

PHARMACOLOGICAL CHARACTERIZATION OF CANINE
MELANOCORTIN-4 RECEPTOR AND ITS
NATURAL VARIANT V213F

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THESIS ABSTRACT

PHARMACOLOGICAL CHARACTERIZATION OF CANINE MELANOCORTIN-4 RECEPTOR AND ITS NATURAL VARIANT V213F

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Obesity is becoming one of the most significant public health problems all over the world, especially in some developed countries. During the past decade, genetic factors associated with obesity have gained more attention. Rodent and human genetic studies showed that leptin-regulated melanocortin circuit plays an important role in regulating food intake and body weight. As one of the components in the circuit, melanocortin-4 receptor (MC4R) was shown to play a critical role in regulation of food intake and energy expenditure. Mutations in *MC4R* were identified to be the most common monogenic form of obesity in humans. Recently, studies on role of MC4R in regulation

of food intake have been extended to other mammalian species, such as pigs.

In the current study, the newly identified canine MC4R (cMC4R) natural variant V213F was generated by site-directed mutagenesis using WT cMC4R as the template. Both WT receptor and the natural variant were expressed in HEK293T cells. Pharmacological characteristics of these receptors were analyzed by binding and signaling assays upon the stimulation of three agonists: [Nle⁴, D-Phe⁷]- α -melanocyte stimulating hormone (NDP-MSH), α -MSH, and β -MSH. WT human MC4R (hMC4R) was included in the experiments for comparison. NDP-MSH was shown to be the most potent agonist for cMC4R and therefore would be better suited for further *in vivo* studies. Both WT cMC4R and its natural variant functioned normally in terms of binding and signaling, similar to WT hMC4R. In conclusion, our results showed that cMC4R functioned normally upon stimulation with three MC4R agonists. Its natural variant V213F does not have any functional defect and therefore is not likely to cause obesity in dogs. NDP-MSH would be a better ligand for the further *in vivo* studies. Further studies may focus on whether cMC4R respond to analog that has been tested in human medicine similarly as hMC4R.

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INTRODUCTION

Overview of melanocortin system

The melanocortin system includes neurons that express pro-opiomelanocortin (POMC), agouti-related peptide (AgRP) and melanocortin receptors whose activity are affected by peptides released from POMC and AgRP neurons.

As a prohormone gene, POMC is extensively processed in a highly tissue-specific manner to yield several peptides that are involved in regulating different physiological processes. Encoding a 31-36-KDa pre-prohormone, the *POMC* gene consists of three exons in which the exon 3 encodes most of translated mRNA (1). The *POMC* gene was found to be expressed at significant levels in a number of mammalian tissues including skin, pituitary gland, hypothalamus and the immune system (2, 3). POMC mRNA is synthesized in the anterior and neurointermediate lobes of the pituitary. POMC undergoes post-translational processing in a tissue-specific manner (2). In the anterior pituitary, it is processed predominantly by prohormone convertase 1 (PC1) to adrenocorticotrophic hormone (ACTH), β -lipotropin (LPH), N-terminal peptide and joining peptide. In the hypothalamus and the neurointermediate lobe of pituitary where both PC1 and PC2 are expressed, these products are further processed to α -, β -, and

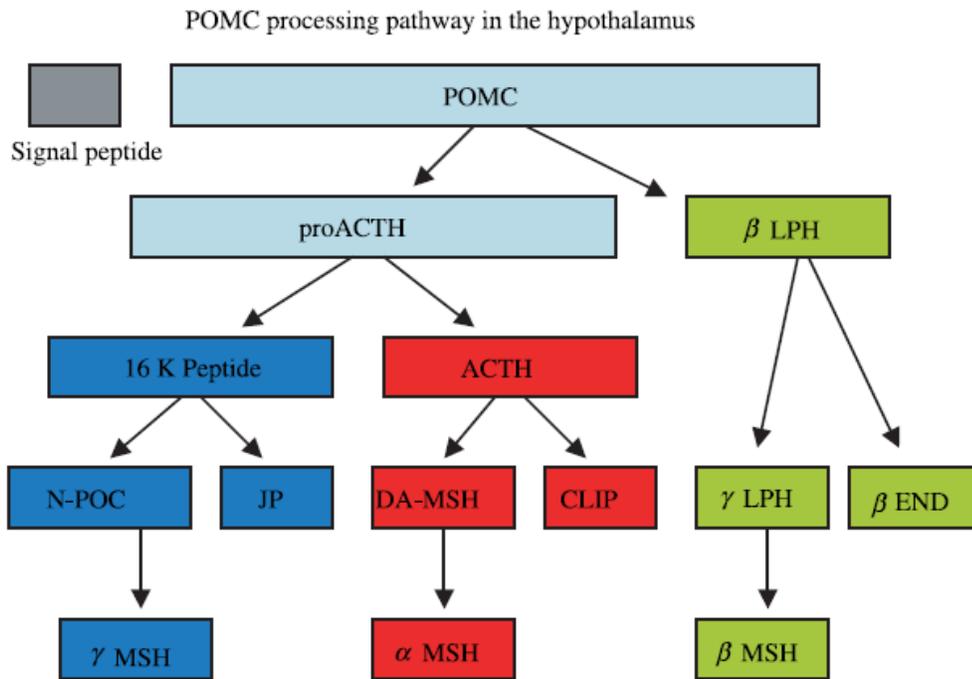


Figure 1: POMC processing pathway in the hypothalamus. N-POC, N-terminal fragment of POMC; JP, junction peptide; DA-MSH, deacetyl MSH; CLIP, corticotropin-like intermediate lobe peptide; LPH= lipotropin; END, endorphin. (Shimizu H., Inoue K. & Mori M. 2007 The leptin-dependent and -independent melanocortin signaling system regulation of feeding and energy expenditure. Journal of Endocrinology 193: 1-9)

γ -melanocyte-stimulating hormone (MSH). Together with ACTH, these four POMC derived peptides are called melanocortins. They share the core sequence His-Phe-Arg-Trp (3) that is required for their binding to specific melanocortin receptors (MCRs) to exert biological effects. There are five known MCRs, MC1-5R, according to the order in which they were cloned (4). The MC1R is largely expressed in melanocytes in skin and hair and stimulates eumelanin pigmentation in a number of species including humans (5). The MC2R, also called ACTH receptor, is expressed on the adrenal cortex and plays a critical role in regulating the hypothalamic-pituitary-adrenal axis (HPA) to control adrenal steroidogenesis and growth. MC5R was identified to be mainly expressed in exocrine glands including the lacrimal and sebaceous glands in which it appears to regulate the synthesis and secretion of exocrine gland products (8). MC3R and MC4R are known as neuronal MCRs as they are both primarily expressed in the central nervous system. They are involved in energy homeostasis as well as a number of other physiological processes.

Molecular cloning revealed that MCRs are members of Family A G protein-coupled receptor (GPCR) and have the typical structure of GPCRs. MCRs couple to the stimulatory heterotrimeric G protein (Gs) upon stimulation with agonist and subsequently activate the adenylyl cyclase to elevate the intracellular cAMP levels (4). MCRs are unique among the GPCR family in that they also have two endogenous antagonists named Agouti and Agouti-related peptide (AgRP). Agouti is a protein

secreted within the hair follicles and act in a paracrine manner to antagonize the action of α -MSH at MC1R expressed on the surface of melanocytes. Therefore, it is involved in regulating pigmentation (6). Known as one of the oldest genetic models of obesity, studies with agouti mouse showed that widespread ectopic expression of agouti protein resulted in a phenotype of obesity, hyperphagia and yellow coat color (6). The cause of obesity of agouti mouse was clarified by the identification of agouti as an antagonist for MC4R (10). AgRP was identified in the hypothalamus and named due to its homology to agouti. It acts as an antagonist at MC4R expressed in hypothalamus. Transgenic mice with ubiquitous expression of human AgRP developed hyperphagia and obesity but not yellow fur (7, 8).

The leptin-regulated melanocortin circuit

Over the last decade, an overriding theme has been established that the central nervous system plays an important role in coordinating metabolic functions in peripheral tissues. The hypothalamus was identified to receive and integrate neural, metabolic and humoral signals from the periphery (9). The arcuate nucleus within the hypothalamus was considered to be the primary sensor of alteration of food intake and energy expenditure. It can be divided into two subsets of neurons, one of which coexpress POMC and anorectic peptide CART (cocaine and amphetamine-regulated transcript), the other coexpress the potent orexigenic peptides neuropeptide Y (NPY) and AgRP.

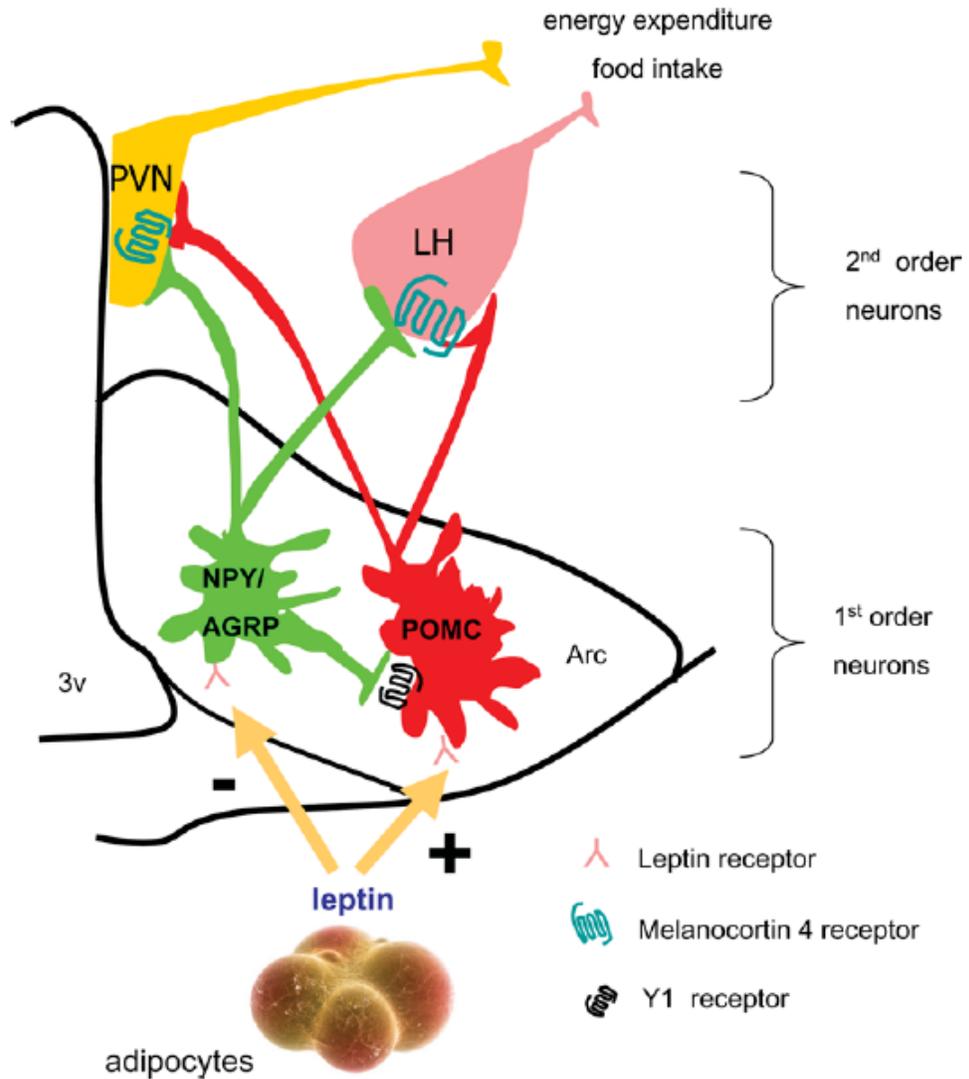


Figure 2: Hypothalamic melanocortin system. 3v, third ventricle; PVN, paraventricular nucleus; LH, lateral hypothalamus; ARC, arcuate nucleus. (Coll AP. 2007 Effects of pro-opiomelanocortin (POMC) on food intake and body weight: mechanisms and therapeutic potential? *Clinical Science* 113: 171-82)

Expression on these two subsets of neurons is dependent on leptin signaling because leptin receptors are expressed on both of them. Leptin is secreted by adipocytes and can cross the blood-brain barrier. The leptin signal to the hypothalamus is obligatory for regulation of food intake and body weight. Excess energy causes high levels of leptin which activate POMC neurons by binding to its receptor OB-Rb and stimulates the release of melanocortin. It in turn goes to activate MC4R expressed on second-order neurons resulting in suppressed food intake and increased energy expenditure. In the mean time, leptin suppresses the activity of NPY/AgRP by binding to its receptor in these neurons. Therefore, AgRP antagonism of α -MSH on MC4R is decreased. The overall effect is decreased food intake and body weight. In contrast, low levels of leptin appearing during the energy depletion have opposite effects on these two subsets of neurons.

The leptin-regulated melanocortin circuit has been proved to be essential for energy homeostasis (9). Therefore, any mutation impairing the functions of components in this circuit would disrupt energy balance and cause obesity. These components include leptin, leptin receptor, POMC, PC1 and MC4R. Human and rodent genetic studies have shown that mutations in all these components lead to monogenic obesity (10-16). Mutations occurring in *MC4R* appear to be the most common form of monogenic obesity.

MC4R and energy homeostasis

The anatomical, pharmacological and rodent genetic studies have shown that MC4R plays a critical role in regulating food intake and energy homeostasis. In situ hybridization showed that MC4R was localized in a number of nuclei in the rat brain, including cortex, thalamus, hypothalamus, and brainstem, with the highest expression in the brainstem (17). As one of the post-translational products of POMC, α -MSH has been shown to reduce food intake in rats by central administration (18). Furthermore, MC4R agonist melanotan II (a cyclic heptapeptide) was shown to suppress food intake and decrease body weight in mouse when administered into brain ventricles of rodents (19, 20), while MC4R antagonist SHU 9119 was shown to stimulate feeding and reverse the suppressive effects of melanotan II on food intake (19).

The role of MC4R in regulation of food intake was also supported by studies on agouti and AgRP. The agouti (A^y) mice overexpressing agouti ectopically in many tissues resulted in a distinct phenotype characterized by hyperphagia, hyperinsulinemia, hypometabolism and increased linear growth (6). Agouti was shown to be specific high-affinity antagonist of MC4R by antagonizing α -MSH (21). Therefore, overexpression of agouti would antagonize the suppressive effect of α -MSH on food intake. As a neuropeptide, AgRP can also act as an endogenous antagonist on MC4R expressed in hypothalamus. Rodent studies showed that overexpression of AgRP in transgenic mice resulted in obesity (7, 8).

MC4R knockout mice provided solid evidence that MC4R is obligatory in regulating food intake and energy expenditure. The mice that were homozygous for MC4R deficiency (*Mc4r*^{-/-}) were shown to have increased food intake and body weight, increased linear growth, and hyperinsulinemia (22). Animals with heterozygous deficiency in MC4R (*Mc4r*^{+/-}) showed an intermediate phenotype compared to *Mc4r*^{-/-} and wild-type littermates, indicating that two copies of the gene are required for normal energy homeostasis. Furthermore, either peripheral or central administration of the non-specific melanocortin agonist melanotan-II did not cause the inhibition of food intake in *Mc4r* knockout mice, suggesting that activation of MC4R is critical for the anorectic action of melanocortin receptor agonists.

Objective of this study

Obesity has emerged as a worldwide epidemic health problem, especially in developed countries like the US. According to reports of National Center for Health Statistics, 61% of adults in the US are overweight and 26% are obese (23). It was also reported by the National Health and Nutrition Examination Surveys that 33.3% of men and 35.3% of women are obese in the US (24). The World Health Organization has proclaimed obesity to be a global epidemic as 310 million people worldwide are obese.

Obesity is strongly associated with type II diabetes mellitus, dyslipidemia, hypertension, cardiovascular disease, and certain types of cancers. Therefore, there are increased morbidity and mortality occurring in obese people. It has been estimated that

there are 300,000 deaths per year being attributable to obesity in the United States.

It is now well accepted that the development of common obesity derives from the interaction of multiple environmental factors including overeating and reduction in physical activity with genetic factors. Environmental factors, such as overeating, physical(3) inactivity and socioeconomic conditions would affect an individual's risk for obesity (25). However, it was reported that the mean weight of an American adult increased by about 4kg between 1991 and 2000 while American adults vary in weight between 50 and 300kg. This indicated that there are huge differences in terms of susceptibilities to weight gain among individuals within the same environment (26).

In addition to several environmental factors, genetic factors also play important roles in pathogenesis of obesity. Obesity can be classified into three main categories on the basis of genetic etiology: monogenic, syndromic and polygenic obesity. Rodent genetic studies contributed enormously to the field of human obesity. During 1990s, a series of murine obesity genes were identified including leptin (10), leptin receptor (27), carboxypeptidase E (28), agouti (29), Mc4r (22) and Agrp (7). After these discoveries, mutations in homologous genes or pathways causing human obesity were also identified. Currently, there are six forms of monogenic obesity in humans and mutations in these genes include leptin (*LEP*), leptin receptor (*LEPR*), *PC1*, *POMC*, *MC4R* and single-minded homolog 1 (*Sim1*) (30). Of these genes, mutations in *MC4R* were the most common form of human monogenic obesity.

MC4R is a protein of 332 amino acids encoded by a single exon localized on chromosome 18q 22 (31). It belongs to Family A of GPCR and transduces its signal by coupling to the heterotrimeric Gs leading to activating adenylate cyclase. As one of the neural MCRs, expression of MC4R is primarily in the brain, such as hypothalamus, which is critically involved in regulation of food intake. MC4R regulates food intake by integrating signals provided by its agonist α -MSH and its antagonist AgRP. Some studies also showed that MC4R exhibits constitutive activity therefore can potentially exert an inhibitory effect on food intake in the absence of ligand (32, 33). In this case, AgRP acts as an inverse agonist. The importance of naturally occurring mutation in *MC4R* in the regulation of food intake and body weight first became apparent when frameshift mutations in *MC4R* gene were first identified in patients with severe early onset obesity in 1998 (34, 35). Since then, more than 150 mutations have been reported in various patient cohorts. These include frameshift, inframe deletion, nonsense and missense mutations. Over 100 residues in MC4R were identified to be mutated, representing approximately 31% of the receptor. In some cohorts, up to 6% of patients with early onset obesity have *MC4R* mutations (36).

To elucidate a causal relationship between the mutation and increased food intake and body weight, many groups also performed detailed functional studies on some of the mutants. Measurement of cAMP levels in cells transfected with WT and mutant MC4Rs would tell if the mutants are defective in signaling. Binding assays of both WT

and mutant MC4Rs indicate if these mutations can cause decreased or absent binding. Further confocal microscopy studies on cells stably transfected with WT MC4R and mutants would reveal if the mutants are expressed on cell surface. These series of experiments can identify the molecular defects of mutants. Intracellularly trapped mutants that do not reach the cell membrane represent the largest set of MC4R mutations reported (37).

Dogs have become one of most important companion animals in the modern society. However, recently it was estimated that 20-40% of owned dogs are obese (38) indicating that obesity has become one of the most important canine health problem. In addition, obesity in dogs also leads to type II diabetes mellitus as it does to humans (39). Since MC4R has been studied extensively in human obesity and naturally occurring mutations in *MC4R* has been suggested as the most important contributor for monogenic human obesity, it is worth studying the role of MC4R in dog obesity.

Recently, canine MC4R (cMC4R) was cloned and a missense variant V213F was identified (40). According to the sequence deposited in Genebank (accession number DQ084210), we designed the primers and successfully cloned cMC4R and inserted into pcDNA 3.1, a mammalian expression vector. The objective of this study was to investigate the pharmacological characteristics of cMC4R and its natural variant V213F. As the central melanocortin system has been suggested to play an important role in regulation of energy balance in humans, we propose that it is also critical for energy

homeostasis of dogs. Therefore, determination of pharmacology of cMC4R and its natural variant is a crucial initial step for our long-term goal of understanding the regulation of energy balance in dog. Once the pharmacological characteristics of cMC4R is known, new drugs that were found to be effective in human medicine would be tested for their potential utility in treating dog obesity.

In this study, the variant V213F was generated by site-directed mutagenesis and detailed pharmacological characterization of WT cMC4R and its natural variant V213F was performed by measuring ligand binding and signaling (cAMP generation in response to ligand stimulation). The natural ligands α -MSH, β -MSH, and the superpotent agonist [Nle⁴, D-Phe⁷]-MSH (NDP-MSH), were used. Human MC4R (hMC4R) was also included in the experiments so that pharmacology of cMC4R can be compared with that of hMC4R. Another aim of this study was to see whether the natural variant V213F is defective in binding and signaling therefore contributing to obesity.

LITERATURE REVIEW

Gene structure and tissue distribution of MC4R

Using polymerase chain reaction (PCR) and genomic cloning, Gantz et al. first cloned the hMC4R in 1993 (41). The human *MC4R* is an intronless gene. The open reading frame (OFR) of the human *MC4R* gene is 999 bp and encodes a protein of 332 amino acids (41). Alignment of cloned hMC4R amino acid sequence with the other MCRs revealed that it was structurally most similar to the MC3R because it shared 58% and 76% overall amino acid identity and similarity, respectively (41). By using fluorescent in situ hybridization technique, they showed that the human *MC4R* gene was localized to chromosome 18q21.3 (41).

Since the cloning of hMC4R, MC4Rs from other mammalian species were also cloned including MC4R of mouse (42), rat (17), pig (43) and cattle (44). In recent years, with increasing interests in role of MC4R in energy homeostasis of lower vertebrates, MC4Rs of some nonmammalian species including trout (45), flounder (46), goldfish (47) and shark (spiny dogfish) (48) were also cloned. The results showed that mammalian MC4Rs all share the same gene structure and have the coding region of the same size compared to that of hMC4R. However, MC4Rs of some nonmammalian species were

identified to have a coding region of different size although they contain a single exon as the hMC4R. For instance, compared to a 332 amino acid protein encoded by human *MC4R* gene, the *MC4R* gene from trout and flounder encode a protein of 339 amino acids and 325 amino acids, respectively (45, 46).

In mammals, MC4R is predominantly expressed in the brain (49). Rodent tissue analysis revealed that MC4R is expressed in multiple sites in the brain including cortex, thalamus, hypothalamus, brainstem, and spinal cord (17). In particular, MC4R is highly expressed in the paraventricular nucleus (PVN) of the hypothalamus in the brain which serves as an important site of action of anorexigenic effects of MCR ligands. The PVN receives input from arcuate nucleus (ARC) where MCR ligands are produced. Pharmacological studies showed that local injection of MC4R agonist MTII (19) and α -MSH into PVN reduced food intake in rodents while administration of antagonist AgRP (50) and SHU9119 (51) into PVN stimulated feeding. The other sites expressing MC4R in the brain, such as brainstem (52), were also implicated in regulating food intake (49). In nonmammalian species, MC4R is also expressed in the brain. However, studies have shown that MC4R is also expressed peripherally in some species. For instance, MC4R was found to be expressed in ovary of goldfish and flounder (46, 47). In addition, MC4R was also expressed in liver and testis of the flounder (46). These results indicated that besides regulation of food intake, MC4R may be involved in the gonadal development and steroidogenesis in nonmammalian species.

Role of MC4R in energy homeostasis

Rodent genetic studies provided several lines of evidences that MC4R plays a critical role in regulation of food intake and energy homeostasis. The agouti (Ay) mice, which ectopically overexpress agouti protein in many tissues including hypothalamus give rise to the phenotype of obesity, hyperphagia and yellow coat color. The linkage between the yellow coat and obesity was later explained by identification of agouti as an antagonist for MC4R (21). Identification of AgRP extended the antagonism of MC4R (7, 53). It encodes a protein nearly identical in size and genomic structure to agouti (7). Its mRNA was identified primarily expressed in adrenal gland and hypothalamus and expression levels were high in ob/ob mice (7). Overexpression of AgRP in transgenic mice led to the phenotype of obesity without altering pigmentation (7, 8). In addition, studies on *Pomc*-null mice and *Mc4r*-deficient mice also greatly supported the role of MC4R in regulation of feed intake and energy homeostasis. Both *Pomc*-null mice and *Mc4r*-deficient mice developed a marked obesity syndrome associated with hyperphagia, hyperinsulinemia, hyperglycemia and an increase in linear growth (22, 54). Furthermore, heterozygous mice (*Mc4r*^{+/-}) had an intermediate phenotype between the WT and homozygous null mice (22). This indicated a clear gene dosage effect. Later studies on *Mc4r*-deficient mice showed that the obesity in *Mc4r*-null mice resulted from defective regulation of energy expenditure as well as hyperphagia (55). In conclusion, lacking either MC4R or its endogenous agonist (POMC-derived MSH) or up-regulation of

expression of its antagonist all result in the same obese phenotype, supporting a critical role of MC4R in regulation of food intake and body weight in rodents.

Pharmacological studies in rodents also support an important role of MC4R in regulation of feeding in rodents. The synthetic MC4R agonist, MTII, when administered intracerebroventricularly (i. c. v.) (19, 20, 56) or into PVN (50, 51, 57), potently suppressed food intake and decreased body weight in mice and rats. Many studies indicated that the PVN is an important site of action of melanocortins on food intake. It was identified as one of the maximally responsive sites in terms of feeding response to injection of α -MSH and its antagonist, AgRP (57). It is also one of the sites of integration of input from ligands of MC4R. Studies showed that co-administration of MTII and NPY to the PVN of mice blocked NPY-induced stimulating feeding response (51). On the contrary, the MC4R synthetic antagonist SHU9119, when delivered into the PVN of the hypothalamus by i.c.v. administration, not only stimulated feeding but also reversed the suppressive effects of MTII on food intake in rodents (19). In addition to the PVN of hypothalamus, brainstem may also be a possible site of action for these melanocortin analogs and involved in the control of feeding. It was identified that the dorsal motor nucleus of the vagus nerve (DMX) had the highest MC4R density found in the brain (17). In 1998, Grill et al. (52) investigated contribution of the caudal brainstem to central melanocortin system in regulation of food intake by administering MTII and SHU9119 into the fourth ventricle of rats respectively. They showed that the agonist reduced food

intake and body weight while the antagonist had the opposite effects, indicating that brainstem might be the one of sites of action of MC4R in controlling food intake.

Human genetic studies also supported the role of MC4R in regulation of food intake and body weight. In 1998, Krude et al. (15) first reported two patients with mutations in *POMC*. One patient was a compound heterozygote for two nonsense mutations in exon 3 of *POMC* and the second patient was homozygous for a mutation introducing an additional out-of-frame start site and interfered with POMC translational initiation. Since then, new mutations in *POMC* continued to be identified to cause obesity. In addition, in 1997, Jackson et al. (16) identified a woman with severe early-onset obesity to be a compound heterozygote for mutations in *PC1*, an important enzyme for post-translational processing for POMC. The phenotypes of obesity caused by mutations in *POMC* and *PC1* were similar to those resulted from *Pomc*- and *Mc4r*-knockout mice. Since POMC and PC1 are associated with generation of melanocortins including the endogenous ligands for MC4R α -MSH and β -MSH, it is reasonable to deduce that role of MC4R in regulation of food intake and energy expenditure could be one of the reasons for the obesity caused by mutations in *POMC* and *PC1*.

The importance of MC4R in regulation of food intake and human body weight first became apparent in 1998 when two groups (34, 35) first identified heterozygous frameshift mutations in *MC4R* that cosegregated with dominantly inherited severe early-onset obesity. Since then, numerous mutations in human *MC4R* have been

identified from different cohorts and various ethnic groups. To establish a causal relationship between obesity and receptor mutations, functional analysis of hMC4R mutants were also performed. Results showed several defects that disrupted function of hMC4R including truncated protein, intracellular retention, no ligand binding and/or no response to ligand stimulation. Just as the *Mc4r*^{+/-} mice showed an intermediate obese phenotype between that of WT and *Mc4r*^{-/-} mice, Cody et al. (58) demonstrated that the mutations found in *MC4R* of human subjects resulting in obesity is due to haploinsufficiency not dominant negative activity.

Naturally occurring *MC4R* mutations

In 1998, two groups (34, 35) first identified frameshift mutation in *MC4R* gene associated with dominant-inherited morbid obesity in humans, establishing causative relationship between mutations in *MC4R* and morbid early onset obesity. Since then, more than 150 mutations have been identified in *MC4R* gene from different patient cohorts and various ethnic groups (36, 59-96). These mutations include frameshift, inframe deletion, nonsense and missense mutations, scattered throughout the coding region of *MC4R* gene. In most of the earlier reports of human *MC4R* mutations, functional analysis of the mutant receptors were not performed, thus only an association rather than causative relationship was established between mutations and the obesity. Later functional analysis of human *MC4R* showed that the majority of mutations lead to receptor defects including receptor biosynthesis, cell surface expression, ligand binding

or signaling (97, 98). The prevalence of human *MC4R* mutations reported from different studies varied from different ethnic groups. In one large study, 5.8% of subjects with severe early onset obesity were identified to have mutations in *MC4R*. Therefore, *MC4R* deficiency represents the most common form of monogenic obesity (36).

Loss-of-function mutations of *MC4R* gene were found mostly inherited in an autosomal dominant manner (75). As the rodent genetic studies showed that *Mc4r*^{+/-} mice have an intermediate phenotype between WT and *Mc4r*^{-/-} mice, it is widely accepted that haploinsufficiency resulted in the obese phenotype in heterozygous *MC4R* loss-of-function mutation carriers. Molecular characterization showed that most of human *MC4R* mutations identified are heterozygous and co-transfection studies showed that they do not have dominant negative activity (63, 71, 99). Although the dominant negative effect was excluded at least for these loss-of-function *MC4R* mutations, we need to be careful when investigating other identified heterozygous missense *MC4R* mutations. It is generally accepted that GPCR can function as dimers. In 2003, Biebermann et al. (75) showed that the *MC4R* mutant D90N had dominant negative activity. The mutation was located in transmembrane 2 of the receptor where the most conserved Asp residue was mutated to Asn. They demonstrated that the dominant negative activity of this mutant was due to its heterodimerization with WT receptor. This finding provided alternative explanation why a particular heterozygous inactivating *MC4R* mutation led to the development of extreme obesity.

Based on rodent and human genetic studies, it is assumed that haploinsufficiency is the major cause for obesity resulted from heterozygous mutation in *MC4R* gene. Therefore reduced gene transcription caused by mutations in essential regions of the *MC4R* promoter could also be a cause of obesity in humans. Several groups have tried to identify mutations or variants in the regulatory sequences of the *MC4R* gene, especially promoter region, in obese subjects. One group defined a 80bp long region as the minimal core promoter region which is essential for the transcriptional activity of MC4R (100). They then screened 431 obese children and adults for mutations in the coding sequence and the minimal core promoter of MC4R. Three variants were detected in 5'UTR and were not associated with the obesity (100). In another study on MC4R variants associated with severe obesity in Pima Indians, 3 upstream variants were detected and none of them were associated with obesity (101). Recently, a two-base deletion mutation –439delGC in the *MC4R* promoter was reported to be associated with early-onset obesity (77, 102). However, further studies are needed to clarify whether the –439delGC mutation is associated with early-onset obesity in other populations as well.

Recently, genome-wide association scans identified three single nucleotide polymorphisms (SNPs) localized at 109-188kb downstream of MC4R (SNP rs 17782313, rs 12970134, and rs17700663) (103-105). Human subject studies showed that these SNP are associated with increased risk for obesity and influence fat mass and weight (103-105). Further studies on these SNPs showed that rs17782313 was significantly

associated with high intakes of total energy, total fat and protein (104). However, SNP rs1770833 was not significantly associated with either dietary intakes or obesity traits (104). The exact mechanism for the effects of these SNPs is not clear so far. One hypothesis is remote effects of these SNPs on translation or transcription of *MC4R* gene.

Molecular classification of the *MC4R* mutations identified from obese human subjects

The functional studies of human MC4R mutants showed they might be defective either in biosynthesis, cell surface expression, ligand binding or signaling. To classify the human MC4R mutations into categories in terms of different defects, Tao and Segaloff proposed a molecular classification system (37), modeled after the classification of mutations in the low density lipoprotein receptor and cystic fibrosis transmembrane conductance regulator (CFTR).

The Class I was named null mutations. Mutants belonging to this class are either defective in protein synthesis and/or accelerated protein degradation, resulting in no receptor protein present in the cell. These might be due to the production of a premature stop codon in coding region of the *MC4R* gene or a frameshift that caused the generation of a premature receptor protein. Human MC4R mutants L64X (69), W16X (60), Y35X (62, 106), Δ CTCT at codon 211 (99), the TGAT insertion at codon 244 (99) and Δ 750-751GA (78) are likely to belong to this class.

The Class II are intracellularly trapped mutants. The mutants belonging to this class are produced but are retained intracellularly, most likely in the endoplasmic reticulum due to misfolding detected by the cell's quality control system. This class comprises the largest set of MC4R mutations reported. These MC4R mutants include I317T (70, 107), I316S (71), P299H (70), C217Y (37, 71), C271R (71, 73), Y287X (71), L250Q (70), N240S (108), R165W (70), R165Q (109), I125K (71), L106P (71), I102S (70), G98R (37), N97D (71), P78L (37, 70, 109), N62S (37, 71) and S58C (37, 70).

Class III was defined as binding defective mutants. This group of mutants are expressed on the cell surface normally, but are defective in ligand binding with either decreased binding capacity and/or affinity, resulting in impairments in hormone-stimulating signaling. MC4R mutants I316S (71), I102S (108), I102T (108), Δ 88-92 (74), I137T (61), I125K (71), L106P (71) and N97D (71) belong to this class. One of the major differences of MC4R from other GPCRs is that its activity can be also inhibited by the endogenous antagonist AgRP. Therefore, mutants that are more sensitive to inhibition of AgRP are also functionally defective. Mutants that alter the relative affinities of the receptor for its endogenous agonist and antagonist can be classified as a subclass within this class. As most of functional studies to date did not include AgRP in binding studies, it is not known how prevalent this kind of defect is. The only mutant identified for this subclass is MC4R mutation I316S (71), which was shown to have altered relative affinities for its endogenous agonist (α -MSH) and

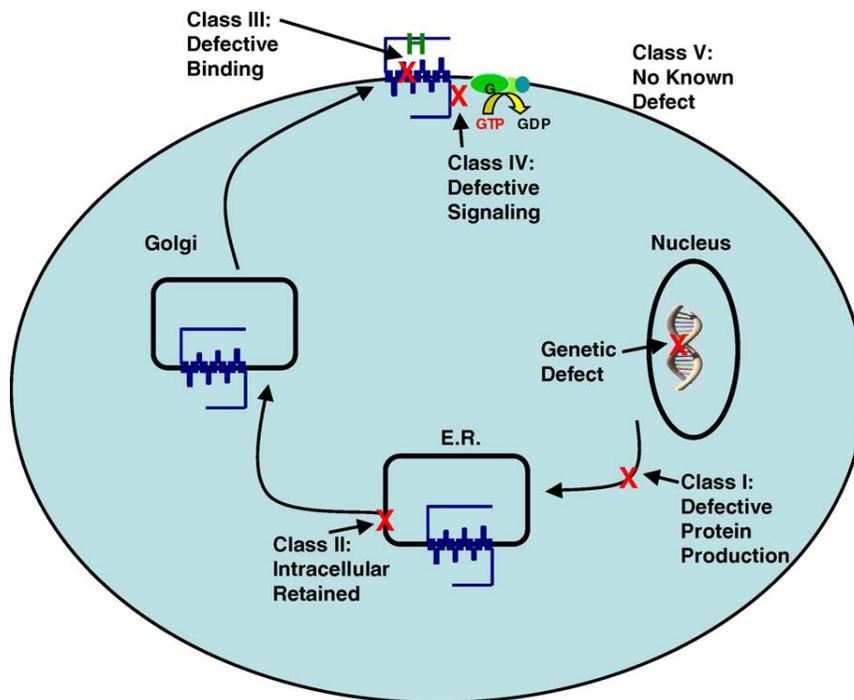


Figure 3: Molecular classification of naturally occurring MC4R mutations in early onset severe obesity. (Tao YX, 2005 Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity. *Molecular and Cellular Endocrinology* 239: 1-14.)

antagonist (AgRP).

Class IV mutants include MC4R mutants D90N (75), A175T (71), I137T (61) and V253I (71). The mutants of this class are expressed on the cell surface and also bind ligands with normal affinity. However, they are defective in agonist-stimulated signaling with decreased efficacy and/or potency.

Class V are variants with apparently normal function. Functional studies showed that these variants were similar to WT MC4R in terms of receptor biosynthesis, cell surface expression, ligand binding and signaling in heterologous expression systems. But they differ from the common polymorphisms because they are not assumed to be present in normal subjects, such as V103I (63) and I125L (62). The other mutants in this class include I170V, M200V, N274S, S295P, T11A, D37V, P48S, V50M, T112M and A154D (37, 62, 63). Whether and how these variants cause energy imbalance and therefore obesity is not clear.

Therapeutic implications

According to a molecular classification of inactivating GPCR mutation (98), GPCR including MC4R mutants could be corrected via different approaches. For instance, nonsense mutations in Class I could potentially be rescued by aminoglycoside antibiotics (98). These mutants are defective in receptor biosynthesis and aminoglycoside antibiotics could bind to the decoding site on the ribosome, which could decrease the codon-anticodon proofreading efficiency resulting in read-through of the premature stop

codon (98). This strategy has been investigated in several diseases including muscular dystrophy (110), cystic fibrosis (111) and nephrogenic diabetes insipidus (NDI) (112). However, no MC4R mutants have been reported to be rescued via this method.

In addition, some of the intracellularly retained MC4R mutants have residual activity in terms of hormone-stimulated cAMP production. Therefore, approaches that result in increased expression on cell surface could potentially be of therapeutic value. It was reported that the C-terminal peptide of AgRP can increase the cell surface expression of MC4R (113). Therefore, some antagonist developed for MC4R sometimes might act as pharmacological chaperones, which would increase the cell surface expression of the receptor and be of potential therapeutic value. Tao et al. (37) identified four MC4R mutants N62S, I102S, Y157S and C271Y that responded to NDP-MSH stimulation with increased cAMP production in spite of minimal binding capacity (less than 5% of WT). This indicated that these mutants are competent in G protein coupling and effector activation. Therefore, development of strategies that increase their expression could be useful for treating obesity in the subjects that carry these mutant *MC4R* genes.

Chemical chaperones are low molecular weight compounds, such as glycerol, dimethyl sulfoxide, and trimethylamine N-oxide, that can help protein folding by influencing the rate or fidelity of the folding reaction (114). It has been reported that chemical chaperones could improve the maturation of a number of proteins including CFTR (115, 116). However, they are less effective in promoting maturation of

ER-retained vasopressin 2 receptor (V2R) mutants compared to pharmacological chaperones (117). In addition, high concentration of chemical chaperones is toxic to cells and their effect is nonspecific. Therefore, compared with pharmacological chaperones, they are not practical options for clinical treatments.

Pharmacological chaperones are defined as small molecules that can correct and stabilize protein misfolding, resulting in recovery of function (118). In 2000, Bouvier and colleagues (119) first investigated the potential utility of pharmacological chaperones to increase the cell surface expression of mutant GPCRs. They showed that selective, membrane-permeable nonpeptidic V2R antagonist could dramatically increase cell surface expression of eight mutant V2Rs associated with NDI (119). Since then, pharmacological chaperones have been reported to increase cell surface expression of mutants of several other GPCRs, including MC1R (120), V1a and V1b (121-123) vasopressin receptors, gonadotropin-releasing hormone receptor (124, 125) and the prototypical GPCR, rhodopsin (126, 127). Recently, studies on pharmacological chaperones for V2R have been extended to *in vivo*. In a clinical trial, patients treated with the nonpeptide antagonist SR495909 had decreased urine volume and water intake and increased urine osmolality (128). Fan & Tao (129) recently showed that a small molecule MC4R antagonist ML00253764 was able to rescue two intracellularly retained MC4R mutants, C84R and W174C, indicating that a small molecule MC4R ligand could act as a pharmacological chaperone assisting intracellularly retained mutants trafficking to cell

surface. Therefore, they are of potential value for treating patients harboring these *MC4R* mutations.

Although pharmacological chaperones may be used for rescuing intracellularly retained *MC4R* mutants that are defective in trafficking, they are not useful for the mutants that are already transported to the plasma membrane but defective in ligand binding or G protein coupling/activation. Haskell-Luevano et al. (130) provided evidences that some of the synthetic ligands, including peptides and small molecules, such as THIQ and MTII, could stimulate *MC4R* mutants with decreased endogenous agonist potency. These synthetic ligands might bind to residues on the receptor different from endogenous ligand. Therefore, development of synthetic ligands could be a potential strategy to rescue the mutants with this defect.

Conclusion

Obesity is becoming one of the most significant public health problems all over the world, especially in developed countries. Due to its profound effects on lipid and glucose homeostasis, obesity is strongly associated with dyslipidemia, hypertension, type-II diabetes mellitus, atherosclerosis and cardiovascular diseases and therefore caused an increased risk of mortality and morbidity (25, 131). It was estimated that obesity and its associated disorders are at epidemic levels in the US and other developed countries (132).

During the past decade, considerable progress has been made on studies on genetic factors associated with obesity. Rodent and human genetic studies have provided multiple lines of evidences that leptin-regulated melanocortin circuit plays an important role in regulating food intake and body weight. They showed that genetic disruption of a single element of a homeostatic system regulating energy balance would lead to obesity. So far, mutations in six genes including *LEP*, *LEPR*, *PC1*, *POMC*, *MC4R* and *SIMI* have been found to cause monogenic early-onset obesity (133). However, naturally occurring mutations in *MC4R* have been identified to be the most common form of monogenic obesity (97). Since the first identification of frameshift mutations (34, 35) in *MC4R* associated with severe early-onset obesity, more than 150 mutations have been found in various patient cohorts. In a study in which 500 subjects with severe early-onset obesity were screened for mutations in *MC4R*, approximately 6% (36) of patients were found to harbor mutations in *MC4R*, emphasizing its importance in obesity pathogenesis.

Although identification of *MC4R* variants from obese patients provided evidences that these mutations might be associated with obesity, it does not necessarily prove that the mutation is the cause of the obesity. Detailed functional studies of these mutants are required to build the causative relationship between mutations and obesity. In addition, it also reveals the molecular defects of these mutants and therefore suggests how these defects cause loss-of-function. Functional studies also help us understand the mechanism of how these mutants cause the obesity. For instance, most of *MC4R* mutations identified

from obese patients are heterozygous, therefore they can cause the obesity by either haploinsufficiency or dominant negative activity. Functional studies showed that most mutants do not have dominant negative activity (63, 71, 99) except for D90N (75) and S136F. Therefore, obesity associated with MC4R mutations is likely caused by haploinsufficiency. So far, about 31% of the residues of the MC4R have been found to be mutated naturally and novel mutations continue to be identified through new screening reports. Therefore, it is imperative to perform detailed functional studies of these identified mutants 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 also necessary to identify the exact defect of the mutants as the potential therapeutic targets so that we can develop agents to correct them.

MATERIALS AND METHODS

Cells, plasmids and peptides

HEK293T cells were obtained from American Type Culture Collection (Manassas, VA). These cells were incubated at 5% CO₂ in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% newborn calf serum, 10mM HEPES, and 100 units/ml of penicillin and 100ug/ml streptomycin (all from Invitrogen, Carlsbad CA). The N-terminal myc-tagged hMC4R was described previously (37). Canine MC4R was cloned in our lab and primers were designed based on the sequence deposited in GenBank (accession number DQ084210). The cloned fragment was inserted into pcDNA3.1, a mammalian expression vector. The superpotent agonist of α -MSH, NDP-MSH and the MC4R natural agonists α -MSH and β -MSH were purchased from Phoenix Pharmaceuticals (Belmont, CA). ¹²⁵I-labelled NDP-MSH was obtained from Peptide Radioiodination Service Center at The University of Mississippi (University, MS).

Site-directed mutagenesis of cMC4R mutant

cMC4R mutant V213F was generated by site-directed mutagenesis using WT cMC4R as the template by QuikChange site-directed mutagenesis kit (Stratagene, La

Jolla, CA). Primer pairs were designed to introduce the desired mutations in the coding region of cMC4R: V213F forward (5'- GCTTCTCTCTACTTCCACATGTTCC -3') and reverse (5'- GGAACATGTGGAAGTAGAGAGAAGC-3'). PCR amplification was performed in a 50µl mixture containing 5µl of 10 x pfu Turbo DNA polymerase buffer, 1µl WT cMC4R (10ng /µl), 15µl of reverse primer (20ng/µl) and 15µl of forward primer (20ng/µl), 1µl of dNTP (12.5mM) and 0.5µl of pfu turbo polymerase with the following cycling parameters: 30s at 95 °C for one cycle and 30s at 95 °C, 1min at 55 °C and 12 min at 68°C for 18 cycles followed by a final cycle of extension at 68°C for 7min. Oligonucleotide primers designed to introduce the point mutation were extended during temperature cycling of PCR by *Pfu Turbo* polymerase. Following PCR, the product was digested by Dpn I which is an endonuclease specific for methylated DNA. Therefore, methylated parental DNA was digested and mutation-containing synthesized DNA was retained. The digested product was transformed into JM109 competent cells. Cells were grown overnight on LB agar plates containing ampicillin and 4 clones were selected for growing in LB medium. Plasmid DNA was extracted with Mini Isopure™ DNA purification kit (Denville Scientific Inc, Metachen, NJ) to screen clones with expected size after digestion with EcoRI and XbaI. The nucleotide sequence of the cMC4R mutant was determined by sequencing three independent plasmids performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center. Plasmid DNA containing the cMC4R V213F of correct sequence was prepared with Maxi Isopure™

DNA purification kit (Denville Scientific Inc.)

Cell plating and transfection

For transient expression of the MC4Rs, cells were plated on gelatin coated 35-mm 6-well plates and transfected using the calcium precipitation method (134). One-microgram plasmid in 2ml media was used per 35-mm well. The transfection cocktail included 86 μ l water, 10 μ l 2.5M CaCl₂, 4 μ l of plasmid DNA, and 100 μ l of 2 \times BSS (consisting of 280 mM NaCl, 1.5 mM Na₂HPO₄, 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.

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 ¹²⁵I-NDP-MSH (50 μ l) was added to each well, with or without different concentrations of unlabeled NDP-MSH or α -MSH or β -MSH. The final concentration of unlabeled ligands ranged from 10⁻¹⁰ to 10⁻⁵ M (for α -MSH and β -MSH) or 10⁻¹¹ to 10⁻⁶ M (for NDP-MSH). After 1 h incubation, cells were washed twice with cold Hanks' balanced salt solution (Sigma-Aldrich) modified to

contain 1mg/ml BSA (referred to as HBSS/BSA). Then 100 μ l of 0.5N 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 (Bmax) and IC₅₀ values were calculated using Prism software version 4 (GraphPad Software, San Diego, CA).

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 NDP-MSH or α -MSH or β -MSH were added. The final concentrations ranged from 10⁻¹² to 10⁻⁶M (for NDP-MSH) or 10⁻¹¹ to 10⁻⁵M (for α -MSH or β -MSH). After incubation at 37 °C for 1 h, cells were then placed on ice, media aspirated, and intracellular cAMP extracted by the addition of 0.5 N perchloric acid containing 180 μ g/ml theophylline, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramine T method. Polyclonal antibody against cAMP was obtained from Strategic Biosolutions (Newark, DE). The radioimmunoassay was performed as described before (135) except that polyethylene glycol 8000 was used to precipitate the antibody-bound fraction of cAMP instead of a second antibody in the original publication (136). Maximal responses (Rmax) and EC₅₀ values were calculated using Prism software

version 4 (GraphPad Software).

Statistical analysis

Statistical calculations were performed using Prism 4.0. Maximal binding (Bmax), IC₅₀, maximal responses (Rmax), and EC₅₀ were calculated using Prism 4.0 (GraphPad, San Diego, CA, USA). Data were presented as the mean and SEM. For comparisons on maximal binding and signaling, one sample t-test was used. For comparisons on IC₅₀ and EC₅₀, an unpaired t-test was used.

RESULTS

Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R 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 hMC4R, WT cMC4R and the natural variant V213F cMC4R were transiently transfected into HEK293T cells and their ligand binding and signaling properties were analyzed. As it is widely used in MCR studies, the superpotent analogue of α -MSH, NDP-MSH, was used in these experiments. As shown in TABLE 1 and Figure 4, WT hMC4R, WT cMC4R and V213F cMC4R bound NDP-MSH with an IC_{50} of 55.59, 37.21, and 37.68 nM, respectively. Therefore these three receptors had similar binding affinities to NDP-MSH. Maximal binding (B_{max}) of WT and V213F cMC4Rs were reduced to 59% and 79% of that of WT hMC4R.

The signaling properties of these receptors were analyzed upon stimulation with NDP-MSH in HEK293T cells transiently transfected with these receptor constructs. Dose-dependent increases of intracellular cAMP were induced by NDP-MSH in all groups. As shown in TABLE 1, NDP-MSH stimulated cAMP production with EC_{50} of

0.92, 1.12, and 1.13 nM for WT hMC4R, WT cMC4R and V213F cMC4R, respectively. The maximal responses (Rmax) of WT cMC4R and V213F cMC4R were 78% and 84% of that of WT hMC4R.

Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using α -MSH as the ligand

As NDP-MSH is a superpotent long-lasting analog of the natural agonist α -MSH, different pharmacological properties of MC4R constructs might be observed when it is used. Therefore, it is necessary to measure ligand binding and signaling properties of MC4R constructs using α -MSH as the ligand. We did binding and signaling assays on these three receptors using α -MSH as the ligand. As shown in TABLE 2 and Figure 5, WT hMC4R, WT cMC4R, and V213F cMC4R had IC₅₀ of 520.33, 1687.37 and 880.20 nM when α -MSH was used as the competitor. Bmax of WT and V213F cMC4Rs were 54% and 65% of that of WT hMC4R respectively. EC₅₀ and Rmax are also shown in TABLE 2. WT cMC4R and cMC4R V213F had similar EC₅₀s. Rmax of WT and V213F cMC4Rs were reduced to 70% and 80% of that of WT hMC4R.

Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using β -MSH as the ligand

In research on body weight regulation, α -MSH has been the focus of the majority of studies because rodents lack the N-terminal cleavage site for β -MSH and are therefore deficient in β -MSH (137). However, the amino acid sequence of β -MSH is highly

conserved across species (138) and most species contain both cleavage sites for β -MSH, therefore β -MSH may have a key role in the control of energy homeostasis in humans and other species. Hence, we also investigated the ligand binding and signaling properties of these MC4R receptors using β -MSH as the ligand. As shown in TABLE 3 and Figure 6, WT hMC4R, WT and V213F cMC4Rs bound β -MSH with an IC_{50} of 883.13, 481.07, and 90.37 nM respectively. B_{max} of WT and V213F cMC4Rs were reduced to 50% and 60% of that of WT hMC4R. The three receptors showed similar signaling response efficiency with similar EC_{50} values (TABLE 3). The maximal response (R_{max}) was similar for WT and V213F cMC4Rs. The R_{max} of WT cMC4R was reduced to 60% of that of WT hMC4R (TABLE 3).

TABLE 1: NDP-MSH-Stimulated cAMP Production and Ligand Binding of WT hMC4R and cMC4R and natural variant V213F cMC4R

MC4R	n	NDP-MSH-stimulated cAMP		NDP-MSH binding	
		EC ₅₀ (nM)	Rmax (%WT)	IC ₅₀ (nM)	Bmax (%WT)
WT hMC4R	3	0.92 ± 0.01	100	55.59±5.36	100
WT cMC4R	3	1.12 ± 0.50	78±26	37.21±1.47	59±11 ^a
V213F cMC4R	3	1.13 ± 0.26	84± 38	37.68 ±2.31	79±32

^a significantly different from WT hMC4R receptor, $p < 0.05$

The data are expressed as the mean ± SEM of three independent experiments. The maximal response (Rmax) under NDP-MSH stimulation was 3740 ± 672 pmol cAMP/10⁶ cells for WT hMC4R. IC₅₀ is the concentration of NDP-MSH that is needed to cause 50% inhibition in the binding assay. EC₅₀ is the concentration of NDP-MSH that results in 50% stimulation of the maximal response.

TABLE 2: Alpha-MSH-Stimulated cAMP Production and Ligand Binding of WT hMC4R and cMC4R and natural variant V213F cMC4R

MC4R	n	Alpha-MSH-stimulated cAMP		Alpha-MSH binding	
		EC ₅₀ (nM)	Rmax (%WT)	IC ₅₀ (nM)	Bmax (%WT)
WT hMC4R	3	1.90 ± 0.28	100	520.33±196.19	100
WT cMC4R	3	4.20 ± 0.92	70±14	1687.37±281.25	54±6 ^a
V213F cMC4R	3	4.81 ± 1.76	80± 11	880.20 ± 363.64	65±30

^a significantly different from WT hMC4R receptor, $p < 0.01$

The data are expressed as the mean ± SEM of three independent experiments. The maximal response (Rmax) under Alpha-MSH stimulation was 1732 ± 553 pmol cAMP/ 10^6 cells for WT hMC4R.

TABLE 3: Beta-MSH-Stimulated cAMP Production and Ligand Binding of WT hMC4R and cMC4R and natural variant V213F cMC4R

MC4R	n	Beta-MSH-stimulated cAMP		Beta-MSH binding	
		EC ₅₀ (nM)	Rmax (%WT)	IC ₅₀ (nM)	Bmax (%WT)
WT hMC4R	3	3.19 ± 2.03	100	883.13±464.12	100
WT cMC4R	3	2.26 ± 0.75	60±19	481.07±246.87	50±5 ^a
V213F cMC4R	3	4.87 ± 2.65	93± 15	90.37 ± 44.46	60±1 ^b

^a significantly different from WT hMC4R receptor, $p < 0.01$

^b significantly different from WT hMC4R, $p < 0.001$

The data are expressed as the mean ± SEM of three independent experiments. The maximal response (Rmax) under Beta-MSH stimulation was 2094 ± 709 pmol cAMP/10⁶ cells for WT hMC4R.

Figure 4: Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC4R and cMC4R constructs and binding and signaling assays were performed as described in Materials and Methods. In binding assay, different concentration of unlabeled NDP-MSH were used to displace the binding of ^{125}I -NDP-MSH to MC4Rs on intact cells. Results shown are expressed as the mean \pm SEM from duplicate determinations within one experiment. In signaling assays, HEK293T cells transiently transfected with the indicated MC4R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean \pm SEM of triplicate determinations within one experiment. All experiments were performed three times.

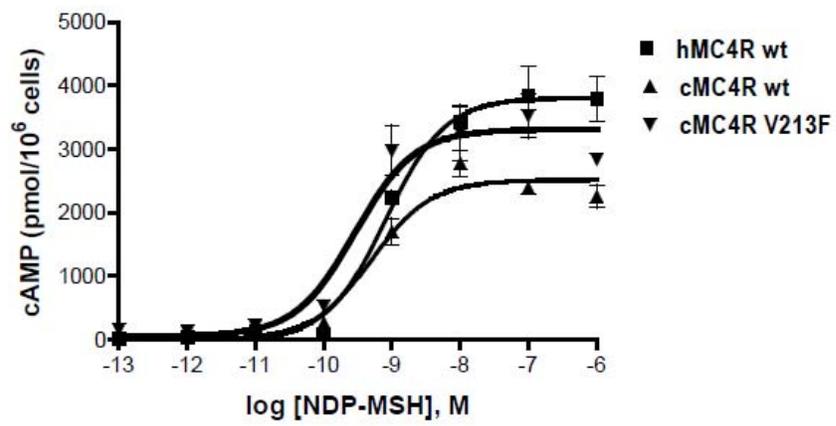
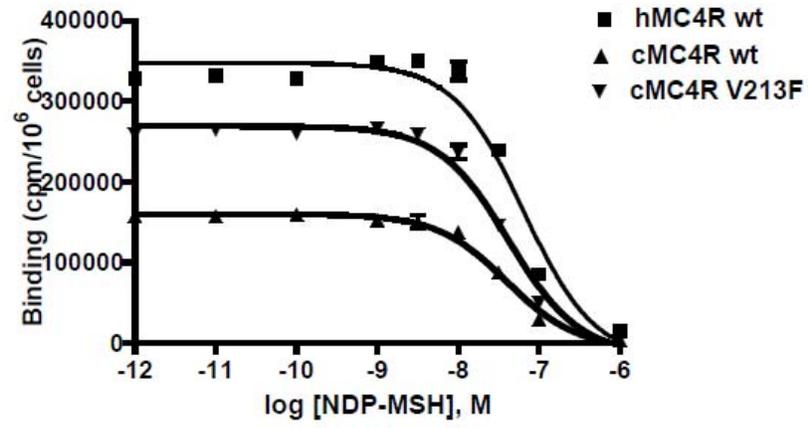


Figure 5: Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using α -MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC4R and cMC4R constructs and binding and signaling assays were performed as described in Materials and Methods. In binding assay, different concentration of unlabeled α -MSH were used to displace the binding of 125 I-NDP-MSH to MC4Rs on intact cells. Results shown are expressed as the mean \pm SEM from duplicate determinations within one experiment. In signaling assays, HEK293T cells transiently transfected with the indicated MC4R constructs were stimulated with different concentrations of α -MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean \pm SEM of triplicate determinations within one experiment. All experiments were performed three times.

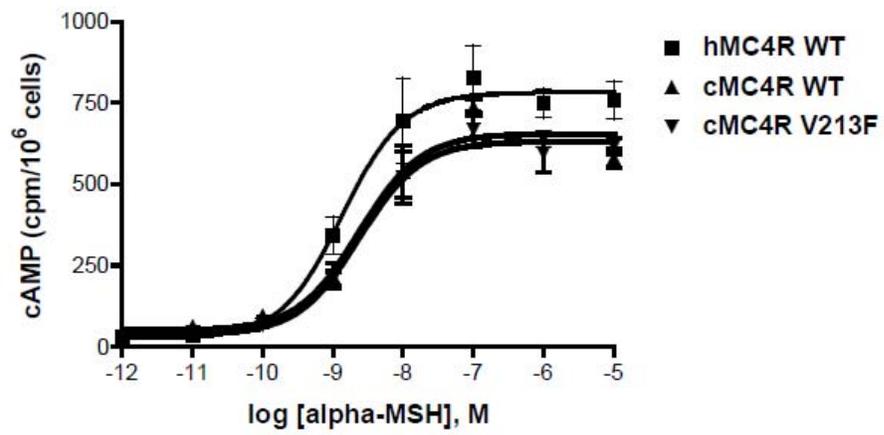
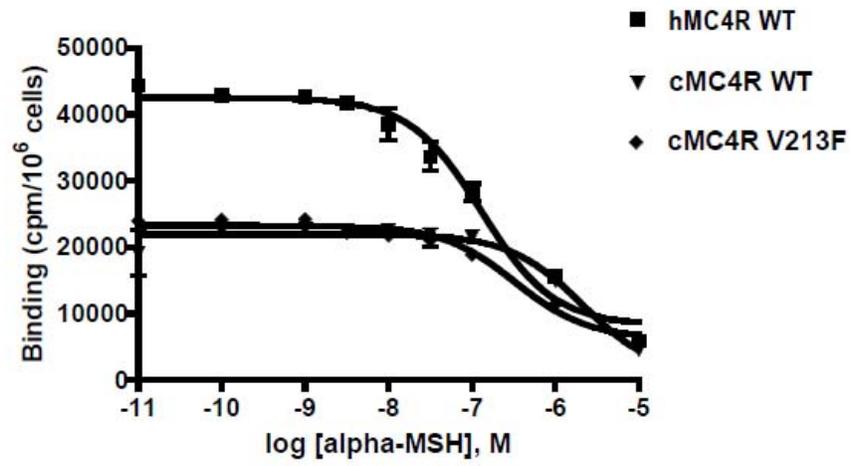
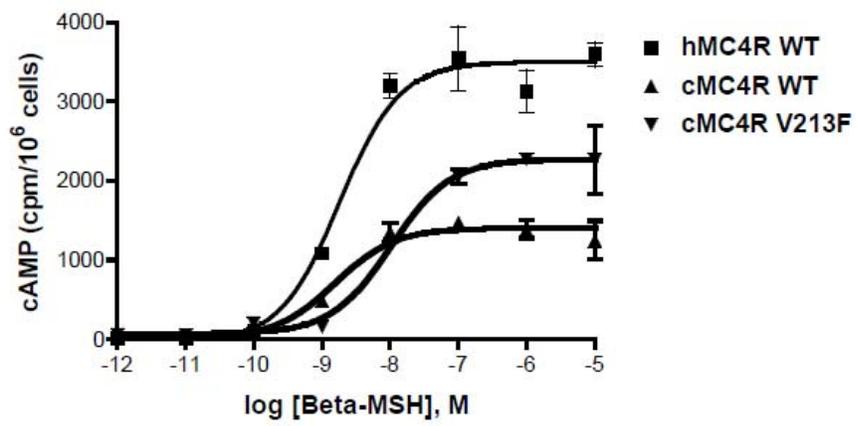
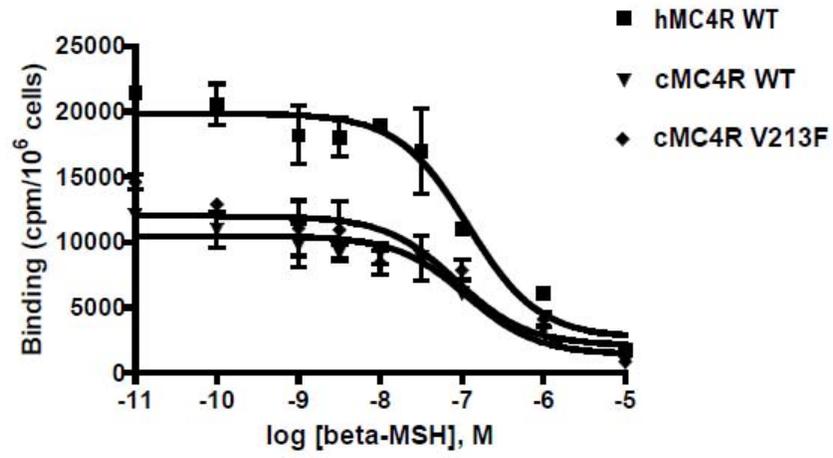


Figure 6: Ligand binding and signaling properties of the WT hMC4R, WT cMC4R and natural variant V213F cMC4R using β -MSH as the ligand. HEK293T cells were transiently transfected with the indicated hMC4R and cMC4R constructs and binding and signaling assays were performed as described in Materials and Methods. In binding assay, different concentration of unlabeled β -MSH were used to displace the binding of 125 I-NDP-MSH to MC4Rs on intact cells. Results shown are expressed as the mean \pm SEM from duplicate determinations within one experiment. In signaling assays, HEK293T cells transiently transfected with the indicated MC4R constructs were stimulated with different concentrations of β -MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean \pm SEM of triplicate determinations within one experiment. All experiments were performed three times.



DISCUSSION

Although obesity is a multifactorial disease associated with genetic, behavioral and environmental components, studies have shown that humans can become severely obese directly resulting from genetic disruption of a single element of a homeostatic system regulating energy balance (139).

Ever since leptin was discovered, researchers have made tremendous progress in elucidating the neural pathways regulating energy homeostasis during the last 15 years. The leptin-regulated melanocortin circuit was identified to be essential for energy homeostasis. Replicating rodent genetic studies, human genetic studies showed that mutations in several components of this circuit, including *LEP*, *LEPR*, *POMC*, *PC1*, and *MC4R* lead to monogenic obesity (97). Of all these genes, mutations in *MC4R* gene were the most common form of human monogenic obesity (36). In some cohorts, up to 6% of early onset morbidly obese patients carried *MC4R* mutations (36). Up to now, more than 150 mutations in *MC4R* have been identified from various patient cohorts and at least 103 residues were mutated, representing 31% of the receptor. Functional studies have been performed on some of these mutants and molecular defects were found associated with loss-of-function of these mutants. Scientists tried also to identify ways to correct

these defects so that personalized treatment can be developed for obese patients. Pharmacological chaperones were shown to be a promising approach.

Ever since human MC4R was cloned in 1993 (41), MC4R have been cloned in several other mammalian species and some nonmammalian species. Extensive studies have been done on role of porcine MC4R (pMC4R) in regulation of energy balance. Several groups have reported that MC4R genotypes are associated with backfat, growth rates and feed intake in a number of breeds and lines of pigs (43, 140-142). However, there are also studies that could not replicate this association. One functional study showed D298N pMC4R had normal function (143).

Recently, canine MC4R was cloned and a missense variant V213F was identified (40). We hypothesized that the central melanocortin system is also critical for regulating energy balance in dogs. Therefore analogs activating the central melanocortin system can be used for treating dog obesity. We investigated the pharmacological characteristics of WT cMC4R and the natural variant V213F. Three ligands were used and human MC4R was included in all experiments for comparison. We were interested in whether cMC4R has similar pharmacology as hMC4R. Therefore, ligands developed for human medicine could potentially be used for treating obese dogs. We were also interested in whether the missense variant responds normally to these ligands so that we can determine whether this mutation in cMC4R causes obesity in dogs.

In the present study, as the first step towards investigating role of MC4R in dog energy homeostasis, WT cMC4R was cloned from canine genomic DNA by Dr. Zhenchuan Fan in our lab. Primers were designed based on sequence deposited in GenBank and coding region of WT cMC4R was amplified by PCR and inserted into pcDNA 3.1. The variant V213F was generated by QuikChange™ site-directed mutagenesis kit using WT cMC4R as the template.

Three constructs were used for pharmacological analysis using NDP-MSH, α -MSH, and β -MSH as ligands. In ligand binding studies, we showed that of the three agonists tested, the rank order in terms of affinity is: NDP-MSH > α -MSH = β -MSH for hMC4R; NDP-MSH > β -MSH > α -MSH for WT cMC4R and V213F cMC4R. In signaling experiments, we showed that the rank order in terms of potency for these three agonists is: NDP-MSH > α -MSH = β -MSH for WT hMC4R, WT cMC4R and V213F cMC4R. From these results, we showed that as a commonly used analog for MCR studies, NDP-MSH is the most potent agonist compared to natural ligands β -MSH and α -MSH, therefore would be better for further *in vivo* studies to elucidate the functional importance of cMC4R in energy homeostasis in dogs.

From our results, we found that cMC4R is fully functional as it binds and signals normally when stimulated by these three agonists. Although WT cMC4R had a different rank order in terms of affinity and potency for these three agonists compared to hMC4R, these two receptors had similar EC₅₀ when stimulated by these agonists. We also found

no overt functional defects for V213F cMC4R upon the stimulation of these three agonists. Therefore it is not likely to cause obesity in dogs.

From our data, we also observed that cMC4R bound β -MSH with higher affinity and responded with higher potency compared to α -MSH. Most research on role of MC4R in regulating food intake has concentrated on the α -MSH, in part because the animal models, rodents, lack the N-terminal cleavage site for β -MSH and are therefore β -MSH deficient (137). Recently, scientists began to realize that β -MSH may play a critical role in the hypothalamic control of body weight in humans, mostly due to the identification of a missense mutation within the coding region of the POMC-derived peptide β -MSH (Y5C- β -MSH) and its association with early-onset human obesity (144). One study also found that β -MSH has significantly higher affinities than α -MSH at both human and rodent MC4R (145). Therefore β -MSH might also be an important endogenous ligand for activation of MC4R and its role in regulation of energy homeostasis. Here, our data indicated that β -MSH might be more important than α -MSH in activating cMC4R. However, these *in vitro* data should be treated with caution and should be only used as the starting point for *in vivo* studies. For instance, previous studies on pMC4R showed pMC4R responds to AgRP and SHU9119 normally *in vitro*. However, these antagonists fail to increase food intake *in vivo* (146). Therefore, further *in vivo* studies are required to verify whether β -MSH acts as a better endogenous agonist than α -MSH to activate cMC4R.

In summary, we generated natural variant V213F cMC4R using cMC4R as the template and expressed them in HEK293T cells. Pharmacological characterization using three agonists revealed that both WT cMC4R and natural variant V213F are functional in terms of binding and signaling. Compared to WT hMC4R, they bind and signal similarly upon the stimulation of each agonist. However, three receptors have different rank orders regarding affinity and potency for these three ligands. NDP-MSH was shown to be the most potent ligand and thus would be better for further *in vivo* studies than the other two agonists.

In conclusion, our results showed cMC4R is fully functional upon stimulation with three MC4R agonists *in vitro*. The natural variant cMC4R V213F does not have any functional defects and therefore is unlikely to cause obesity in dogs. As many analogs targeting MC4R are being developed for human obesity, further studies will focus on whether cMC4R bind and respond to these analogs similarly as hMC4R, thus indicating whether it is practical to use these analogs to treat dog obesity.

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