
10000
5000
0
Binding (cpm/10^6 cells)
pMC4R WT
pMC4R R236H
pMC4R D298N
0
-10
-9
-8
-7
-6
-5
log [α-MSH], M

B

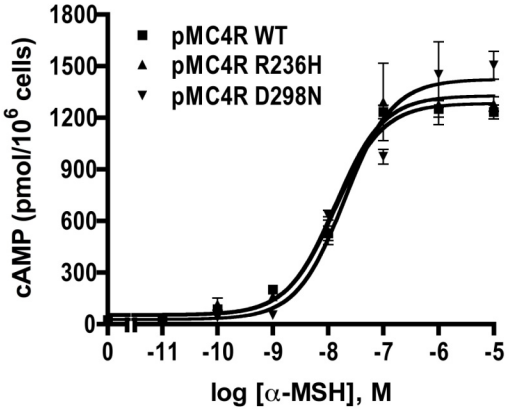
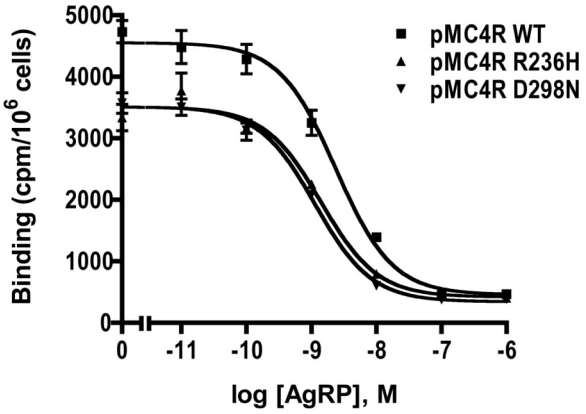
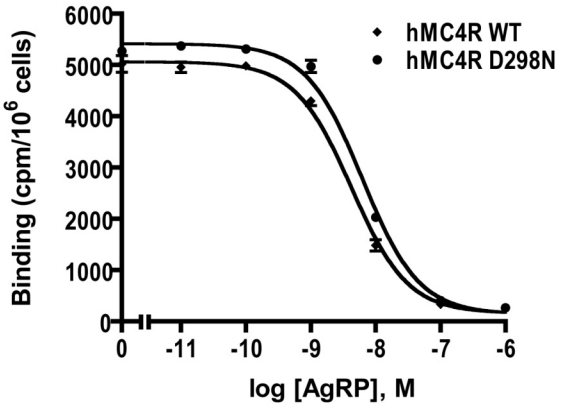

1800
1500
1200
900
600
300
0
cAMP (pmol/10^6 cells)
pMC4R WT
pMC4R R236H
pMC4R D298N
0
-11
-10
-9
-8
-7
-6
-5
log [α-MSH], M

Figure III-4. Ligand binding properties of the WT and mutant MC4Rs on AgRP. HEK293T cells were transiently transfected with the indicated pMC4R and hMC4R constructs and binding assays were performed using AgRP as the competitor. Different concentrations of AgRP were used to displace the binding of ^{125}I-NDP-MSH to pMC4Rs or hMC4Rs on intact cells. Results shown are expressed as the mean ± SEM from duplicate determinations within one experiment. All experiments were performed three times.


pMC4R WT
pMC4R R236H
pMC4R D298N
Binding (cpm/10^6 cells)
5000
4000
3000
2000
1000
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M


hMC4R WT
hMC4R D298N
Binding (cpm/10^6 cells)
6000
5000
4000
3000
2000
1000
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M

CHAPTER IV

FUNCTIONAL CHARACTERIZATION AND PHARMACOLOGICAL RESCUE OF THE HUMAN MELANOCORTIN-4 RECEPTOR VARIANTS IDENTIFIED FROM OBESE PATIENTS

4.1. Introduction

During the past decade, mouse and human genetic studies together with detailed pharmacological investigation revealed that the leptin-regulated melanocortin circuit plays a critical role in regulating food intake and energy expenditure. To date, it has been demonstrated that mutations in six genes including leptin (*LEP*) (Montague, et al. 1997), leptin receptor (*LEPR*) (Clement, et al. 1998), prohormone convertase 1 (*PC1*) (Jackson, et al. 1997), pro-opiomelanocortin (*POMC*) (Krude, et al. 1998), melanocortin-4 receptor (*MC4R*) (Vaisse, et al. 1998; Yeo, et al. 1998), and single-minded homolog 1 (*SIM1*), a critical factor for neuronal cell developement (Holder, et al. 2000), cause monogenic obesity (Rankinen, et al. 2006). Of these molecules, the MC4R has been identified as a key component in the leptin-regulated pathway (Huszar, et al. 1997; Ollmann, et al. 1997; Graham, et al. 1997; Fan, et al. 1997; Thiele, et al. 1998). Mutations in the MC4R gene represent the most common monogenic form of human obesity (Farooqi, 2003a; Cone, 2005; Tao, 2005). It was estimated that up to 6% of early-onset morbid obesity was caused by mutated hMC4Rs (Farooqi, et al. 2003a).

As a member of the G protein-coupled receptors (GPCRs) superfamily, the MC4R shares the common structure of GPCRs including three extracellular loops, seven transmembrane domains (TMs), and three intracellular loops with its N- and C- termini facing the outside and inside of the cell, respectively. The MC4R couples primarily to the stimulatory G protein (Gs). Therefore hormone stimulation activates the adenylyl cyclase to increase the intracellular cAMP level (Gantz and Fong, 2003). The endogenous hormones that activate the MC4R are α-melanocyte stimulating hormone (α-MSH) and β-MSH. These hormones together with γ-MSH and ACTH are all derived from POMC through a tissue-specific, posttranslational processing (Smith and Funder, 1988). Agouti-related protein (AgRP) serves as an endogenous antagonist to inhibit the MC4R activity.

So far, about 110 mutations in the coding region of the hMC4R have been identified from populations of various ethnic backgrounds. The mutants contain frameshift, in frame deletions, or nonsense and missense mutations. Many of the mutant hMC4Rs have been functionally characterized in detail (Tao, 2005). Previously, several groups reported the identification of ten novel hMC4R mutants in obese children or adults of diverse ethnic groups. They are R7C in the N-terminus (Hinney, et al. 2006), C84R (Hainerová, et al. 2007), S127L (Hinney, et al. 2003; Valli-Jaakola, et al. 2004) and S136F (Rettenbacher, et al. 2007) in TM2, W174C (Buono, et al. 2005) in TM4; A219V (Larsen, et al. 2005) and P230L (Hinney, et al. 2003) in IL3, F261S (Shao, et al. 2005) in TM6, I317V (Buono, et al. 2005) and L325F (Larsen, et al. 2005) in the C terminal tail. The functional properties of these mutants were either not investigated (W174C and I317V) or only partially determined (the other eight mutants). In this study, we set out to

quantitatively measure not only the cell surface expression but also the total receptor expression levels of these mutants, to analyze their functional properties, including ligand binding (using both NDP-MSH and AgRP(86-132)) and signaling responses upon stimulation by NDP-MSH. Since the final signaling is the net result of stimulation by agonists and inhibition by the antagonist AgRP, it is important to investigate whether the mutations affect their binding affinity for AgRP. Previous studies suggested that loss of basal signaling might be one defect of the receptor in MC4R mutants identified from obese subjects (Srinivasan, et al. 2004). Therefore, the constitutive activities of the mutant hMC4Rs were also determined in this study.

The majority of the functional defects of hMC4R mutants identified and characterized so far are caused by intracellular retention of the misfolded receptor. It was already know that some of the mutations in V2 vasopressin receptor (V2R) that cause nephrogenic diabetes insipidus also result in the mutant receptor trapped intracellularly (Morello and Bichet, 2001). Some small molecules that bind V2R can cross the cell membrane and act as pharmacological chaperones increasing the cell surface expression of the mutant V2R (Morello, et al. 2000). Similar results were achieved recently in δ-opioid receptor (Petaja-Repo, et al. 2002), gonadotropin-releasing hormone receptor (Janovick, et al. 2002) and rhodopsin (Noorwez, et al. 2003).

Since most of the defective hMC4R mutants tested to date showed small and discrernible hormone-stimulated cAMP generation, approaches that result in increased expression of the mutant receptor on the cell surface could potentially be of great therapeutic value. It was known that the C-terminal peptide of AgRP can increase the cell surface expression of MC4R (Shinyama, et al. 2003). Thus, among the numerous

antagonists developed for MC4R, some might act as pharmacological chaperones. In this study, we planed to test the effect of a small antagonist of MC4R, ML00253764 (Vos, et al. 2005; Nicholson, et al. 2006), on cell surface expression of the hMC4R mutants that if we could identify from the functional analsysis of the 10 mutant receptors described in the first part of this report.

4.2. Materials and Methods

4.2.1. Cells, plasmid and peptides

Human embryonic kidney (HEK) 293 and 293T cells (ATCC) were incubated at 5% CO_2 in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% newborn calf serum, 10 mM HEPES, and 100 units/ml of penicillin and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). The N-terminal myc-tagged hMC4R was described previously (Tao and Segaloff, 2003). The superpotent agonist of α-MSH, [Nle^4, D-Phe^7]-α-MSH (NDP-MSH), the MC4R antagonist AgRP(86-132), and the ^{125}I-labelled NDP-MSH were purchased from Phoenix Pharmaceuticals (Belmont, CA), Peptides International (Louisville, KY), and Peptide Radioiodination Service Center at The University of Mississippi (University, MS), respectively.

4.2.2. Site-directed mutagenesis of human MC4R

Mutant hMC4Rs were generated by site-directed mutagenesis using N-terminal myc-tagged hMC4R as the template. Point mutants were generated by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described in detail previouly

(Tao and Segaloff, 2003). Maxiprep kits from Qiagen (Valencia, CA) were used to prepare the plasmids for transfection. Automated DNA sequencing, performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center, was used to confirm that only the intended mutations were introduced in the constructs. Primer pairs used to generate the mutants are available upon request from the authors.

4.2.3. Plasmid transfection and establishment of stable cell lines

For transient expression of the hMC4Rs, HEK293T cells were plated on gelatin-coated 35-mm six-well plates and transfected with 4 μg of plasmid per well using the calcium precipitation method (Chen, et al. 1987). The cells were then incubated for 48 hrs before being used for radioligand binding and signaling assays. For establishment of stable cells, HEK293 cells were plated on gelatin-coated 100-mm dishes and transfected with 10 μg of plasmid per dish as described above. After 24 hrs incubation, the cells were trypsinized and then transferred to T75 flasks in media containing 250 μg/ml geneticin (Research Products International, Mt. Prospect, IL). Selection was continued for three weeks. The geneticin-resistant cells were maintained in media containing 250 μg/ml geneticin.

4.2.4. Radioligand binding assay

Transfected cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) modified to contain 1 mg/ml BSA (referred to as Waymouth/BSA). Fresh Waymouth/BSA was added to each well, together with 50 μl of ^{125}I-NDP-MSH (~100,000 cpm) with or without 100 μl of different concentrations of

unlabeled NDP-MSH or AgRP(86-132). The total volume in each well was 1 ml. The final concentration of unlabeled ligand ranged from 10^{-12} to 10^{-6} M. After 1 h incubation, cells were washed twice with cold Hanks' balanced salt solution (Sigma-Aldrich) modified to contain 1 mg/ml BSA (referred to as HBSS/BSA). Then, 100 µl of 0.5 N NaOH was added to each well. Cells were collected from each well using cotton swabs, and ligand binding was counted in gamma counter. All determinations were performed in duplicate. Maximal binding capacity (*B*max) and IC_{50} values were calculated using Prism software version 4 (GraphPad Software, San Diego, CA).

4.2.5. NDP-MSH agonist stimulation of intracellular cAMP generation

Transfected cells were washed three times with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) was added to each well. After 15 min incubation, either 100 µl of buffer alone or 100 µl of different concentrations of NDP-MSH, were added. The final concentration of the unlabeled ligand ranged from 10^{-11} to 10^{-6} M. After 1 hr incubation, cells were then placed on ice, media aspirated, and intracellular cAMP were extracted by the addition of 0.5 N percholoric acid containing 180 µg/ml theophylline, and measured using radioimmunoassay (RIA). All determinations were performed in triplicate. Maximal responses (Rmax) and EC_{50} values were calculated using Prism 4. To measure the cAMP generation of the HEK293 stable cells treated with ML00253764 as described below, cells were washed three times with Waymouth/BSA before either 100 µl of buffer alone or 100 µl of NDP-MSH were added into each well. The final concentration of the NDP-MSH was 10^{-6} M. Cyclic AMP accumulation was assayed as described above.

Herein, all determinations were performed in triplicate. Only maximal respons (Rmax) was calculated using Prism 4.

4.2.6. ML00253764 treatment of the HEK293 stable cells

One milliom stable HEK293 cells were plated on gelatin-coated 35-mm six-well plates and allowed to attach for 24 hrs. Cells were then rinsed three times with Waymouth/BSA to wash off the residual serum before 900 µl of Waymouth/BSA were added to each well together with 100 µl of ML00253764 (10^{-4} M, dissolved first in DMSO, then diluted in OG/BSA) was added to each well to reach a final concentration of 10^{-5} M of the drug. The cells then were cultured for another 24 hrs before assayed for expression and signaling.

4.2.7. Fluorescence-activated cell sorting (FACS) assay of HEK293 cells stably expressing WT or mutant hMC4Rs

The day before the experiment, cells were plated into wells of 6-well plates (two wells per mutant). On the day of the experiment, cells were washed once with filtered PBS-IH [in mM, consisting of 137 NaCl, 2.7 KCl, 1.4 KH_2PO_4, and 4.3 Na_2HPO_4 (pH 7.4)], fixed with 4% paraformaldehyde for 30 min, permeabilized (only for determination of total expression) with 1% Triton-X-100 in PBS-IH for 4 min and then incubated with blocking solution (5% BSA in PBS-IH) for 1 hr. Cells were then incubated for 1 hr with FITC-conjugated 9E10 monoclonal antibody (Affinity Bioreagents, Golden, CO) diluted 1:20 in PBS-IH containing 1 mg/ml BSA. Cells were then washed three times with PBS-IH and assayed with a MoFlo7-color flow cytometer & high-performance sorter

(Dakocytomation, Fort Collins, CO). All the steps were performed on ice. The expression level of the mutant receptors was calculated as a percentage of WT expression using the formula: [mutant-pcDNA3]/[WT-pcDNA3] x100%. To measure the effect of the drug on cell surface expression of the mutant receptors, HEK293 cells stably expressing these mutant receptors were treated with ML00253764 as described above before the FACS assay were performed.

4.2.8. Confocal microscopy

The day before the experiment, 8 $x10^4$ of stable HEK293 cells were plated into each well of an 8-well chamber and cultured for 24 hrs. On the day of the experiment, cells were washed three with Waymouth/BSA and 450 µl of Waymouth/BSA was added into each well afterthere. Either 50 µl of buffer alone or 50 µl of ML00235764 (10^{-4} M) were added into each well and the cells were then cultured for another 24 hrs. The cells were then washed three time with filtered PBS-IH, fixed with 4% paraformaldehyde for 30 min, and then incubated with blocking solution (5% BSA in PBS-IH) for 1 hr. then incubated for 1 hr with FITC-conjugated 9E10 monoclonal antibody diluted 1:20 in PBS-IH containing 1 mg/ml BSA. After cells were washed twice with PBS-IH, the coverslipes were mounted to the slides using VECTASHIELD® HardSet™ mounting medium (Alexis, San Diego, CA) and the slides were viewed with a Bio-Rad confocal microscope. Photos were captured using an RT-Slide Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) at 200X amplification with an exposure time of 45 sec. All the steps were performed at room temperature.

4.2.9. Statistical analysis

Data were presented as mean ± SEM. Statistical analyses were performed using Prism software version 4 (GraphPad Software). For comparisons on cell surface and total expression of the WT and mutant hMC4Rs and cell surface maximal binding and maximal signaling, one-sample unpaired *t* test was used. For comparisons of IC_{50} and EC_{50}, a two-sample unpaired *t* test was used. Differences with a $p<0.05$ were considered statistically significant.

4.3. Results

4.3.1. Cell surface expression of the mutant hMC4Rs

From previous functional studies of the naturally occurring hMC4R mutations identified from various patient populations, defects in cell surface expression emerge as the most common defect. Therefore, we performed a quantitative analysss of cell surface expression of the mutant hMC4Rs. Human MC4Rs tagged at the N terminus with a myc-tag were stably expressed in HEK293 cells and FACS assay was used to estimate the cell surface expression levels. The cell surface expression of the WT hMC4R was defined as 100%. As summarized in Fig. IV-1, the mutant hMC4Rs S136F, A219V, I317V, and L325F possessed receptor cell surface expression levels of 76 ± 16%, 67 ± 12%, 80 ± 14% and 72 ±3% relative to the WT hMC4R. Therefore, these four mutant hMC4Rs showed a relatively similar cell surface expression level as that of the WT hMC4R. In contrast, the mutant hMC4Rs R7C, C84R, S127L, W174C, P230L and F261S have receptor cell surface expression levels of 44 ± 2%, 3 ± 3%, 47 ± 8%, 10 ±

8%, 42 ± 11% and 20 ± 7% relative to the WT hMC4R and thus had significantly reduced cell surface expression levels relative to the WT hMC4R. Taken together, these data demonstrated that the mutant hMC4Rs S136F, A219V, I317V, and L325F were relatively normal in cell surface expression compared to the WT hMC4R. However mutant hMC4Rs R7C, C84R, S127L, W174C, P230L and F261S were all defective in cell surface expression to some extent.

4.3.2. Total expression of the mutant hMC4Rs

Decreased cell surface expression of the mutant hMC4Rs might be caused by either decreased total expression or intracellular retention of the mutant receptors or both. Therefore, we further determined the total expression levels of the mutant hMC4Rs by FACS. Cells were permeabilized with Triton X-100 and then stained with anti-myc antibody. As shown in Fig. IV-2, the mutant hMC4Rs R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V, and L325F had receptor total expression levels of 69 ± 6%, 42 ± 9%, 101 ± 5%, 81 ± 4%, 43 ± 9%, 84 ± 18%, 93 ± 13%, 75 ± 13%, 149 ± 31% and 135 ± 22% relative to the WT hMC4R (designated as 100%). This result revealed that the total expression levels of these mutant receptors were relatively similar to that of the WT hMC4R except the mutant hMC4R R7C, C84R and W174C with significantly decreased total expression. Further, we determined the percentage of the WT and mutant hMC4Rs expressed on the cell surface relative to their total expression. For the WT hMC4R, 45 ± 3% was localized on the cell surface. The percentage of cell surface expression of the mutant hMC4Rs R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V, and L325F were 30 ± 4%, 3 ± 3%, 21 ± 4%, 44 ± 11%, 6 ± 7%,

36 ± 2%, 20 ± 3%, 11 ± 3%, 24 ± 1% and 25 ± 4%, respectively. Taken together, these data indicated that the decreased cell surface expression of S127L, P230L and F261S hMC4Rs were caused by intracellular retention of the mutant receptors, not decreased total expression. The total expression levels of the mutant hMC4R R7C, C84R and W174C were significantly decreased compared to the WT hMC4R.

4.3.3. Ligand binding properties of the mutant hMC4Rs

We further investigated the ligand binding profiles of the WT and mutant hMC4Rs using whole-cell binding assays. For this study, both the superpotent agonist of a-MSH, NDP-MSH, and the natural antagonist AgRP(86-132), were used to compete with [^{125}I]-NDP-MSH. As summarized in Table 3, when the cell surface maximal binding (Bmax) of the WT hMC4R to NDP-MSH was defined as 100%, the mutant hMC4Rs R7C, S136F, I317V, and L325F had the Bmax levels of 99 ± 3%, 101 ± 9%, 94 ± 8%, and 92 ± 6% relative to the WT hMC4R. The mutant hMC4Rs C84R, S127L, W174C, A219V, P230L, and F261S had the reduced cell surface binding. They were only 3 ± 1%, 32 ± 2%, 1 ± 0%, 40 ± 3%, 62 ± 6%, and 15 ± 1%, respectively, of the WT hMC4R maximal binding. There is no significant difference in the binding affinities between the WT and the mutant hMC4Rs. For the human AgRP(86-132) binding, it was also observed that binding affinities of the mutant hMC4Rs were at the same level as that of the WT hMC4R except for the mutant hMC4Rs S136F, S127L, and A219V that either has a 3-fold increase (S127L, IC_{50} of 18.25 ± 4.85 nM and A219V, IC_{50} of 12.83 ± 0.33 nM) or 10-fold decrease (S136F, IC_{50} of 0.41 ± 0.05%) IC_{50} compared to the WT hMC4R (IC_{50} of 4.16 ± 0.70 nM).

4.3.4. Signal properties of the mutant hMC4Rs upon NDP-MSH stimulation

To investigate the signaling properties of the hMC4Rs, HEK293T cells transiently transfected with WT or mutant hMC4Rs were stimulated with different concentrations of NDP-MSH, and accumulation of intracellular cAMP was measured. A representative experiment is shown in Fig. IV-5. Results from several experiments are summarized in Table 3. When the maximal response of WT hMC4R was defined as 100%, the mutant hMC4Rs R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V, and L325F had the maximal response levels of 102 ± 4%, 20 ± 1%, 57 ± 3%, 11 ± 1%, 31 ± 3%, 56 ± 3%, 122 ± 4%, 49 ± 1%, 100 ± 2%, and 94 ± 4% relative to the WT hMC4R. However the signaling efficacy of the mutant hMC4Rs were at the same level as that of the WT hMC4R except for the mutant W174C, A219V, and P230L that had EC_{50s} of 14.80 ± 0.35, 24.19 ± 1.27, and 12.31 ± 0.67 nM, respectively, about 12- to 20-fold higher than that of the WT hMC4R, respectively (Fig. IV-5 and Table 3). In addition, when the basal (constitutive) activity of WT hMC4R was defined as 100%, R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V, and L325F were the constitutive activity of 105 ± 6%, 78 ± 5%, 262 ± 19%, 79 ± 4%, 47 ± 2%, 58 ± 3%, 199 ± 5%, 137 ± 9%, 134 ± 4%, and 124 ± 5% of the WT hMC4R basal activity (Fig. IV-6). Therefore, R7C, S127L, S136F, W174C, A219V and F261S had decreased basal (constitutive) signaling compared to the WT hMC4R whereas other mutants had normal (W174C and A219V) or increased (S127L and P230L) constitutive activity.

4.3.5. Amino acid requirement at codon 136 of the hMC4R for NDP-MSH binding

As shown above, for S136F, although its total and cell surface expression and ligand binding to NDP-MSH are normal compared to the WT hMC4R, it does not exhibit any signaling. To better understand the side-chain requirement at codon 136 of hMC4R for NDP-MSH signaling, we generated six additional mutants. These mutants included S136A, S136C, S136D, S136L, S136T, and S136Y. As shown in Fig. IV-7 and Table 4, all six mutants had normal binding affinity and maximal binding compared to the WT hMC4R. All the mutants also showed EC_{50s} and maximal responses at the same levels as the WT hMC4R except mutant S136C that showed a deceased maximal resonse (Fig. IV-8). In summary, all mutants bound NDP-MSH with normal affinity and capacity and signal normal relative to the WT hMC4R except S136C that shows a decreased signaling ability. Amino acid requirement at codon 136 for normal hMC4R function is not critical unless phenylalanine is substituted at this site. The Phe substitution results in a loss-of-function phenotype and a cysteine substitution causes an impaired-function phenotype of the receptor.

4.3.6. Functional rescue of hMC4R C84R and W174C

As described above, mutant receptors C84R and W174C only showed minimal cell surface expression compared to the WT hMC4R. Their maximal ligand binding was almost undetectable although both mutant receptors had residuel signaling as compared to that of the WT hMC4R. To investigate the effect of the MC4R antagonist, ML00253764, on the cell surface expression of these two mutatnt receptors, the HEK293 cells stably expressing WT, C84R and W174C hMC4Rs were treated with 10^{-5} M

ML00253764 for 24 hrs and confocal microscopy and FACS assays were then perfomed. As shown in Fig. IV-9, the cell surface expression levels of both mutant hMC4Rs were increased after drug treatment. FACS assay further confirmed that the cell surface expression of C84R and W174C were increased to ~35% of that of the WT hMC4R after treatment with ML00253764 (Fig. IV-10). Even the cell surface expression of the WT hMC4R was increased to 152% after ML00253764 treatment. In addition, after treatment, the mutant hMC4Rs were functional. Cyclic AMP generation of the C84R and W174C hMC4Rs was increased to up to 62% of that of the WT hMC4R when the receptors were stimulated with 10^{-6} M NDP-MSH (Fig. IV-11). In summary, we showed that small molecule MC4R antagonist ML00253764 acts as a pharmacological chaperone, increasing cell surface expression of MC4Rs.

4.4. Discussion

Mutations in the hMC4R gene were identified as a cause for obesity in 1998 (Yeo, et al. 1998; Vaisse, et al. 1998). Since then, studies from numerous groups have identified 110 distinct mutations in the coding region of the MC4R gene from both obese and normal weight subjects. Based on the life cycle of GPCRs and modeled after low-density lipoprotein receptor and cystic fibrosis transmembrane conductance regulator (Hobbs, et al. 1990; Welsh, et al. 1993), we proposed that mutations in the hMC4R could be divided into five classes (Tao and Segaloff, 2003). According to this system, the class I mutants are defective in receptor biosynthesis, including truncation mutants such as W16X (Marti, et al. 2003), Y35X (Hebebrand, et al. 2002), and L64X (Jacobson, et al. 2002).

The class II mutants are synthesized normally but are defective in trafficking onto the cell surface. It was estimated that about 70% of the mutant hMC4Rs belong to this class. The class III mutants can be synthesized and transported onto the cell surface normally. However, these mutants are defective in ligand binding. Examples include N97D, L106P, I125K, I316S (Yeo, et al. 2003), I137T (Gu, et al. 1999) and Δ88-92 (Donohoue, et al. 2003). The class IV mutants are defective only in signaling but relatively normal in receptor synthesis, cell surface expression and ligand binding. Currently, no such a mutant is identified naturally. Mutants that are normal in cell surface expression, ligand binding and signaling are class V mutants. The identified mutants that belong to this class include V103I (Hinney, et al. 2003; Geller, 2004), Y35C, C40R, M218T (Rong, et al. 2006), D37V, P48S, V50M, I170V, N274S (Tao and Segaloff, 2003), T11A, F51L, T112M, A154D, M200V, and S295P (Tao and Segaloff, 2005). We proposed that this system could be used to classify all naturally occurring inactivating GPCR mutants that cause diseases (Tao, 2006).

In this study, we functionally characterized ten mutant hMC4Rs including R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V and L325F. According to our classification scheme described above, C84R, S127L, W174C, P230L and F261S are class II mutants. A219V belongs to class III. S136F is a class IV mutant. R7C, I317V, and L325F are class V mutants.

The mutant W174C and I317V hMC4Rs were identified from morbidly obese Italian adults and were not functionally characterized (Buono, et al. 2005). Our data showed that W174C has a significantly decreased total expression and cell surface expression compared to the WT hMC4R (Fig. IV-1 & IV-2). This mutant receptor also showed

barely undetectable ligand binding for both NDP-MSH and AgRP and significantly decreased cAMP response and basal activity (Fig. IV-3, -4, -5 & -6). In contrast, the cell surface expression, ligand binding and cAMP response of I317V are similar to the WT hMC4R. Its total expression is even higher than that of the WT hMC4R. Therefore, I317V might represent a polymorphism with no overt functional defect. This might be due to the similar structures of isoleucine and valine.

Mutant R7C was previously shown normal in term of its cell surface expression and cAMP response upon stimulation by NDP-MSH (Hinney, et al. 2006). Our data showed similar results as that reported by Hinney and coworkers in term of signaling. In addition, R7C showed a normal ligand binding for both NDP-MSH and AgRP although its cell surface expression is dramatically decreased.

The mutant hMC4R S127L has been identified independently in several ethnic populations (Lubrano-Berthelier, et al. 2003; Hinney, et al. 2003; Valli-Jaakola, et al. 2004). Previous reports showed that S127L is normal in cell surface expression (Lubrano-Berthelier, et al. 2003; Hinney, et al. 2003) whereas its cAMP response is impaired with decreased Rmax and increased EC_{50} upon stimulation by either α-MSH (Lubrano-Berthelier, et al. 2003; Hinney, et al. 2003; Valli-Jaakola, et al. 2004), β-MSH (Valli-Jaakola, et al. 2004) or γ-MSH (Valli-Jaakola, et al. 2004). Partially in line with these results, our data showed that S127L results in a significantly decreased Rmax. However, EC_{50} is normal compared with the WT hMC4R. This might be caused by the usage of NDP-MSH but not α-MSH, β-MSH or γ-MSH as shown above. Furthermore, S127L is normal in total expression but defective in cell surface expression compared to the WT hMC4R. S127L also showed a significantly decreased Bmax whereas its IC_{50} is

normal. Thus, the mutant hMC4R S127 is a class II mutant. Its function is impaired mainly due to the intracellular retention of the receptor. In addition, S127L was a constitutively active mutant (Fig. IV-6).

P230L was reported as a constitutively active mutant with increased Rmax and decreased EC_{50} upon stimulation of α-MSH (Hinney, et al. 2003). Our data confirmed that P230L has significantly increased basal activity and showed that P230L has a normal total expression level compared with the WT hMC4R. However, its cell surface expression was significantly decreased. Low cell surface expression thus results in a significantly decreased Bmax. Interestingly, our data showed that P230L has normal EC_{50} and Rmax.

F261S was the first MC4R mutation identified from obese Chinese and preliminary characterization showed that it has decreased Rmax and increased EC_{50} (Shao, et al. 2005). Our data are consistent with these observations. Furthermore, we showed that the cell surface expression of F261S was significantly decreased to 35% of the WT hMC4R, although the total expression is similar to the WT MC4R. Lower cell surface expression resulted in corresponding decreases in Bmax and Rmax whereas the IC_{50} and EC_{50} were not significantly altered. Thus, F261S is defective in intracellular trafficking, hence a class II mutant.

Mutants A219V and L325F were identified from German patients presented with juvenile-onset obesity (Larsen, et al. 2005). Our data showed that total expression levels of the two mutants are similar as the WT hMC4R. However, cell surface expressions of the mutants are either slightly decreased (L325F) or significantly decreased (A219V). Ligand binding assay showed that A219V has a normal binding affinity but a

significantly decreased Bmax whereas L325F is normal in term of both binding affinity and capacity. Further, A219V showed a significantly decreased Rmax and a 20-fold increased EC_{50} compared to the WT hMC4R whereas L325F are relatively normal in terms of both Rmax and EC_{50}. This result is totally different from Larsen's report that L325F was a loss-of-function mutant due to its defect in ligand binding.

Mutant S136F was reported as a loss-of-function mutant with normal cell surface expression compared with the WT hMC4R (Rettenbacher, et al. 2007). Our data are consistent with these observations. We further showed that total expression and ligand binding property of S136F are also normal compared with the WT hMC4R. Therefore, S136F is a class IV mutant that is normal in receptor biosynthesis and ligand binding but defective in cAMP response. In this study, multiple mutageneses demonstrated that several mutations at codon 136 of hMC4R can be tolerated. However, it is detrimental to receptor function if a phenylalanine is introduced at this site.

In this study, we reported for the first time that treatment of the mutant hMC4R C84R and W174C with a MC4R pharmacological chaperone ML00253764 increased the cell surface expression of the mutant receptors. Although pharmacological chaperones may be used to correct transport defective mutants, they are not of therapeutic value for the mutants that are transported to the plasma membrane but defective in ligand binding or G protein coupling/activation. For the mutants that are defective in G protein coupling/activation, perhaps the most promising option would be the introduction of a normal gene through gene therapy (Schoneberg, et al. 1997).

TABLE 4. **Ligand binding and agonist-stimulated cAMP generation of WT and mutant hMC4Rs**

hMC4R	n	NDP-MSH binding		n	NDP-MSH-stimulated cAMP		n	AgRP binding
		IC_{50} (nM)	Bmax (% WT)		EC_{50} (nM)	Rmax (% WT)		IC_{50} (nM)
WT	9	13.16 ± 2.24	100	3	1.22 ± 0.19	100	4	4.16 ± 0.70
R7C	3	12.32 ± 0.99	99 ± 3	3	3.06 ± 0.06	102 ± 4	3	3.60 ± 0.36
C84R	3	N/A	3 ± 1 [c]	3	8.73 ± 0.43	20 ± 1 [b]	3	N/A
S127L	3	14.99 ± 2.57	32 ± 2 [b]	3	1.78 ± 1.07	57 ± 3 [a]	3	18.25 ± 4.85
S136F	3	3.72 ± 0.77	101 ± 9	3	7.61 ± 0.23	11 ± 1 [b]	3	0.41 ± 0.05
W174C	3	N/A	1 ± 0 [c]	4	14.80 ± 0.35	31 ± 3 [a]	3	N/A
A219V	3	20.11 ± 3.55	40 ± 3 [a]	3	24.19 ± 1.27	56 ± 3 [a]	3	12.83 ± 0.33
P230L	3	12.82 ± 1.31	62 ± 6 [a]	3	12.31 ± 0.67	122 ± 4	3	4.01 ± 1.09
F261S	3	2.49 ± 0.94	15 ± 1 [c]	3	1.48 ± 0.12	49 ± 1 [a]	3	1.33 ± 0.59
I317V	3	18.44 ± 2.53	94 ± 8	3	3.60 ± 0.14	100 ± 2	3	3.56 ± 0.19
L325F	3	5.12 ± 0.42	92 ± 6	4	2.62 ± 0.06	94 ± 4	3	6.17 ± 0.12

[a] Significantly different from corresponding WT receptor, $p<0.05$
[b] Significantly different from corresponding WT receptor, $p<0.01$
[c] Significantly different from corresponding WT receptor, $p<0.001$

The data are expressed as the mean ± SEM of three independent experiments. The maximal binding (Bmax) of intact HEK293T cells expressing WT hMC4R was 155601 ± 1568 cpm bound/10^6 cells. The maximal response (Rmax) was 2520 ± 59 pmol cAMP/10^6 cells for WT hMC4R. IC_{50} is the concentration of NDP-MSH that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of NDP-MSH that results in 50% stimulation of the maximal response.

TABLE 5. **Ligand binding and NDP-MSH-stimulated cAMP generation of WT and mutant hMC4Rs**

hMC4R	n	NDP-MSH binding		n	NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	Bmax (% WT)		EC_{50} (nM)	Rmax (% WT)
WT	13	16.87 ± 2.24	100	13	2.68 ± 0.46	100
S136A	4	24.84 ± 3.60	108 ± 10	4	9.93 ± 2.00	142 ± 50
S136C	4	37.37 ± 6.26	116 ± 8	4	5.41 ± 2.74	37 ± 11 [a]
S136D	4	26.74 ± 8.65	132 ± 6	4	6.76 ± 2.06	90 ± 9
S136L	4	21.91 ± 3.79	103 ± 5	4	9.83 ± 2.82	122 ± 17
S136T	4	43.77 ± 5.64	116 ± 7	4	3.57 ± 1.15	99 ± 31
S136Y	4	43.64 ± 9.26	104 ± 6	4	8.95 ± 0.60	130 ± 42

[a] Significantly different from corresponding WT receptor, $p<0.05$

The data are expressed as the mean ± SEM of four independent experiments. The maximal binding (Bmax) of intact HEK293T cells expressing WT hMC4R was 165601 ± 2568 cpm bound/10^6 cells. The maximal response (Rmax) was 1520 ± 39 pmol cAMP/10^6 cells for WT hMC4R. IC_{50} is the concentration of NDP-MSH that is needed to cause 50% inhibition in the binding assay. EC_{50} is the concentration of NDP-MSH that results in 50% stimulation of the maximal response.

Figure IV-1. Cell surface expression of WT and mutant hMC4Rs stably expressed in HEK293 cells. Construction of the stable cell lines was performed as described in *Materials and Methods*. Cell surface expression levels of the WT and mutant hMC4Rs were quantitatively determined by FACS. The results are expressed as percentage of cell surface expression level of the WT hMC4R. Shown are mean ± SEM of three experiments. Star (*) indicates significantly different from the WT hMC4R.

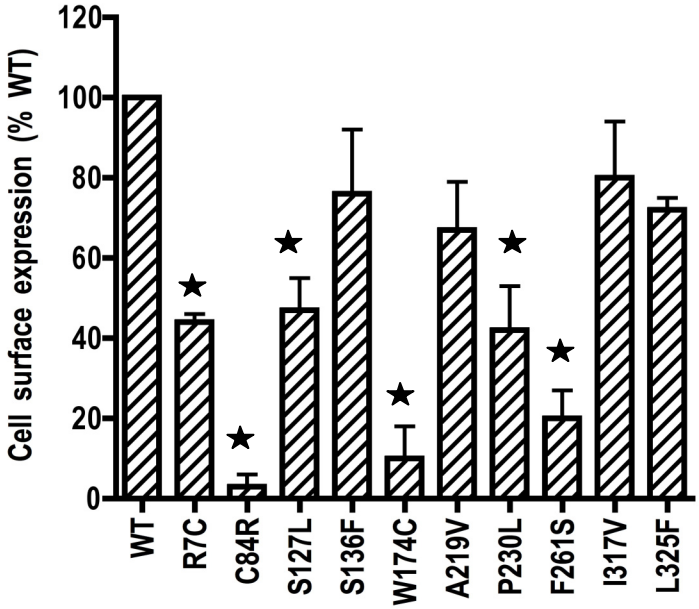

Cell surface expression (% WT)
120
100
80
60
40
20
0
WT
R7C
C84R
S127L
S136F
W174C
A219V
P230L
F261S
I317V
L325F

Figure IV-2. Total expression of WT and mutant hMC4Rs stably expressed in HEK293 cells. Construction of the stable cell lines was performed as described in *Materials and Methods*. Total expression levels of the WT and mutant hMC4Rs were quantitatively determined by FACS. The results are expressed as percentage of total expression level of the WT hMC4R. Shown are mean ± SEM of three experiments. Star (*) indicates significantly different from the WT hMC4R.

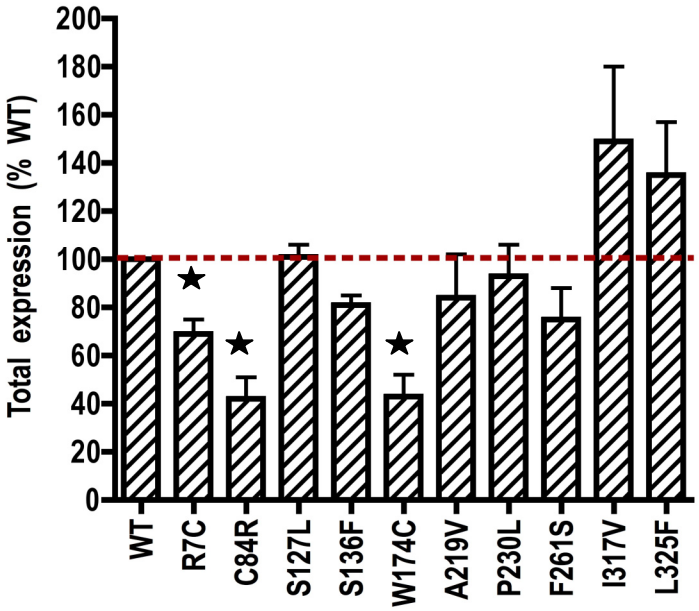
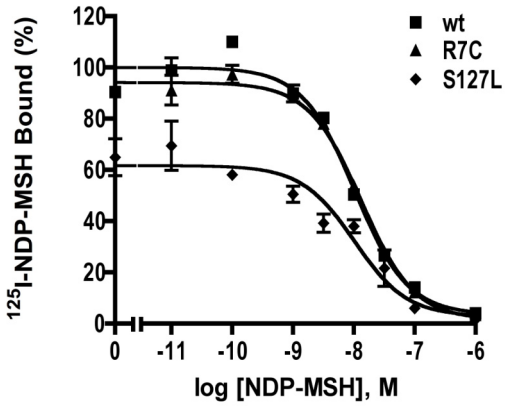
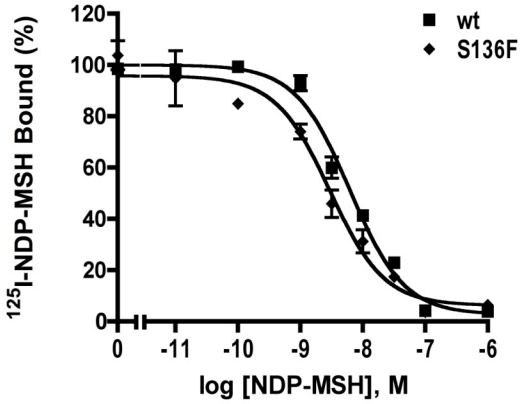
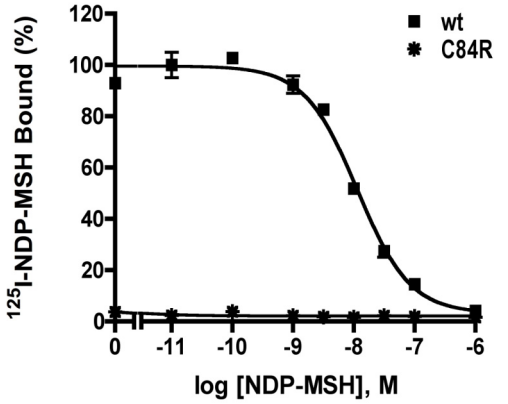
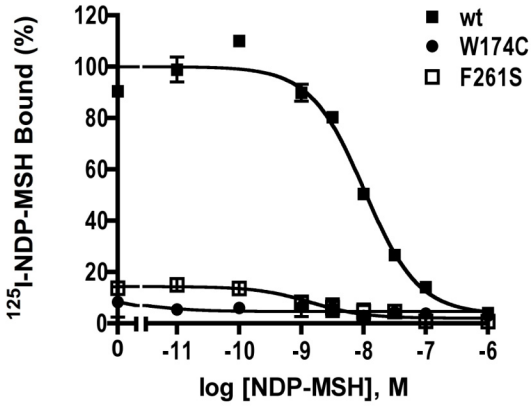

Total expression (% WT)
200
180
160
140
120
100
80
60
40
20
0
WT
R7C
C84R
S127L
S136F
W174C
A219V
P230L
F261S
I317V
L325F

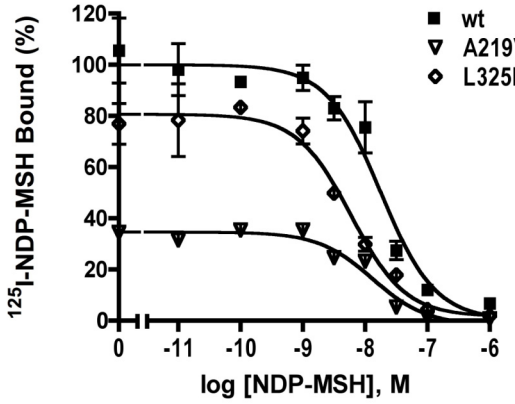
Figure IV-3. NDP-MSH binding assay of the WT and mutant hMC4Rs transiently expressed in HEK293T cells. HEK293T cells were transiently transfected with the indicated hMC4R constructs and binding assays were performed as described in *Materials and Methods*. Different concentrations of unlabeled NDP-MSH were used to displace the binding of ^{125}I NDP-MSH to hMC4R constructs on intact cells. Results are expressed as percentage of the maximal binding for WT hMC4R from duplicate determinations within one experiment. Shown are mean ± SEM. All experiments were performed at least three times. A representative experiment is shown herein.


wt
R7C
S127L
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
S136F
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
C84R
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
W174C
F261S
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
A219V
L325F
125I-NDP-MSH Bound (%)
log [NDP-MSH], M

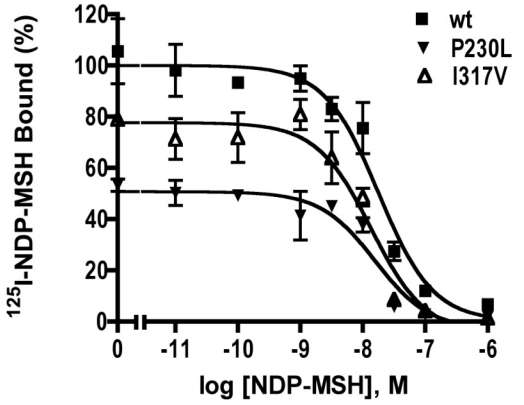
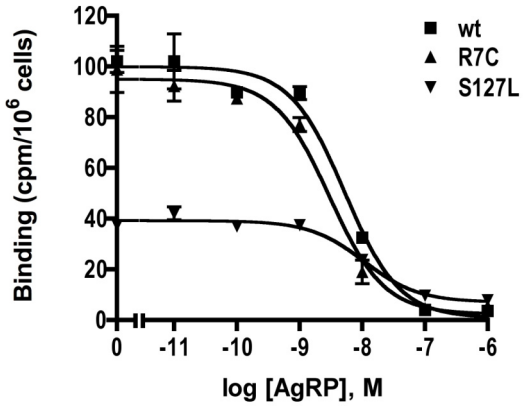
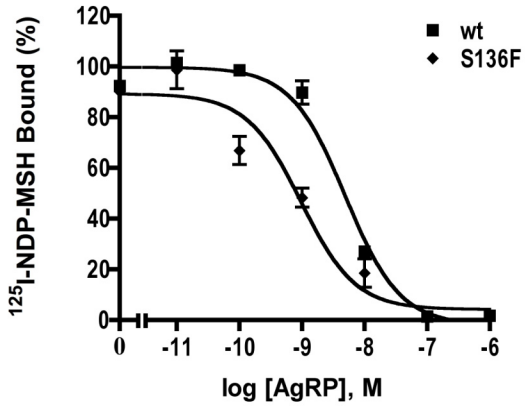
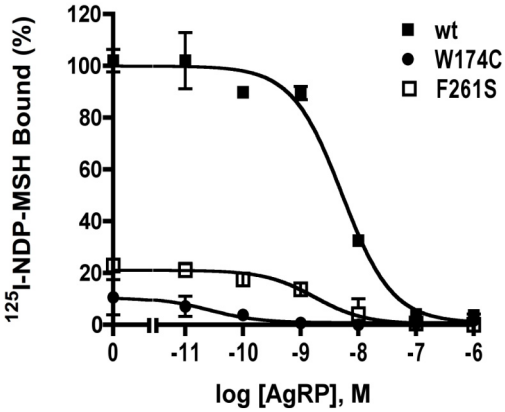
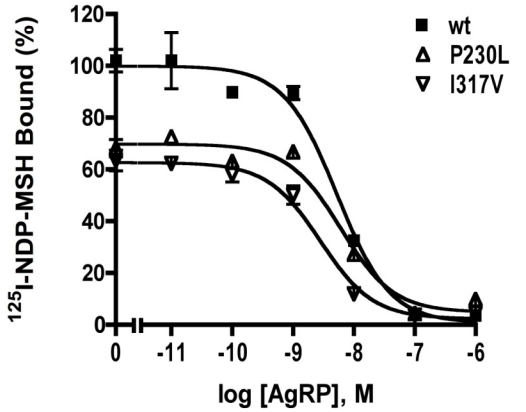

wt
P230L
I317V
125I-NDP-MSH Bound (%)
log [NDP-MSH], M

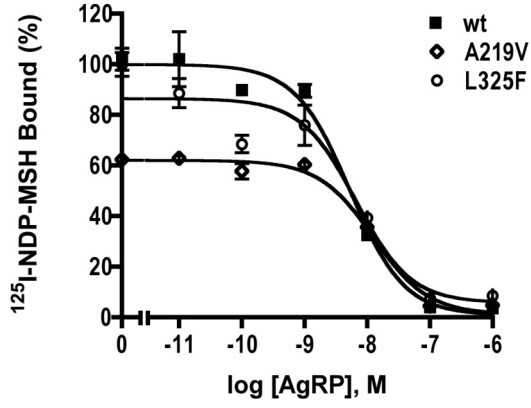
Figure IV-4. AgRP binding assay of the WT and mutant hMC4Rs transiently expressed in HEK293T cells. HEK293T cells were transiently transfected with the indicated hMC4R constructs and binding assays were performed as described in *Materials and Methods*. Different concentrations of unlabeled AgRP were used to displace the binding of ^{125}I NDP-MSH to hMC4R constructs on intact cells. Results shown are expressed as mean ± SEM of the maximal binding from duplicate determinations within one experiment. All experiments were performed at least three times. A representative one is shown herein.


Binding (cpm/10^6 cells)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
R7C
S127L


125I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
S136F


125I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
W174C
F261S


125I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
P230L
I317V


125I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
A219V
L325F

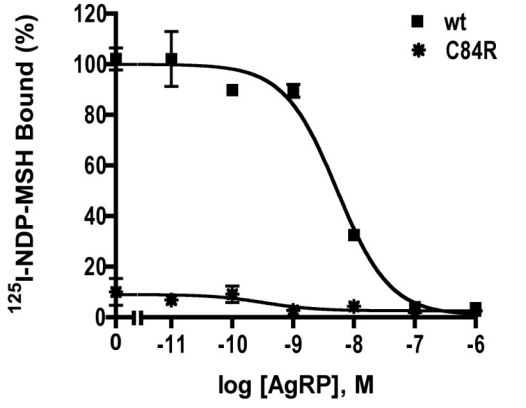
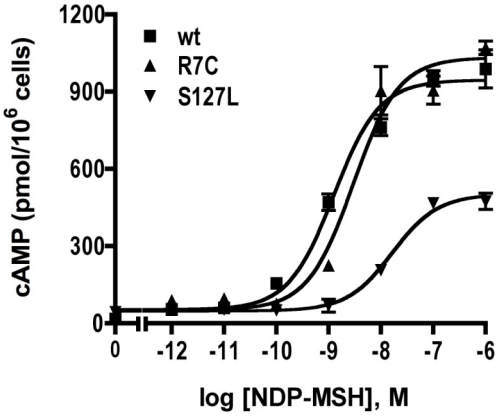
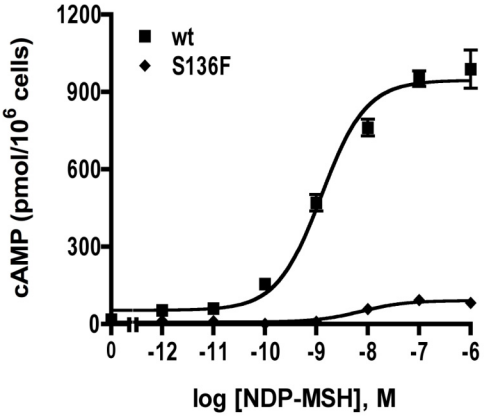
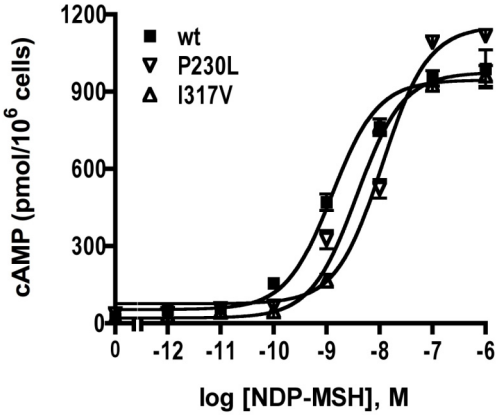
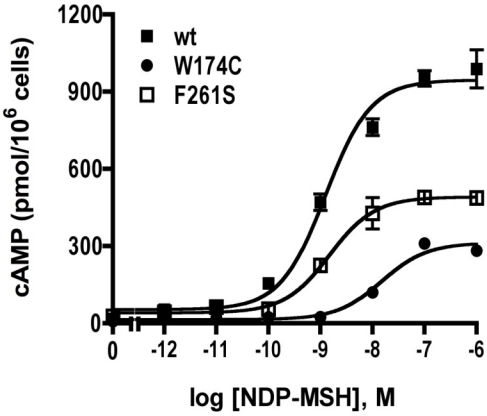

125I-NDP-MSH Bound (%)
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
log [AgRP], M
wt
C84R

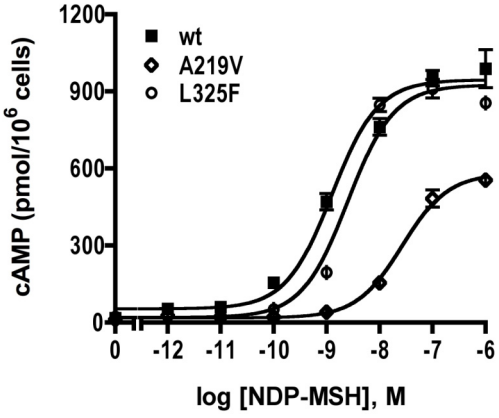
Figure IV-5. cAMP response of the WT and mutant hMC4Rs transiently expressed in HEK293T cells. HEK293T cells were transiently transfected with indicated hMC4R constructs and stimulated with various concentrations of NDP-MSH and intracellular cAMP levels were measured as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment and all experiments were performed at least three times. Results from a representative experiment are shown herein.


wt
R7C
S127L
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
S136F
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
P230L
I317V
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
W174C
F261S
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
A219V
L325F
cAMP (pmol/10^6 cells)
log [NDP-MSH], M

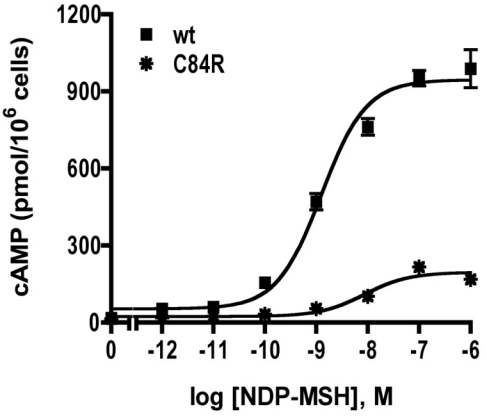

wt
C84R
cAMP (pmol/10^6 cells)
log [NDP-MSH], M

Figure IV-6. Basal (constitutive) activities of the WT and mutant hMC4Rs. The basal activities of WT and mutant hMC4Rs were assessed by measuring intracellular cAMP levels in HEK293T cells transiently expressing WT or mutant hMC4Rs without hormone stimulation. The results are expressed as percentage of basal cAMP level of WT hMC4R-expressing HEK293T cells. Shown are mean ± SEM of three or more experiments. The basal cAMP level in the WT hMC4R was 18.9 ± 6.9 pmol/10^6 cells (mean ± SEM of six experiments). Star (*) indicates significantly different from the WT hMC4R.

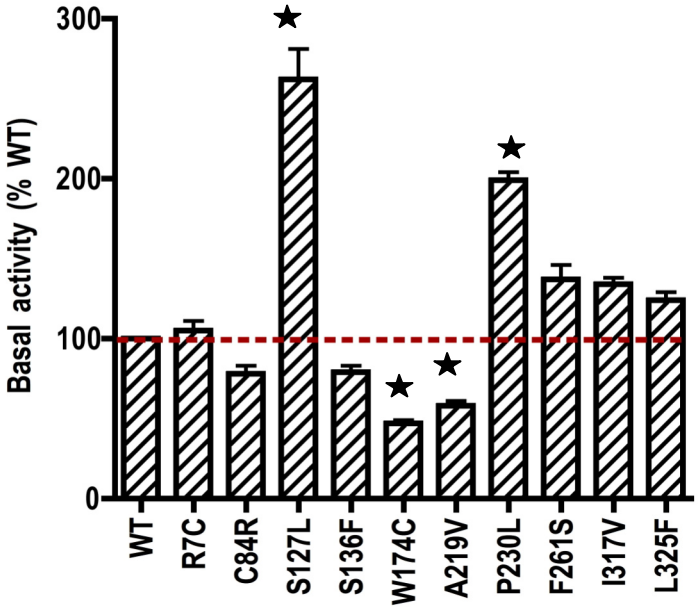
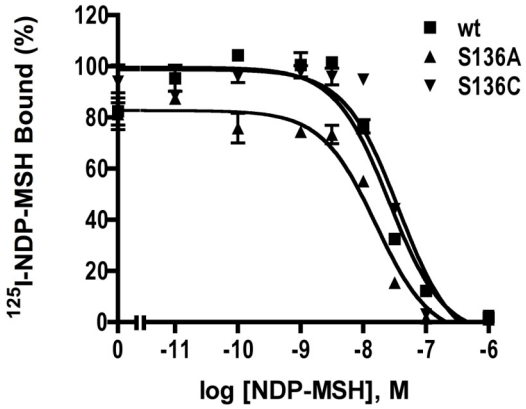
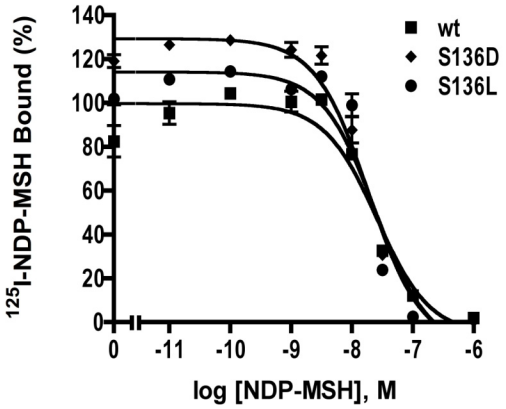
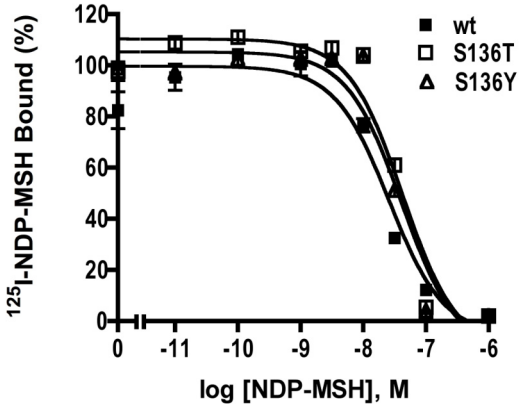

Basal activity (% WT)
300
200
100
0
WT
R7C
C84R
S127L
S136F
W174C
A219V
P230L
F261S
I317V
L325F

Figure IV-7. NDP-MSH binding assay of the WT and mutant hMC4Rs transiently expressed in HEK293T cells. HEK293T cells were transiently transfected with indicated hMC4R constructs and binding assays and cAMP response were performed as described in *Materials and Methods*. Results are expressed as the mean ± SEM of duplicates determinations within one experiment and all experiments were performed at least three times. Results from a representative experiment are shown herein.


wt
S136A
S136C
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
S136D
S136L
125I-NDP-MSH Bound (%)
log [NDP-MSH], M


wt
S136T
S136Y
125I-NDP-MSH Bound (%)
log [NDP-MSH], M

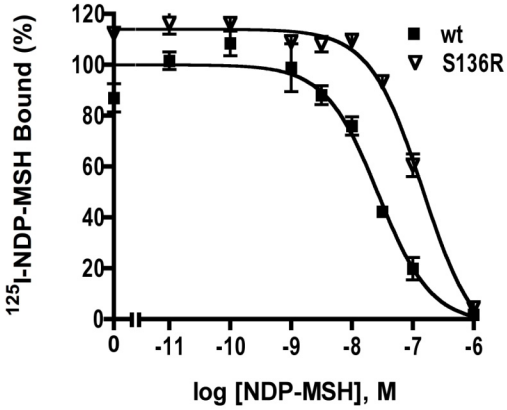
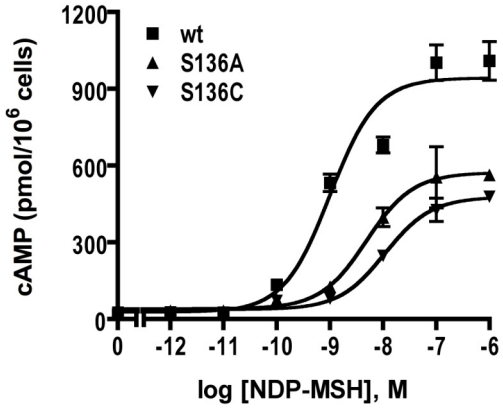
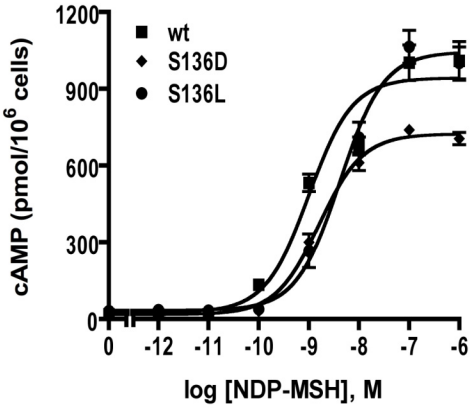
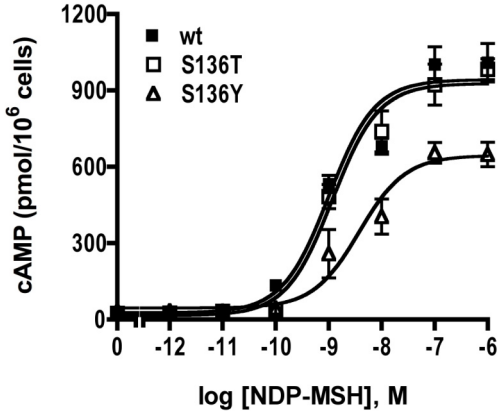

wt
S136R
125I-NDP-MSH Bound (%)
log [NDP-MSH], M

Figure IV-8. cAMP response of the WT and mutant hMC4Rs transiently expressed in HEK293T cells. HEK293T cells were transiently transfected with indicated hMC4R constructs and binding assays and cAMP response were performed as described in *Materials and Methods*. Results are expressed as the mean ± SEM of triplicate determinations within one experiment and all experiments were performed at least three times. Results from a representative experiment are shown herein.


wt
S136A
S136C
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
S136D
S136L
cAMP (pmol/10^6 cells)
log [NDP-MSH], M


wt
S136T
S136Y
cAMP (pmol/10^6 cells)
log [NDP-MSH], M

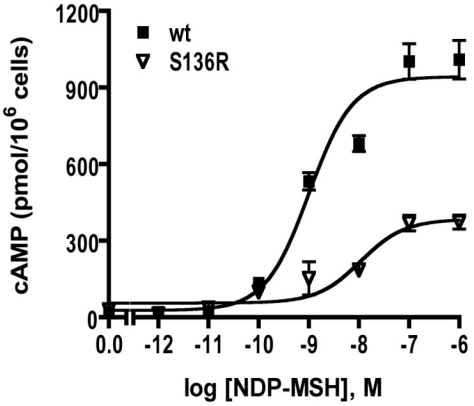

wt
S136R
cAMP (pmol/10^6 cells)
log [NDP-MSH], M

Figure IV-9. Confocal microscopy assay of the effect of ML00253764 on cell surface expression of C84R and W174C hMC4Rs. HEK293 cells stably expressing hMC4R C84R and W174C were treated with ML00253764 and cofocal microscopy was performed as described in *Materials and Methods*. All experiments were performed three times. The typical pictures were shown here. + and – represent that the cells were treated with or without ML00253764, respectively.

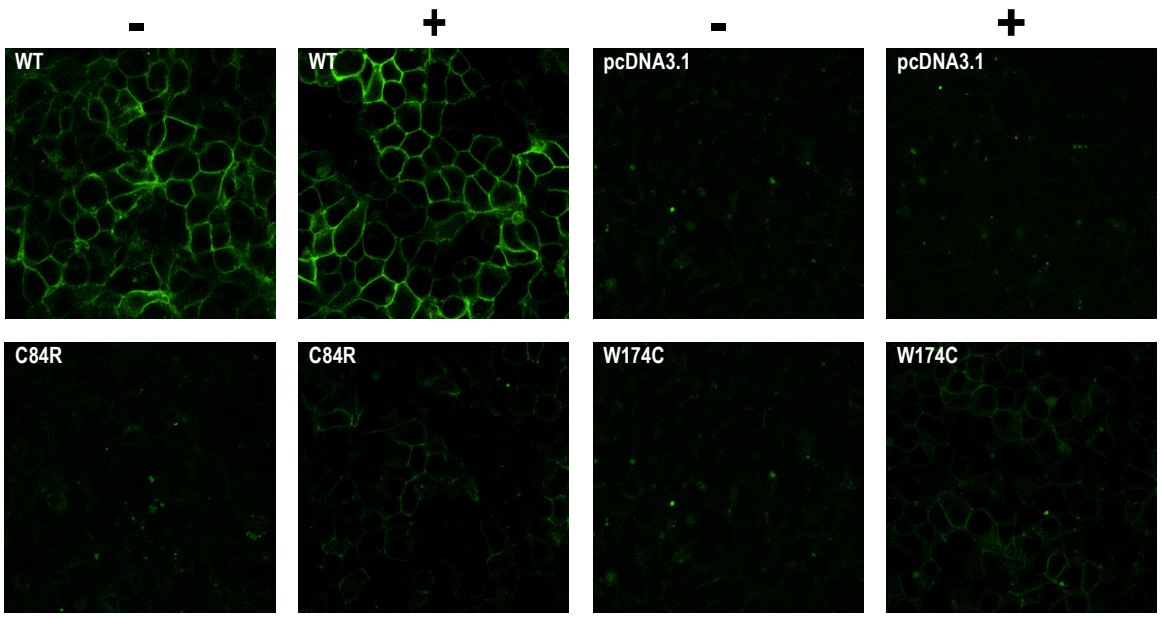

-
+
-
+
WT
WT
pcDNA3.1
pcDNA3.1
C84R
C84R
W174C
W174C

Figure IV-10. FACS assay of the effect of ML00253764 on the cell surface expression of WT and mutant hMC4Rs. HEK293 cells stably expressing hMC4R C84R and W174C were treated with ML00253764 and stained with FITC-cojungated 9E10 monoclonal antibody. FACS assay of the receptor cell surface expression was described in *Materials and Methods*. Results are expressed as percentage of WT expression levels ± SEM from three independent experiments.

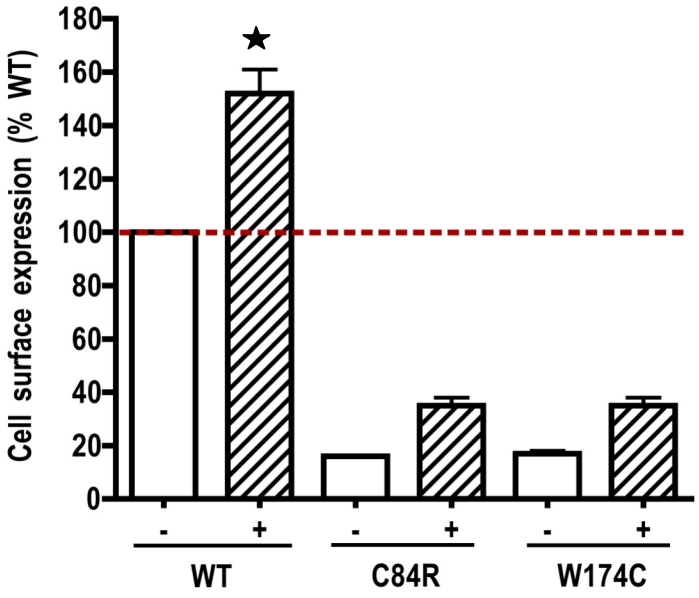
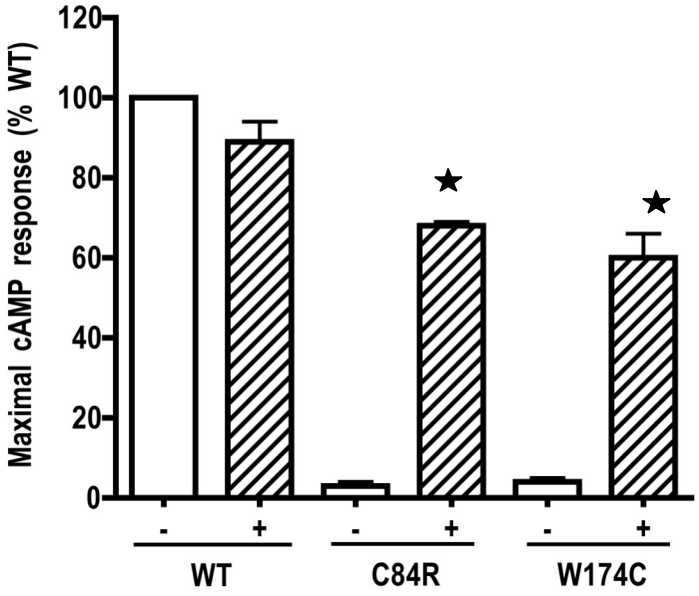

Cell surface expression (% WT)
180
160
140
120
100
80
60
40
20
0
-
+
-
+
-
+
WT
C84R
W174C

Figure IV-11. cAMP response of the WT and mutant hMC4Rs after ML00253764 treatment. HEK293 cells stably expressing hMC4R C84R and W174C were treated with ML00253764. cAMP response was performed as described in *Materials and Methods*. Results are expressed as percentage of WT maximal response ± SEM from three independent experiments. Star (*) indicates the value after the drug treatment was significantly different from that before drug treatment.


Maximal cAMP response (% WT)
120
100
80
60
40
20
0
-
+
-
+
-
+
WT
C84R
W174C

CHAPTER V

CONCLUSIONS

Mouse gene targeting studies revealed that the melanocortin-3 receptor (MC3R) affected feeding efficiency and fat storage in mice. The functions of the MC3R in other mammalian species remain to be investigated. We are interested in exploring the functions of the porcine MC3R (pMC3R) in regulating fat storage because of the economical importance of swine industry. Although nucleotide sequences of MC3Rs from several species have been reported, pMC3R had not been cloned and sequenced. We reported herein the molecular cloning and pharmacological analysis of the pMC3R. Sequence analysis revealed that pMC3R was highly homologous (>80%) at nucleotide and amino acid sequences to human, rat, and mouse MC3Rs. With human MC3R (hMC3R) as a control, the binding and signaling properties of pMC3R were investigated using several agonists including α- and γ-melanocyte stimulating hormone (α-MSH and γ-MSH), D-Trp^{8}-γ-MSH, and [Nle^{4}-D-Phe^{7}]-MSH (NDP-MSH) and the natural antagonist agouti-related protein (AgRP). The results showed that pMC3R bound NDP-MSH with the highest affinity followed by D-Trp^{8}-γ-MSH, γ-MSH and α-MSH. The same ranking was also found for hMC3R, although pMC3R had 2-9 fold higher affinities for these ligands. Both pMC3R and hMC3R bound AgRP with high affinity. D-Trp^{8}-γ-MSH was the most potent agonist to stimulate cAMP generation followed by NDP-MSH, γ-MSH, and α-MSH. This ranking was the same as that of hMC3R. The

availability of pMC3R and its pharmacological characteristics will facilitate the investigation of pMC3R in regulating food intake and fat storage.

The melanocortin-4 receptor (MC4R) is critical in regulating mammalian food intake and energy expenditure. Numerous mutations in the MC4R gene have been identified from obese humans. So far two naturally occurring porcine MC4R (pMC4R) mutations, D298N and R236H, have been identified from various strains of pigs and D298N is being utilized as a genetic marker to screen performance traits of pigs. In this study, we performed functional analyses of pMC4R D298N and R236H, including their ligand binding and signaling properties in transiently transfected HEK293T cells. Ligand binding assays showed that both D298N and R236H pMC4Rs had similar binding capacities and affinities for the natural agonist α-MSH and the natural antagonist Agouti-related protein as WT pMC4R. In signaling assays, both mutants had normal EC_{50} and maximal signaling to α-MSH. In summary, pMC4R mutants D298N and R236H do not have any overt functional defects; therefore we suggest caution using these mutations as selection markers in breeding programs.

In this study, we reported detailed functional characterization of ten novel human MC4R (hMC4R) mutations including R7C, C84R, S127L, S136F, W174C, A219V, P230L, F261S, I317V, and L325F. We also reported the functional rescue of the loss-of-function mutants due to intracellular retention. The results showed that R7C had decreased cell surface expression although its binding capacity and signaling potency were comparable to WT hMC4R. C84R, S127L, W174C and F261S were defective in either total or cell surface receptor expression or both resulting in mutants with impaired function. A219V had normal total and cell surface expressions; however, its ability for

binding and signaling were significantly reduced. I317V and L325F had similar binding and signaling properties as WT hMC4R and thus only represent polymorphism of hMC4R. P230L had decreased cell surface expression and maximal binding although it had similar signaling potency as the WT hMC4R. S136F had normal total and cell surface expression and ligand binding. However, it was defective in signaling. Based on the observation that C84R and W174C were intracellularly retained, we tested a small molecule inverse agonist of the MC4R, ML00253764, to rescue the cell surface expression of these two mutants. The results showed that the cell surface expression of C84R and W174C were increased to 35% of that of the WT hMC4R after treatment with ML00253764. Even the cell surface expression of the WT hMC4R was increased to 152% after ML00253764 treatment. In addition, cAMP generation of the C84R and W174C MC4Rs was increased to 62% of that of the WT hMC4R after treatment with ML00253764. Thus, it was clear that ML00253764 acts as a pharmacological chaperone, increasing the cell surface expression of the hMC4R.

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Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmquist JK & Lowell BB 2005 Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* 123 493-505

Barb CR, Robertson AS, Barrett JB, Kraeling RR & Houseknecht KL 2004 The role of melanocortin-3 and -4 receptor in regulating appetite, energy homeostasis and neuroendocrine function in the pig. *Journal of Endocrinology* 181 39-52

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Biebermann H, Krude H, Eisner A, Chubanov V, Gudermann T & Gruters A 2003 Autosomal-dominant mode of inheritance of a melanocortin-4 receptor mutation in a patient with severe early-onset obesity is due to a dominant-negative effect caused by receptor dimerization. *Diabetes* 52 2984-2988

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