Human melanocortin-4 receptor: structure-function relationship of transmembrane domain 3 and biased signaling

by

Xiu-Lei Mo

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 3, 2013

Keywords: G protein-coupled receptor, Melanocortin-4 receptor, Transmembrane domain 3, Constitutively active mutant, Inverse agonist, Biased signaling

Approved by

Ya-Xiong Tao, Chair, Associate Professor of Physiology
Juming Zhong, Associate Professor of Veterinary Histology
Timothy D. Braden, Associate Professor of Reproductive Physiology
Vishnu Suppiramaniam, Associate Professor of Pharmacal sciences

Abstract

The melanocortin-4 receptor (MC4R) is a G protein-coupled receptor critical for maintaining energy homeostasis. Transmembrane domain 3 (TM3) of MC4R contains residues that are suggested to be essential in ligand binding and signaling. Several MC4R mutations in TM3 are associated with human obesity. To gain a better understanding of the functions of TM3, we analyzed the functions of 26 residues in TM3 using alanine-scanning mutagenesis. We showed that all 26 TM3 mutants had normal cell-surface expression. Four mutants were defective in ligand binding and signaling, and six mutants had normal ligand binding, but impaired cAMP production. L140A had increased basal cAMP level.

To further characterize the function of L140, we generated 17 additional L140 mutants. Fifteen L140 mutants had significantly decreased cell surface expression, with L140R and L140V expressed normally. Ten L140 mutants had increased basal cAMP activities. Four L140 mutants were defective in ligand-stimulated cAMP generation. Interestingly, with the extracellular signal-regulated kinase (ERK) 1/2 pathway, we showed that nine constitutively active mutants had similar levels of basal pERK1/2 as that of wild type (WT) receptor, and two signaling defective mutants had similar levels of pERK1/2 as that of WT upon agonist stimulation, different from their cAMP signaling properties, suggesting biased signaling in these mutant receptors. In summary, we identified 13 residues in TM3 that were essential for ligand binding and/or signaling. Moreover, L140 was critical for locking MC4R in an inactive

conformation and several mutants showed biased signaling in cAMP and ERK1/2 signaling pathways.

The MC4R is constitutively active in vivo and its basal activity has been suggested to be important in obesity pathogenesis. Theoretically, it is expected that constitutively active MC4R mutants are associated with a lean phenotype, even anorexia nervosa. However, there are several constitutively active MC4R mutants identified from obese patients, and the mechanism of how these mutations associate with obesity still needs further investigation.

In addition to the conventional Gs-stimulated adenylyl cyclase pathway, it has been recently demonstrated that MC4R also activates mitogen-activated protein kinases, ERK1/2. Herein, we investigated the potential of four MC4R ligands that are inverse agonists at the Gs-cAMP signaling pathway, including agouti-related peptide (AgRP), MCL0020, Ipsen 5i, and ML00253764, to regulate ERK1/2 activation (pERK1/2) in wild type and six naturally occurring constitutively active mutant (CAM) MC4Rs. We showed that these four inverse agonists acted as agonists for the ERK1/2 signaling cascade in wild type and CAM MC4Rs. Three mutants (P230L, L250Q and F280L) had significantly increased pERK1/2 level upon stimulation with all four inverse agonists, with maximal induction ranging from 1.6 to 4.2 fold. D146N had significantly increased pERK1/2 level upon stimulation with AgRP, MCL0020 or ML00253764, but not Ipsen 5i. The pERK1/2 levels of H76R and S127L were significantly increased only upon stimulation with AgRP or MCL0020. In summary, our studies demonstrated for the first time that MC4R inverse agonists at the Gs-cAMP pathway could serve as agonists in the MAPK pathway. These results suggested that there were multiple activation states of MC4R with ligand-specific and/or mutant-specific conformations capable of differentially coupling the MC4R to distinct signaling pathways.

Acknowledgments

I would like to thank my mentor, Dr. Ya-Xiong Tao, for his great support, constant encouragement and endless enthusiasm even in the midst of negative results, and his willingness to place his students' wellbeing above his own. I could not have this done without him. I'm also indebted to my advisory committee, Drs. Juming Zhong, Timothy D. Braden and Vishnu Suppiramaniam for sharing their wisdom, advice, and expertise throughout the years. I appreciate Dr. Douglas White for working as my outside reader and providing valuable guidance. Last, but not the least, I would like to acknowledge my lab fellows and alumni, Hui Huang, Zhao Yang, Drs. Menghong Dai, Rui Yang, Zhiqiang Wang and Fan Yang for their valuable contributions and discussions to these research projects. I'm also thankful for the support from my wife Jing Li, my parents and parent-in-law. Without their unselfish support and encouragement, I would not be able to finish my graduate study.

Finally, my warm thanks go to Dr. Edward Morrison for his great effort on helping me transferring to Dr. Tao's Lab, to Dr. Frank Bartol for the precious five-year financial support for my PhD study from his graduate study office. I would also like to thank the financial support from National Institutes of Health grant R15DK077213, American Diabetes Association grant 1-12-BS212, Auburn University Intramural Grant Program and Interdisciplinary Grant of College of Veterinary Medicine, which make my experiments possible.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AC	Adenylate Cyclase
ACTH	Adrenocorticotrophic Hormone
AgRP	Agouti-related Protein
ARC	Arcuate Nucleus
BAT	Brown Adipose Tissue
Bmax	Apparent maximal binding
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CART	Cocaine and Amphetamine Regulated Transcript
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle's Medium
DMH	Dorsomedial Hypothalamus
EGFP	Enhanced Green Fluorescent Protein
ERK	Extracellular Signal-regulated Kinase
FOS-IR	FOS-immunoreactivity
GABA	γ-Aminobutyric Acid
GPCR	G Protein-coupled Receptor
GWAS	Genome-wide Association Study
HEK	Human Embryonic Kidney

IGF-1	Insulin-like Growth Factor 1
JAK	Janus Kinase
MAPK	Mitogen-activated Protein Kinase
MC3R	Melanocortin-3 Receptor
MC4R	Melanocortin-4 Receptor
MCR	Melanocortin Receptor
MSH	Melanocyte Stimulating Hormone
NHANES	National Health and Nutrition Examination Survey
NPY	Nuropeptide Y
ob	Obese
ObR	Leptin Receptor
PBS-IH	PBS for Immunohistochemistry
PH	Posterior Hypothalamic
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
POMC	Proopiomelanocortin
PTP1B	Protein-tyrosin Phosphatase 1B
PVN	Paraventricular Nucleus
Rmax	Maximal Response
SCN	Suprachiasmatic Hypothalamic Nuclei
SNP	Single Nucleotide Polymorphism
SOCS3	Suppressor of Cytokine Signaling 3
STAT	Signal Transducer and Activator of Transcription

VMH	Ventromedial Hypothalamic Nucleus
VMN	Ventromedial
WHO	World Health Organization
WT	Wild Type

Chapter 1

Introduction

Obesity, by definition, results from calorie intake in excess of energy expenditure. Clinically, people are considered obese when their body mass index (BMI) is more than 30 kg/m^2. BMI is a measurement obtained by dividing a person's body weight in kilograms by square of the person's height in meters. Obesity is rapidly becoming a critical health problem in the United States, with nearly 70% of adults considered overweight (BMI $\geq$ 25 kg/m^2) or obese compared with less than 25% forty years ago (Flegal, et al. 2012). In fact, obesity and associated comorbidities have reached epidemic proportions in the U.S and other developed countries. Although environmental and lifestyle factors contribute to obesity pathogenesis, there is mounting evidence demonstrating the importance of genetic contributions in the pathogenesis of obesity. Therefore, there is enormous interest in understanding the molecular mechanisms underlying the regulation of energy homeostasis, and the defects that result in obesity.

The melanocortin-4 receptor (MC4R) is a member of family A, rhodopsin like G protein-couple receptor, which has been shown to be a critical regulator of energy homeostasis, regulating both energy intake and expenditure (Tao 2005, 2010). Human genetic studies have demonstrated that defects in *MC4R* are the most common monogenic form of obesity, characterized by its early-onset and severity (Farooqi, et al. 2003; Lindgren, et al. 2008; Loos, et al. 2008). About 170 *MC4R* mutations, including nonsense, missense, frameshift, and inframe deletions, have been identified from obese patient cohorts of different ethnic origins (Hinney, et al. 2013; Tao 2005, 2010).

This chapter presents an overview of the obesity epidemic and current treatments. The regulation of energy homeostasis by central nervous system and gastrointestinal hormone are discussed. Finally, the importance of MC4R in regulating energy homeostasis, including its cloning and tissue distribution, naturally occurring mutations and molecular mechanism are highlighted.

1.1 Obesity

1.1.1 The epidemic of obesity

Obesity, by definition, is a medical condition in which a person's BMI is more than 30 kg/m^2. Obesity cannot be simply considered as a cosmetic problem (Friedman 2004), since it is closely related to various diseases, including type 2 diabetes mellitus, dyslipidemia, hypertension, arteriosclerosis, cardiovascular disease, polycystic ovarian syndrome, and certain types of cancer (Desvergne, et al. 2004). Therefore obesity should be considered as a disease (Allison, et al. 2008). Indeed, overweight (BMI between 25 and 29.9) and obesity now ranks as the fifth leading global cause of death.

Obesity has become an epidemic in developed countries. In the United States, nearly 70% of adults are considered as overweight or obese compared with less than 25% forty years ago (Flegal et al. 2012). According to the report from World Health Organization, globally, more than 1 billion adults are overweight and at least 300 million adults are clinically obese. According to this report (http://www.who.int/dietphysicalactivity/media/en/gsfs_obesity.pdf, accessed 16 May 2013), the obesity epidemic is not only restricted to developed countries, but also is increasing at an alarming rate in developing countries. For example, one survey shows that the prevalence of overweight, general obesity, and abdominal obesity among Chinese adults has increased significantly from 1993 to 2009 (Xi, et al. 2012).

There has been a dramatic increase in childhood obesity over the past decades. Therefore, childhood obesity is also becoming a significant public health crisis. It was estimated that 43 million children under age of 5 years were overweight in 2010, with a quarter of which are obese (Lobstein, et al. 2004). These overweight children will have more risk to have obesity comorbidities such as type 2 diabetes and heart disease during or even before early adulthood

(Lobstein et al. 2004). Obesity has a direct effect on longevity. It is estimated that people with severe obesity (BMI >45 kg/m^2) will have the maximum life loss of 13 years, representing a 22% reduction in life expectancy (Fontaine, et al. 2003).

In addition to the health problem, obesity is associated with significant economic burden. It has been reported that, in the United States, about 21% of adult medical expenditures are imposed from the health care resources associated with obesity (Finkelstein, et al. 2004). In 2006, it is reported that obese people had medical spending that was $1,429 greater than spending for normal-weight individual. In 2008, obesity associated medical costs are $147 billion per year in the United States according to the National Health Expenditure Accounts estimates (Finkelstein, et al. 2008). The majority of these medical expenditures are financed by taxpayers (Finkelstein et al. 2008), with roughly half financed by Medicare and Medicaid (Finkelstein et al. 2008). The lifetime costs of obesity are still positive even taking into account the shortened lifespan for the obese (Finkelstein et al. 2008).

1.1.2 Current treatment for obesity

The heart of the obesity epidemic is largely due to that few effective therapeutic options are available to the obese patients, despite that considerable scientific progress on the physiological system regulating energy homeostasis has been made during past decades. Current treatments for obesity includes lifestyle intervention, pharmacotherapy and bariatric surgery for the morbidly obese patients, with BMI of 40 kg/m^2 or more. Traditionally, lifestyle intervention, such as increased exercise, dieting, and behavioral counseling, can succeed in short-term for the majority of obese patients. However, the drawback to this option is that it does not produce sustained weight loss. The failure of long-term effects of lifestyle intervention reflects the

strongly conserved nature of the biological defense of body fat mass, accounting for the largest obstacle in preventing weight regain (Guyenet and Schwartz 2012; Woods, et al. 2000).

Pharmacotherapy, such as sibutramine (a serotonin–norepinephrine reuptake inhibitor that reduces appetite) and orlistat (a triacylglycerol lipase inhibitor that reduces dietary fat absorption), was used as an adjunct to facilitate weight loss and prevent weight regain for some patients under lifestyle interventions (Eckel 2008). However, current pharmacotherapies have inadequate efficiency, for example, it is reported that the average weight loss for sibutramine and orlistat is 4.45 kg and 2.89 kg over 12 months treatment, respectively. Another obstacle of using pharmacotherapy is attributing to the adverse effect and imperfect safety record (Nguyen, et al. 2012). Indeed, sibutramine was withdrawn in Europe and North America in 2010 because it has been shown that patients undergoing long-term sibutramine treatment are more likely to have increased risk of nonfatal myocardial infarction and nonfatal stroke (James, et al. 2010).

Bariatric surgeries, such as Roux-en-Y gastric bypass, vertical sleeve gastrectomy and adjustable gastric banding, have increased in popularity in the treatment of obesity due to its superior ability to produce effective long-term weight loss in both magnitude and durability, resulting in an average of 61.2% of weight loss (Buchwald, et al. 2004; Stefater, et al. 2012). It has also been reported that individuals receiving bariatric surgery have reduced incidence of diabetes (Buchwald et al. 2004), heart disease (Pontiroli and Morabito 2011), and cancer (Ashrafian, et al. 2011), independent of weight loss (Buchwald et al. 2004; Chambers, et al. 2011; Stefater et al. 2012). However, only a small fraction of obese population undergoes these procedures due to the significant risks during and after operation, and the high cost (DeMaria 2007; Nguyen et al. 2012; Terranova, et al. 2012). It is reported that the overall rate of death for bariatric surgery was 0.3%, and major complications occurred in 4.1% of the patients even

operations are performed by the best surgeons (Flum, et al. 2009). Several mechanisms, including gastric volume restriction, delayed gastric emptying, increased energy expenditure and central nervous system (CNS) control, have been proposed for the metabolic benefits of bariatric surgery to help the choice of better bariatric procedure for individual patients and to develop more efficient and less invasive procedures (Stefater et al. 2012).

1.1.3 Regulation of energy homeostasis by the central nervous system

Energy homeostasis refers to the balance between energy intake, including the intake through the form of food and drinks, and energy expenditure, including resting energy expenditure (basal metabolism) and physical activity over a prolonged period in adult animals. In normal adult animals, the energy homeostasis is precisely maintained, therefore, there is no significant change in body weight during adulthood (Guyenet and Schwartz 2012).

An 'adiposity negative-feedback' system, formed by the integration of several peripheral signals and central nervous system, has been proposed more than 50 years ago to clarify the physiological mechanism of energy homeostasis (Kennedy 1953). The hypothalamus plays a critical role in the 'adiposity negative-feedback' system. It receives and integrates multiple neuronal and hormonal signals about energy homeostasis through several nuclei, particularly the ventro medial, paraventricular (PVN) and arcuate nuclei (ARC). The ARC contains two subset of neurons, one of which is orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neuron, and another is anorexigenic proopiomelanocotin (POMC)/cocaine- and amphetamine-related transcript (CART) neuron (Cone 2005; Schwartz, et al. 2000; Tao 2010).

Four criteria have been proposed for being a negative-feedback signal. These criteria are (1) that it crosses the blood brain barrier; (2) that its circulation level is proportional to the adiposity level; (3) that it exerts weight-loss function through acting on neuronal systems

involved in energy homeostasis; and (4) that inhibition of these neuronal actions results in obesity. Some hormones (such as glucocorticoids), cytokines (such as interleukin-6, tumour necrosis factor-α) and nutrients (such as free fatty acids and glucose), fulfill some criteria, however, leptin and insulin are the only two hormones that satisfy all of these criteria (Morton, et al. 2006; Niswender and Schwartz 2003; Schwartz et al. 2000; Woods et al. 2000).

The discovery of leptin by Douglas Coleman and Jeffrey Friedman was a landmark event in modern biomedical research, for which they received the Albert Lasker Basic Medical Research Award in 2010 (Flier and Maratos-Flier 2010). Leptin is an adipose tissue derived hormone produced by adipocytes (Zhang, et al. 1994) that informs the brain about the status of energy stores in the organism through the 'adiposity negative-feedback system'. In mammals, leptin decreases food intake through inhibiting the orexigenic NPY/AgRP and activating POMC/CART neurons (Cone 2005; Schwartz et al. 2000; Tao 2010). These two types of neurons express leptin receptors (Cheung, et al. 1997). Leptin, by binding to the leptin receptors in POMC/CART neurons, stimulates ARC POMC gene expression (Schwartz, et al. 1997; Thornton, et al. 1997). POMC-derived α-melanocyte-stimulating hormone (α-MSH) is an agonist of the MC4R, whereas AgRP is an antagonist of the MC4R. MC4R signaling is an important downstream mediator of leptin's effects on energy homeostasis (Seeley, et al. 1997). Deficiencies in leptin signaling are responsible for the profound genetic obesity present in rodent obesity models, including *ob/ob* mice (leptin deficiency) (Zhang et al. 1994), *db/db* mice (leptin receptor deficiency) (Chen, et al. 1996), and Zucker *fa/fa* rat (leptin receptor deficiency) (Phillips, et al. 1996).

Since leptin plays critical role in regulating energy homeostasis, initially it was highly expected that leptin would have significant therapeutic potential towards curing obesity.

However, disappointing efficacy in clinical trials (Heymsfield, et al. 1999) diminished the initial passion. Indeed, human obesity is associated with leptin resistance rather than leptin deficiency (Caro, et al. 1996; Maffei, et al. 1995; Rohner-Jeanrenaud and Jeanrenaud 1996). Several mechanisms for leptin resistance have been proposed, including impaired transport of leptin across the blood–brain barrier, endoplasmic reticulum stress, inflammation, perturbations in developmental programming, and leptin-signaling defects (Myers, et al. 2008; St-Pierre and Tremblay 2012). The exact mechanism of leptin resistance are not clarified yet, and relevant studies are extremely active at the cellular, physiological, and behavioral levels (Myers, et al. 2010; St-Pierre and Tremblay 2012).

There are several nuclei in the hypothalamus, including the ARC, the PVN, the dorsomedial nucleus, and the preoptic area, that are critical for regulating energy homeostasis. These nuclei perform different functions. ARC neurons respond to various hormones and nutrients, including adiposity signals (leptin and insulin), gastrointestinal (GI) hormones (such as ghrelin), gonadal steroids, and glucose (Cone, et al. 2001). There are transport mechanisms that carry these molecules through the blood–brain barrier. In addition, some neurons in the ARC have cell bodies or projections in the median eminence, where there is no blood–brain barrier, therefore capable of sensing nutrient changes in the blood. Neurons in other parts of the hypothalamus, such as the PVN and the dorsomedial nucleus are also critical for regulating leptin-mediated energy homeostasis (Myers, et al. 2009). The preoptic area controls energy expenditure and core body temperature regulation (Nakamura and Morrison 2008).

In addition to the hypothalamus, the midbrain (Morley 1987) and the brainstem are also involved in the central regulation of energy homeostasis. For example, the ventral tegmental area and striatum, which are the main components of the mesolimbic dopamine system, play essential

roles in mediating the brain reward circuit for the preference of palatable food (Myers et al. 2009). These reward circuits interacting with the circuits controlling energy homeostasis to generate food intake signals (Guyenet and Schwartz 2012). The brainstem is also critical in regulating energy homeostasis. The dorsal vagal complex comprises: (1) the area postrema (AP), a circumventricular organ lacking a functional blood–brain barrier therefore capable of sensing chemical changes in the blood; (2) the nucleus of the tractus solitarius (NTS), a center that integrates GI distension, mechanosensory, and other inputs from the viscera; and (3) the dorsal motor nucleus of the vagus (DVN), a center that integrates motor and secretory drive to the viscera. NTS receives and integrates vagal afferent satiation, blood-borne energy status signals (such as glucose and fatty acid levels), and GI peptides secreted during a meal, and send signals to the DVN to maintain energy balance (Grill and Hayes 2009). Several peptides, including those secreted in response to meals, such as amylin, are sensed at AP to terminate the meal, generating anorexic responses. Administration of melanin-concentrating hormone into the fourth ventricle targeting the NTS decreases body temperature without changing physical activity and food intake, suggesting that melanin-concentrating hormone inhibits energy expenditure through the hindbrain (Zheng, et al. 2005a).

Neurons in the NTS also integrate leptin and melanocortin signals to control food intake, functioning through elements of leptinergic and melanocortinergic signaling systems (Schwartz 2006). For example, it has been demonstrated that POMC is not only produced in the ARC of the hypothalamus, but also highly expressed in the NTS in the hindbrain (Bronstein, et al. 1992; Palkovits, et al. 1987). The MC4R is also expressed abundantly in the DVN (Mountjoy, et al. 1994), and exerts similar functions in decreasing food intake and increasing energy expenditure upon activation as that in the hypothalamus (Brown, et al. 1998; Grill, et al. 1998; Skibicka and

Grill 2008, 2009; Williams, et al. 2000). Injection of MC4R ligands into the fourth ventricle near the dorsal vagal complex affects meal size, but not meal frequency (Zheng, et al. 2005b). Brainstem melanocortin system is also involved in mediating cholecystokinin (CCK) inhibition of food intake (Fan, et al. 2004).

1.1.4 Regulation of energy homeostasis by GI peptides

In addition to the adiposity signal, leptin and insulin, the GI tract also sends nutritional signals to the brain to control energy homeostasis. Important satiety hormones produced in the GI tract include ghrelin (Li, et al. 2013), obestatin (Zhang, et al. 2013), CCK (Sayegh 2013), peptide tyrosine tyrosine (PYY) (Zac-Varghese, et al. 2011).

Ghrelin is a 28 amino acid peptide produced mainly in the fundus of the stomach. Ghrelin functions as a key modulator of energy homeostasis, acting via the ghrelin receptor to regulate appetite, select fuel substrate, modulate body weight, and maintain glucose/lipid homeostasis (Li et al. 2013). Ghrelin may also sends the peripheral energy storage signal to the brain through the ghrelin receptor, leading to changes in food intake and adiposity (Arvat, et al. 2000; Wren, et al. 2000). Therefore, ghrelin plays an important role in enabling adaptation to changes in nutritional status by controlling metabolic parameters such as energy intake and expenditure.

Ghrelin is the only GI hormone known to stimulate food intake. The orexigenic effect of ghrelin is mainly attributed to the activation of its receptor localized in the hypothalamic nucleus. ICV administration of ghrelin significantly augments feeding in rodents and increases body weight gain. Ghrelin antibody and ghrelin receptor antagonist apparently suppresses feeding behavior. Within the arcuate nucleus, the ghrelin receptor is mainly expressed on almost all NPY /AgRP neurons (Willesen, et al. 1999). Both antibodies against NPY or AgRP, and antagonists of the Y1 NPY receptor have been reported to abolish ghrelin-induced feeding (Toshinai, et al.

2003). In addition, the stimulatory effects of ghrelin on these orexigenic neurons are complemented by reduced POMC neuronal activity via inhibitory β-amino butyric acidergic inputs from NPY/AgRP neurons (Cowley, et al. 2003). Thus, ghrelin may stimulate the food intake not only via activating orexigenic NPY/ AgRP neurons, but also by simultaneously suppressing anorexigenic POMC.

Variations in ghrelin receptor genes have been linked to obesity, disordered eating behavior, hypertriglyceridemia, and insulin resistance. Five single nucleotide polymorphisms (SNPs) has been found to be associated with BMI in 1095 individuals from 178 pedigrees with multiple obese members (Baessler, et al. 2005). F279L and A204E mutations are associated with short stature and obesity, respectively (Wang, et al. 2004). Four naturally occurring missense mutations have been documented in either the National Center for Biotechnology Information SNP database or the literature (Gjesing, et al. 2010; Holst and Schwartz 2006). Two SNPs (rs490683 and rs9819506) in the promoter region of the human ghrelin receptor gene are associated with abnormalities in body weight and BMI (Mager, et al. 2008). Even though common polymorphisms of ghrelin receptor genes might not be strong contributors to polygenic obesity, they definitely modulate obesity-related traits (insulin secretion/resistance) and eating behavior.

Based on bioinformatic prediction, Zhang and colleagues discovered that, in addition to ghrelin, the ghrelin gene encoded another secreted and bioactive peptide. The precursor is encoded by the gene *GHRL*, which is located on chromosome 3, band p25–26.2 and is cleaved by protease into peptide segments. Zhang and colleagues isolated this new 23-amino acid hormone from rat stomach, and it showed actions opposite to ghrelin on food intake, body weight, and gastric emptying and named it “obestatin” (Zhang, et al. 2005).

In the first report of obestatin, Zhang et al. showed that the intraperitoneal injection and intracerebroventricular treatment of obestatin in adult male mice have decreased food intake (Zhang et al. 2005). Then many groups repeated its anorectic effects in a number of experimental paradigms after both peripheral and central administration of obestatin. On the other hand, several researchers were not able to confirm the effect of obestatin on food intake (Gourcerol, et al. 2007a; Kobelt, et al. 2008; Nogueiras, et al. 2007; Seoane, et al. 2006; Unniappan, et al. 2008), thus the role of obestatin as an anorectic hormone was challenged (Gourcerol, et al. 2007b). The reason for the discrepancies is unclear. The different experimental paradigms such as different animal species, methods of administration, and the duration of drug treatment, might have influenced the outcome of the effect of obestatin on food intake in some way. Also, a recent study questioning the purity of synthetic peptides including obestatin might shed a clue on these discrepancies (De Spiegeleer, et al. 2008). In addition to the effect on food intake, obestatin may regulate other physiological processes, including modulating gastrointestinal motility, GH secretion and lipid metabolism.

Accumulating publications have confirmed the role of obestatin in regulating energy homeostasis and its association with obesity, among many other physiological functions. Its receptor(s) involved in these functions remains, however, controversial. Two receptors for obestatin have been proposed: GPR39 and GLP-1R (Granata, et al. 2008; Zhang et al. 2005). Both G protein-coupled receptors (GPCRs) are found to be correlated with the function of obestatin, especially metabolism. GPR39 knockout mice showed accelerated gastric emptying, increased volume of gastric secretion, higher mature body weight and body fat composition, and increased cholesterol levels compared with wild type (Moechars, et al. 2006), supporting the role of obestatin-GPR39 system in regulating energy homeostasis.

CCK is another intestinal peptide hormone regulating energy homeostasis. The main source of peripheral CCK is the endocrine I-cells of the upper small intestine (Buffa, et al. 1976; Larsson and Rehfeld 1978; Polak, et al. 1975). In addition, CCK can be found in enteric neurons and the central nervous system.

There are three possibilities that could explain the mode of action for CCK in regulating energy homeostasis: endocrine, paracrine, and neurocrine (Sayegh 2013). The endocrine route assumes that the I-cells secrete CCK into the blood stream, primarily the circulation of the upper GI tract (the celiac and cranial mesenteric arteries), and activates the DMV, NTS, and AP in the hindbrain through circumventricular organs that lack the blood-brain barrier. The result of this activation is decreased food intake. The paracrine route suggests that CCK secreted by I-cells travels through the interstitial space and activates neighboring vagal afferents, which intensely supply the duodenum and terminate in the DVC to reduce food intake. The neurocrine route suggests that CCK is secreted by neurons in the gut and activates nearby vagal afferents, which in turn activate the DVC to reduce food intake. This route requires intense investigation, as there is not enough data to support or reject it.

PYY is produced by the enteroendocrine L cells in the distal gut that decreases food intake (Zac-Varghese et al. 2011). Two forms of PYY, PYY1-36 and PYY3-36, are released postprandially, with the latter form consisting of the predominant form. PYY3-36 is generated by cleaving PYY1-36 of the tyrosine proline residues at the N-terminus by dipeptidyl peptidase IV, an aminopeptidase that cleaves dipeptides from the N-terminus of proteins containing an alanine or proline at the second position. Postprandial plasma PYY levels are proportional to the amounts of calories consumed. These PYY molecules signal through binding to the G protein-coupled NPY receptors (Y1, Y2, Y4, Y5, and Y6) (Michel, et al. 1998). Of the five subtypes of

NPY receptors, PYY1–36 bind to Y1 and Y5 receptors, whereas PYY3–36 binds selectively to Y2 receptor (Batterham, et al. 2002).

1.2 Melanocortin-4 receptor

1.2.1 Melanocortin system

The melanocortin system consists of several agonists, two antagonists, and five receptors. The agonists, including α-MSH, β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH), are all derived from POMC by posttranslational cleavages (Bertagna 1994; Smith and Funder 1988). In the anterior pituitary gland, POMC is cleaved into ACTH and other peptides by prohormone convertase (PC) 1. In hypothalamus, brainstem, skin as well as hair follicles, POMC is further cleaved by PC2 into MSH. The melanocortin system is unique in having two endogenous antagonists, Agouti and Agouti-related peptide (AgRP). To the best of our knowledge, in other GPCR system, there is no other endogenous antagonists that have been identified.

Five melanocortin receptors (MCRs), numbered as MC1R to MC5R based on the sequence of their cloning, mediate the diverse physiological functions of their ligands. The MC1R primarily expressed in skin and hair follicles regulates pigmentation. The MC2R is the classical ACTH receptor expressed in the adrenal cortex that regulates adrenal steroidogenesis and cell proliferation. The role of the MC3R in regulating food intake is controversial. Data from knockout animals revealed that *Mc3r* knockout results in increased feed efficiency and adiposity (Butler, et al. 2000; Chen, et al. 2000). The MC4R are expressed primarily in the central nervous system, and mounting evidence support its essential role in regulating energy homeostasis. Study of MC5R knockout mice suggests that the MC5R is involved in regulating exocrine gland secretions (Chen, et al. 1997).

1.2.2 Molecular cloning and tissue distribution of MC4R

The first cloning of human (h) MC4R was obtained independently by the groups of Gantz and Cone (Gantz, et al. 1993; Mountjoy et al. 1994) through degenerate PCR and homology screening. The hMC4R is a protein of 332 amino acids encoded by an intronless gene with an open reading frame of 999 bp. Among the MCRs, the MC4R share the highest homology with the MC3R, with 58% identity and 76% similarity. The human *MC4R* gene is located at chromosome 18q21.3 (Gantz et al. 1993; Magenis, et al. 1994).

The MC4R is a member of the family A, rhodopsin like GPCRs with seven transmembrane domains (TMs) connected by alternating extracellular loops (ELs) and intracellular loops, and the extracellular N terminus, and the intracellular C terminus. The MC4R, as well as other MCRs, unlike other family A GPCRs, have some unique features, such as (1) the missing disulfide bond linking the top of TM3 and EL2 which is highly conserved in other family A GPCRs (Tarnow, et al. 2003), (2) the short intracellular loops and ELs, especially EL2, making MC4R one of the shortest members in the GPCR superfamily, and (3) the substitution of the highly conserved Pro in TM5 and Asn in TM7 (in the NPxxY motif, N7.49) in family A GPCRs by a Met and an Asp, respectively, in the MC4R (Tao 2010).

In the original cloning article, Gantz et al. (Gantz et al. 1993) showed that the hMC4R is primarily expressed in brain. They further showed that the MC4R mRNA is expressed in regions of the thalamus, hypothalamus, and hippocampus using in situ hybridization in mouse brain sections. In the hippocampus, only CA1 and CA2 region, but not CA3 and CA4 regions, was shown to have extensive labeling of the MC4R. It has been shown that other regions, including dentate gyrus, cortex, and amygdala, also have the MC4R mRNA expression (Gantz et al. 1993). It was shown that the MC4R mRNA is also widely expressed in adult rat brain, including cortex,

thalamus, hypothalamus, brainstem, and spinal cord (Kishi, et al. 2003; Mountjoy et al. 1994; van der Kraan, et al. 1999). In rat hypothalamus, it is expressed highly in PVN, including both parvicellular and magnocellular neurons. In addition to neurons, astrocytes were also reported to express the MC4R (Caruso, et al. 2007; Selkirk, et al. 2007).

1.2.3 MC4R in energy homeostasis

The critical importance of MC4R in mediating energy homeostasis was not realized until the molecular cloning of the MCRs, although there are earlier studies showing that ICV administration of α-MSH and ACTH decreases food intake in rats (Poggioli, et al. 1986; Vergoni, et al. 1986). In 1997, several studies established the essential role of the MC4R in regulating energy homeostasis.

Cone and colleagues showed that ICV administration of melanotan II (MTII), a superpotent and stable cyclic analog of α-MSH, inhibits the hyperphagia in four different obese mouse models (Fan, et al. 1997). This inhibition was attenuated by coadministration of SHU9119, a high-affinity cyclic antagonist of MC3R and MC4R (Fan et al. 1997). Administration of SHU9119 alone increases feeding (Fan et al. 1997), even in diet-induced obesity, therefore exacerbating the obesity (Hagan, et al. 1999). These results indicated that the disruption of hypothalamic melanocortinergic neurons might be a potential mechanism responsible for the obesity in mice (Fan et al. 1997). More potent effect on alterations in food intake was achieved by direct injection of these compounds into the PVN, which express very high levels of the MC4R (Mountjoy et al. 1994), suggesting that PVN is the primary sites of action in melanocortin regulation of feeding behavior (Giraudo, et al. 1998). The use of more specific MC4R antagonists such as HS014 and HS024 provided further evidence that MC4R is important in

regulating food intake (Conde-Frieboes, et al. 2012; Kask, et al. 1998a; Kask, et al. 1998b; Kask, et al. 1998c; Skuladottir, et al. 1999).

The report of the *Mc4r* knockout mouse model published in 1997 is a landmark study providing the definitive evidence that the MC4R is critical for regulating energy homeostasis in mice (Huszar, et al. 1997). The homozygous knockout mice are severely obese. The heterozygous mice have intermediate body weights compared with the wild-type (WT) littermates and homozygous mice, suggesting that there is a gene dosage effect (Huszar et al. 1997). *Mc4r* knockout mice have also been demonstrated to be hyperphagic, hyperglycemic, hyperinsulinemic, and have delayed meal termination and reduced sensitivity to CCK (Blevins, et al. 2009; Fan et al. 2004). In addition to hyperphagia, the *Mc4r* knockout mice was also shown to have decreased energy expenditure, accounting for 40% of the effect of the MC4R on energy homeostasis (Balthasar, et al. 2005). Recently, a study showed that the ability of regulating food intake is contributed to the MC4R expressed in the PVN and/or amygdala, whereas the effect of controlling energy expenditure is attributed by the MC4R expressed in other neurons (Balthasar et al. 2005).

Another important breakthrough, establishing the importance of MC4R in regulating energy homeostasis, is the cloning of AgRP gene by several groups independently (Ollmann, et al. 1997) (Graham, et al. 1997) (Fong, et al. 1997). AgRP was initially considered as a neutral antagonist for the MC4R. However, subsequent studies demonstrated that it is indeed an inverse agonist for human and rodent MC4Rs decreasing basal signaling of WT (Chai, et al. 2003; Haskell-Luevano and Monck 2001; Nijenhuis, et al. 2001) or constitutively active mutant MC4Rs (Tao, et al. 2010).

It has been shown that AgRP is orexigenic (Ilnytska and Argyropoulos 2008). The inhibitory effect of α-MSH on food intake was blocked after the ICV injection of AgRP, and food intake increases for at least 24 h (Rossi, et al. 1998). Bloom and colleagues (Kim, et al. 2000) further showed that the PVN, the dorsomedial nucleus, and the medial preoptic area were the areas with the greatest response to AgRP, whereas no changes in feeding were seen after the administration of AgRP into the ARC and lateral hypothalamic area (Kim et al. 2000). AgRP also blocks the inhibitory effect of leptin on food intake and body weight in a dose-dependent manner (Ebihara, et al. 1999).

Agrp knockout mice was originally reported to be normal in food intake, body weight, or susceptibility to diet-induced obesity (Qian, et al. 2002). However, a subsequent knockout study on mice with a different genetic background showed that 6-month-old homozygous *Agrp* knockout mice have decreased body weights, due to increased metabolic rate and motor activity (Wortley, et al. 2005). Furthermore, *Agrp* knockout mice have increased circulating thyroid hormones, increased uncoupling protein 1 expression in brown adipose tissue, and longer life span when fed with a high-fat diet (Redmann and Argyropoulos 2006). Finally, human genetic studies suggest that SNPs in AgRP such as A67T might provide protection against obesity, associated with anorexia nervosa and leanness (Marks, et al. 2004; Vink, et al. 2001). However, functional studies on the AgRP variant did not identify any defect in its interaction with the MC4R (de Rijke, et al. 2005). Therefore, the exact mechanism for this protection remains to be investigated.

The MC4R in the hypothalamus has been well accepted to be a critical downstream mediator of leptin (Zhang et al. 1994) in regulating energy homeostasis. Leptin increases POMC mRNA expression (Mizuno, et al. 1998; Schwartz et al. 1997; Thornton et al. 1997) through the

leptin receptor expressed in POMC neurons in the ARC (Cheung et al. 1997), increasing the frequency of action potentials in the POMC neurons (Cowley, et al. 2001), whereas decreases AgRP mRNA expression in *ob/ob* (leptin-deficient) but not *db/db* (leptin receptor-deficient) mice (Ebihara et al. 1999). Fasting, with plasma leptin concentration decreased, results in up-regulation of AgRP mRNA (Ebihara et al. 1999; Hahn, et al. 1998; Mizuno and Mobbs 1999). Furthermore, ICV administration of SHU9119 blocked the anorexigenic effect of leptin on food intake (Seeley et al. 1997). It is noteworthy that although the melanocortin system is the dominant component of leptin signaling, it has been reported that other factors, including age, gender and diet, induce independent and additive effects of the two systems (Boston, et al. 1997; Trevaskis and Butler 2005; Trevaskis, et al. 2008).

The critical importance of leptin-regulated melanocortin circuit in energy homeostasis in humans and rodents is also highlighted by the fact that defects in multiple molecules in this circuit cause obesity. Mutations in humans including leptin (Montague, et al. 1997; Strobel, et al. 1998), leptin receptor (Clement, et al. 1998), *POMC* (Krude, et al. 1998), *PC1* (Jackson, et al. 1997), and *MC4R* (Tao 2010), all result in monogenic obesity. In addition, loss of the transcription factor single-minded 1 in both mice and humans causes obesity (Holder, et al. 2000; Michaud, et al. 2001) due to the disruption of the development of the PVN, which is critical in MC4R regulation of food intake (Balthasar et al. 2005). The rodent models include *ob/ob* mice [deficient in leptin (Zhang et al. 1994)], *db/db* mice (Chen et al. 1996) and Zucker *fatty* rats (Phillips et al. 1996) (deficient in leptin receptor), *Pomc* knockout mice (Challis, et al. 2004; Yaswen, et al. 1999), *fat/fat* mice (deficient in carboxypeptidase E, an enzyme involved in POMC processing) (Naggert, et al. 1995), AgRP-overexpressing mice (Graham et al. 1997; Ollmann et al. 1997), and *Mc4r* knockout mice (Huszar et al. 1997).

1.2.4 *MC4R* mutations in obesity pathogenesis

The first mutations in the *MC4R* gene associated with severe early-onset obesity were identified independently by Froguel and O'Rahilly in 1998 (Vaisse, et al. 1998; Yeo, et al. 1998). Up to now, more than 170 distinct mutations have been identified from patient cohorts of different ethnic origins. These mutations, including missense, nonsense and inframe deletion mutations, are widely spread throughout the MC4R.

Based on the life cycle of the receptor, we proposed a classification scheme for MC4R mutations (Tao 2010): (1) Class I mutations have defective in protein synthesis and/or accelerated protein degradation, and thus the total receptor protein levels are decreased. Nonsense mutants might belong to this class, although this assumption needs to be verified experimentally. It has been shown that R7C, C84R, and W174C, with decreased total expression levels, are class I mutations (Fan and Tao 2009). (2) Class II mutations have normal protein synthesis, but are intracellularly retained, most likely in the endoplasmic reticulum due to misfolding being detected by the cell's quality control system. (3) Class III mutations have normal cell surface receptor expression, but are defective in ligand binding. These mutants are always impaired in intracellular signaling due to the impairment in binding. (4) Class IV mutations have normal cell surface expression and ligand binding, but defective in transducing signaling with decreased efficacy and/or potency. The signaling properties of these mutants are characterized solely by measurement of cAMP signaling. The ability of naturally occurring mutants to activate Gi/o or MAPK has not been explored. (5) Class V mutations are variants with unknown defect. These variants have normal cell surface expression, ligand binding, basal and maximal signaling. Whether and how these variants cause energy imbalance and therefore obesity is unclear.

Mounting evidence have demonstrated that the MC4R is a critical regulator of energy homeostasis in humans, and human genetic studies have identified that defects in *MC4R* are the commonest monogenic form of obesity, characterized by its early-onset and severity (Farooqi et al. 2003). It has been shown that patients carrying the MC4R-deficient mutations are hyperphagic. When they are offered a buffet meal, they have increased caloric intake (Farooqi et al. 2003). The degree of hyperphagia correlates with residual receptor activity. The patients carrying mutations with a complete loss of signaling cause more severe obesity (Farooqi et al. 2003). It has been shown that *MC4R* mutations not only affect food intake but also influence energy expenditure (Farooqi et al. 2003). It was reported that Pima Indians with two pathogenic *MC4R* mutations (R165Q and a frameshift mutation with A insertion at nucleotide 100) have decreased energy expenditure. Similarly, in Hispanic patients, *MC4R* variants are also associated with altered energy expenditure (Cole, et al. 2010). In addition to obesity, *MC4R* mutation carriers were also reported to have increased longitudinal growth (Farooqi et al. 2003), similar to the *Mc4r* knockout mice (Huszar et al. 1997). Patients with homozygous *MC4R* mutations are more severely obese than patients with heterozygous MC4R mutations (Farooqi et al. 2003).

Constitutive activity of MC4R has been suggested to be important in obesity pathogenesis. MC4R mutants with decreased basal activity has been demonstrated to be one explanation how the mutants in MC4R results in obesity. Theoretically, it is expected that constitutively active MC4R mutants are associated with a lean phenotype, even anorexia nervosa. For example, mutant I251L, which has higher constitutive activity, is considered to be the potential reason that this variant is negatively associated with BMI (Xiang, et al. 2006). However, there are several constitutively active MC4R mutants identified from obese patients, and the

mechanism of how these mutations associate with obesity still needs further investigation (Tao 2010).

1.2.5 Signaling pathways of the MC4R

Since the cloning of the MC4R by Gantz et al., in 1993 (Gantz et al. 1993), it has been well accepted that the classical signaling pathway for the MC4R is by coupling to the heterotrimeric stimulatory G protein (Gs). Receptor activation leads to increased adenylyl cyclase (AC) activity and subsequently enhances cyclic 3',5'-adenosine monophosphate (cAMP) production and activity of protein kinase A (PKA).

In addition to coupling with Gs, MC4R activation was also shown to coupling with Gq or Gi/o proteins. In HEK293 cells, MC4R activation was also shown to increase intracellular calcium (Mountjoy et al. 1994; Nickolls, et al. 2005) that was sensitive to cholera toxin (Mountjoy et al. 1994). In GT1-1 cells, a hypothalamic cell line endogenously expressing murine MC4R, it was shown that MC4R can increase intracellular calcium through Gq/phospholipase C-dependent signaling pathway (Newman, et al. 2006). However, in another mouse hypothalamic cell line GT1-7 cells, no increase in intracellular calcium concentrations was observed (Buch, et al. 2009). By measuring GTPγS binding, it was shown that the MC4R can also couple to Gi/o proteins (Buch et al. 2009). In both heterologous cells expressing WT MC4R and GT1-7 cells, MC4R activation stimulates pertussis toxin-sensitive GTPγS binding, indicating the coupling to Gi/o proteins (Buch et al. 2009).

In addition to activation of the G protein dependent signaling pathway, it has been demonstrated recently that MC4R also activates mitogen-activated protein kinases (MAPK), both in vitro and in vivo in rat hypothalamus (Chai, et al. 2006; Daniels, et al. 2003; Vongs, et al. 2004). It has also been demonstrated that ERK1/2 is involved in MTII-induced decreases in food

intake (Sutton, et al. 2005). In COS-1 cells expressing rodent MC4R, MTII induce ERK1/2 activation in time- and dose-dependent manners (Daniels et al. 2003). ICV injection of MTII into rat hypothalamus showed that this ERK1/2 activation also happens in vivo (Daniels et al. 2003). Vongs et al. (Vongs et al. 2004) showed that in CHO cells stably expressing hMC4R, NDP-MSH also induces ERK1/2 activation in time- and dose-dependent manners, and this activation is abolished by SHU9119. The GT1-1 cell line that expresses MC4R endogenously also responds to NDP-MSH stimulation with increased ERK1/2 activation (Chai et al. 2006). In cerebral microvessels, MC4R activation of MAPK leads to potentiation of leptin signaling (Zhang, et al. 2009).

The signaling mechanism leading to ERK1/2 activation is cell-line dependent and still largely undetermined. In CHO cells expressing hMC4R, NDP-MSH-induced ERK1/2 activation was blocked by phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002, but not PKA inhibitor Rp-cAMPS, suggesting the importance of PI3K in ERK1/2 activation. In GT1-1 cells, however, it has been demonstrated that increased ERK1/2 activation is mediated by calcium and protein kinase C (Chai et al. 2006). In vivo, ERK1/2 activation induced by fourth ventricle administration is mediated by the cAMP-PKA pathway (Sutton et al. 2005). In other GPCR family, it has been well established there is G protein-independent ERK1/2 activation signaling pathway mediated by important adaptor proteins arrestins, which was conventionally considered as desensitizers to terminate G protein dependent signalling after agonist binding to the receptor (Violin and Lefkowitz 2007). The role of arrestins in MC4R-mediated ERK1/2 activation is largely undetermined, and therefore, needs further investigation

Mulholland and colleagues (Chai, et al. 2009) recently reported that MC4R activation inhibits the c-Jun N-terminal kinase (JNK), therefore inhibiting Ser307 phosphorylation at the

insulin receptor substrate-1. These effects were blocked by SHU9119. MC4R agonist augments insulin-stimulated AKT phosphorylation both in vitro and in vivo. Increased insulin-stimulated glucose uptake was observed in GT1-1 cells after NDP-MSH stimulation. These observations suggest that MC4R activation can interact with insulin signaling through modulating JNK activity (Chai et al. 2009). This is consistent with an earlier observation that central administration of MTII improves insulin tolerance in diet-induced obese rats (Banno, et al. 2007). In summary, MC4R activation can result in coupling to all three major classes of G proteins, Gs, Gi/o, and Gq, changing second messengers such as cAMP and calcium and activating MAPK including ERK1/2 and JNK.

AgRP was initially identified as an orexigenic stimulus, through competitive binding of melanocortins to the MC4R (Banno et al. 2007). AgRP was later found to be an inverse agonist at the MC4R (Adan and Kas 2003; Haskell-Luevano, et al. 2001; Nijenhuis et al. 2001), suggesting that AgRP might exerts its function independently of melanocortins. Several additional lines of evidence (Tolle and Low 2008; Wu, et al. 2008) revealed that the effects of AgRP on energy homeostasis are independent of melanocortin signaling. Since it has been reported that MC4R can functionally couple with Gi/o proteins, it was speculated that it might be involved in the independent effect of melanocortins. Indeed, a recent study demonstrated that AGRP is able to induce the incorporation of GTPγS35 in PTX-sensitive manner (Buch et al. 2009). However, in the same cells PTX was not able to block AGRP-mediated reduction of forskolin-induced cAMP accumulation. It has also been demonstrated that AGRP causes interactions between the MC4R and arrestins in HEK293 or Cos-1 cells (Breit, et al. 2006). Therefore, AgRP mediated MC4R signaling and its physiological role in mediating energy homeostasis is still largely undetermined and need further investigation.

Chapter 2

2.1 Introduction

Obesity is rapidly becoming a critical health problem in the U.S., with nearly 70% of adults considered as overweight or obese compared with less than 25% forty years ago (Flegal et al. 2012). Obesity and associated comorbidities, such as type 2 diabetes mellitus, hypertension and heart diseases, have reached epidemic proportions in the United States and other developed countries (Bhargava, et al. 2004).

Studies have demonstrated that the melanocortin-4 receptor (MC4R) is critical for regulating food intake and energy expenditure in mice (Huszar et al. 1997). The *Mc4r* knockout mice have maturity-onset obesity, hyperphagia, hyperglycemia and hyperinsulinemia. Human genetic studies have identified that defects in *MC4R* are the commonest monogenic form of obesity, characterized by its early-onset and severity (Farooqi et al. 2003). More than 170 *MC4R* mutations, including nonsense, missense, frameshift, and inframe deletions, have been identified from obese patient cohorts of different ethnic origins (Tao 2005, 2010).

The MC4R is a member of family A G protein-coupled receptors (GPCRs) with seven transmembrane (TM) helices connected by alternating extracellular and intracellular loops. Since the cloning of the MC4R by Gantz et al. in 1993 (Gantz et al. 1993), it has been established that this receptor subtype primarily couples to the stimulatory G protein (G_s), which increases adenylyl cyclase activity, and subsequently leads to increased cyclic adenosine monophosphate (cAMP) production that then enhances the activity of protein kinase A (PKA). The activation of

MC4R by the endogenous agonist, α-melanocyte stimulating hormone (α-MSH), results in decreased food intake and increased energy expenditure, while the inhibition of MC4R by the endogenous antagonist, agouti-related protein, leads to increased food intake and decreased energy expenditure (Fan et al. 1997; Fong et al. 1997; Ollmann et al. 1997). Ligands targeting the MC4R have significant therapeutic potential in obesity treatment. Therefore, a better understanding of the MC4R function at molecular level is critical for the development of effective therapy for human obesity.

In addition to the conventional G_s-cAMP-PKA pathway, it has been demonstrated recently that MC4R also activates extracellular signal-regulated kinase (ERK) 1/2 (Daniels et al. 2003; Vongs et al. 2004) and this signaling pathway is involved in melanocortin-induced decreases in food intake (Sutton et al. 2005). Thus, activation of ERK1/2 pathway is one cellular mechanism that may underlie the regulation of energy homeostasis mediated by the MC4R.

Transmembrane domain 3 (TM3) has been suggested to be important for ligand binding and activation in various GPCRs (Ballesteros, et al. 2001; Farrens, et al. 1996; Gether, et al. 1997; Lu and Hulme 1999; Rasmussen, et al. 1999; Roth, et al. 2008; Sheikh, et al. 1996; Tao, et al. 2000). Studies based on site-directed mutagenesis (Ballesteros et al. 2001; Rasmussen et al. 1999; Roth et al. 2008; Tao et al. 2000), electron paramagnetic resonance spectroscopy (Farrens et al. 1996), fluorescence spectroscopy (Gether et al. 1997) and engineered metal ion-binding site studies (Sheikh et al. 1996) have indicated the potential role of TM3 in GPCR functions. Indeed, direct evidences from high-resolution crystal structures of several GPCRs, such as rhodopsin (Palczewski, et al. 2000), β_2 adrenoceptor (Cherezov, et al. 2007; Rasmussen, et al. 2011; Rasmussen, et al. 2007; Rosenbaum, et al. 2007; Rosenbaum, et al. 2011) and human A (2A)

adenosine receptor (Jaakola, et al. 2008), further confirmed that the movement of TM3 was critical for the conversion of the receptor to the active conformation.

A few recent studies suggest that TM3 of MC4R is important for ligand binding and activation (Chen, et al. 2007; Haskell-Luevano et al. 2001; Lagerstrom, et al. 2003; Pogozheva, et al. 2005; Yang, et al. 2000). Some residues in TM3 have been suggested to be essential in forming the binding pocket that directly interacts with ligand (Yang et al. 2000), and some residues in TM3 have been implicated to be crucial in receptor activation by participating in the conformational switch upon ligand stimulation (Chen et al. 2007). In particular, several naturally occurring MC4R mutants found in TM3 have been associated with human obesity. However, the structure-function relationship of MC4R TM3 has not been fully elucidated. In the current study, pharmacological characterization of 26 amino acid residues (Fig. 1, from V119 to V145, excluding S136 and DRYFTI motif) were performed systematically to gain further insight into the molecular basis involved in cell surface expression, ligand binding and receptor activation.

2.2 Materials and methods

2.2.1 Hormones and supplies

[Nle^4,D-Phe^7]-α-melanocyte stimulating hormone (NDP-MSH) was purchased from Peptides International (Louisville, KY). [^{125}I]-NDP-MSH was prepared using a modified chloramine-T method as previously described (Xiang et al. 2006). Cell culture plates and flasks were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, antibiotics and reagents were obtained from Invitrogen (Carlsbad, CA).

2.2.2 In vitro mutagenesis of MC4R

Wild type (WT) human (h) MC4R was generously provided by Dr. Ira Gantz and tagged with c-myc epitope tag at the N-terminus as described previously (Tao and Segaloff 2003). Mutations were generated using QuikChange^{TM} site-directed mutagenesis kit (Stratagene, La Jolla, CA) with WT myc-hMC4R as template (Tao and Segaloff 2003). Plasmids used for transfection were prepared using IsoPure Maxi Prep Kit (Denville Scientific, Metuchen, NJ). Automated DNA sequencing was performed (University of Chicago Cancer Research Center DNA Sequencing Facility, Chicago, IL) to confirm the intended mutations were introduced in the constructs correctly.

2.2.3 Cell culture and DNA transfection

HEK293T cells were obtained from American Type Culture Collection (Manassas, VA), and maintained at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/mL gentamicin. Cells were plated on gelatin-coated 35 mm 6-well clusters and transfected at 50-70% confluency using calcium phosphate transfection method (Chen and Okayama 1987). Ligand binding assay, cAMP signaling assay and flow cytometry assays were performed 48 h after transfection.

2.2.4 Radioligand binding assay

Ligand binding assays were performed as described in detail previously (Tao and Segaloff 2003). Briefly, 48 hours after transfection, cells were washed twice with warm Waymouth/BSA, and then incubated with 100,000 cpm of ^{125}I-NDP-MSH with or without different concentrations of unlabeled NDP-MSH (from 10^{-11} to 10^{-6} M) at 37 ^{o}C for 1 h. Cells

were then washed twice with cold Hank's balanced salt solution containing 1 mg/ml BSA and subsequently solubilized with 100 μl of 0.5 N NaOH. Cells lysates were then collected using cotton swabs, and counted using a gamma counter. All determinations were performed in duplicate. Apparent maximal binding (B_{max}) (Tansky, et al. 2007) or receptor occupancy (Wang, et al. 2008) and concentrations that result in 50% inhibition (IC_{50}) were calculated using Prism 4.0 software (San Diego, CA)

2.2.5 cAMP signaling assay

Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA and then incubated in fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich, St. Louis, MO) at 37 ºC for 15 min. Subsequently, either buffer alone or different concentrations of NDP-MSH (from 10^{-12} to 10^{-6}) were added and the incubation was continued for another hour. Cells were then solubilized with cold 0.5 N percholoric acid containing 180 μg/ml theophylline (phosphodiesterase inhibitor). The supernatant was collected to measure the level of cAMP production using radioimmunoassay (Fan, et al. 2008). All determinations were performed in triplicate. Maximal response (R_{max}) and concentrations that result in 50% maximal response (EC_{50}) were calculated using Prism 4.0 software.

2.2.6 Flow cytometry assay

The cell surface and total expression level of WT and mutant MC4Rs were quantified using flow cytometry technique as described before (Fan and Tao 2009) except that transiently transfected cells were used. Briefly, 48 h after transfection, cells were placed on ice and washed twice with filtered phosphate buffered saline for immunohistochemistry (PBS-IH, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4), fixed with 4% paraformaldehyde for

30 min, permeabilized (only for measurement of total expression level) with 1% Triton X-100 in PBS-IH for 4 min, and then incubated with blocking solution (PBS-IH with 5% BSA) for 1h. Cells were then incubated with monoclonal anti-myc (9E10) antibody (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA) diluted 1:100 in PBS-IH with 0.1% BSA for 1 h. Cells were then washed thoroughly and incubated for another hour with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:1000 in PBS-IH with 0.1% BSA. Cells were then washed three times with PBS-IH and fluorescence signals were collected with C6 Accuri Cytometer (Accuri Cytometers, Inc., Ann Arbor, MI). Fluorescence from cells transfected with empty vector was used as negative control. The expression level of the mutants was calculated as a percentage of WT MC4R expression using the formula: [mutant fluorescence- pcDNA3 fluorescence]/ [WT fluorescence- pcDNA3 fluorescence] ×100% (Wang et al. 2008). For assessing the effect of proteasome or lysosome inhibitor on total receptor expression, 24 h after transfection, cells were treated with MG132 (25 μM), NH_4Cl (25 mM) or chloroquine (100 μM) for 18 h prior to the measurement.

2.2.7 ERK1/2 signaling assay

For studying ERK1/2 signaling, cells were washed twice and incubated with Waymouth/BSA (Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) containing 1 mg/ml bovine serum albumin (BSA)) 24 h after transfection and starved in Waymouth/BSA for 24 h at 37 ^{o}C before hormone stimulation. Cells were treated for 5 min with either buffer alone or 1 μM NDP-MSH, solubilized in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 1 mM sodium orthovanadate and 1 mM sodium fluoride), and then clarified by centrifugation at 4 ^{o}C, 14,000×g for 15 min. Thirty μg of supernatant proteins were separated on 10% SDS-PAGE gel and then transferred to PVDF membrane. Phosphorylated ERK1/2 and β-

tubulin were detected by immunoblotting with rabbit anti-pERK1/2 antibody (1:1000~1:3000, Cell Signaling Technology, Beverly, MA) and mouse anti-β-tubulin antibody (1:5000~1:20000, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), respectively. Membranes were then washed thoroughly with Tris-buffered saline (TBS, 20 mM Tris, 0.5 M NaCl, pH 7.5) containing 0.1% Tween-20 (TBST), and then probed with horseradish peroxidase (HRP)-conjugated secondary donkey anti-rabbit (1:500~1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA) and HRP-conjugated donkey anti-mouse (1:5000~1:20000, Jackson ImmunoResearch Laboratories) at room temperature for 2 h. The membranes were then washed with TBST and a final wash with TBS. Blots were then visualized using enhanced chemiluminescence reagent (Pierce) and quantified using ImageJ 1.44 software (National Institute of Health, Bethesda, MD) after densitometric scanning of the films. ERK1/2 phosphorylation was normalized according to the loading of proteins by expressing the data as a ratio of pERK1/2 over β-tubulin. The phosphorylated ERK1/2 was expressed as a percent of the basal value of WT hMC4R.

2.2.8 Statistical analyses

The significance of differences in cell surface expression level, signaling and binding parameters, and pERK1/2 activities, between the WT and mutant hMC4Rs were analyzed using Student's *t*-test with Prism 4.0.

2.3 Results

2.3.1 Expression, ligand binding and signaling properties of the TM3 mutant hMC4Rs

To study the importance of TM3 residues in ligand binding and signaling, we mutated 26 TM3 residues as shown in Fig. II-1, excluding previously well-studied S136 (Fan and Tao 2009) and DRYFTI motif (Govaerts, et al. 2005; Tao and Segaloff 2003; Yamano, et al. 2004), by alanine-scanning mutagenesis. For these studies, TM3 residues were mutated to Ala (or substitution of Ala by Gly) to eliminate the side chain interactions. Results obtained from these mutants would reveal which amino acid residues are important for ligand binding and/or receptor activation. A total of 26 TM3 mutants were generated.

To quantitate the cell surface and total receptor expression levels of mutant MC4Rs, flow cytometry technique was used as described in *Materials and Methods*. The results demonstrated that all TM3 mutants were expressed normally on the cell surface with no significant difference compared to WT MC4R (Fig. II-2A). One mutant, I129A, had a small, although statistically significant, decrease in total expression, and the other 25 mutants had normal total expression levels (Fig. II-2B).

Ligand binding and signaling properties of these mutants were assessed using NDP-MSH. Because NDP-MSH is a superpotent analog of α-MSH (Sawyer, et al. 1980), we reasoned that if a mutant cannot bind and respond to NDP-MSH, it would not be able to bind and respond to the endogenous ligands such as α- or β-MSH.

As shown in Fig. II-3 and Table 2.1, the WT hMC4R binds to NDP-MSH with an IC_{50} of 108.83 ± 22.60 nM. Of the 26 mutants, D126A had no measurable binding. Six mutants, D122A, I125A, L133A, L134A, S139A and L140A, had decreased maximal binding. Four mutants,

V124A, I125A, L140A and I143A, had lower IC_{50}s (increased affinities). Upon NDP-MSH stimulation, WT MC4R responded with increases in cAMP accumulation in a dose-dependent manner with an EC_{50} of 0.51 ± 0.07 nM (Fig. II-4 and Table 2.1). D126A had no response to NDP-MSH stimulation, consistent with its lack of detectable binding. Three mutants, D122A, L133A and L140A, had decreased maximal response and/or increased EC_{50}s, consistent with their decreased ligand binding. Five mutants, S127A, V128A, S132A, L141A and I143A, despite normal ligand binding, had decreased maximal response and/or increased EC_{50}s suggesting that these mutants were partially defective in signaling. The other mutants could signal at least as well as WT MC4R in terms of maximal signaling. Three mutants, S142A, A144G and V145A, even had significantly increased maximal response. Several mutants, including N120A, S139A, L140A and L141A, had decreased EC_{50}s (Fig. II-4 and Table 2.1).

Since it was suggested that basal signaling might be important for maintaining energy homeostasis in humans (Srinivasan, et al. 2004), we also measured the basal signaling of the mutant MC4Rs. As shown in Fig. II-5, two mutants, I137A and L140A, had significantly increased basal signaling. Of the remaining 24 mutants, seven had decreased basal signaling and the other mutants had normal levels of basal signaling.

2.3.2 Expression, ligand binding and signaling properties of the L140 mutant hMC4Rs

Since $L140^{3.43}$ (superscript indicates Ballesteros-Weinstein numbering for GPCR residues (Ballesteros and Weinstein 1995)), a highly conserved residues presenting in 74% family A GPCRs (Mirzadegan, et al. 2003), has been suggested to be important in stabilizing the inactive conformation of GPCRs (Baranski, et al. 1999; Kosugi, et al. 2000; Latronico, et al. 1998; Lu and Hulme 1999; Tao et al. 2000) (reviewed in (Tao 2008)), saturation mutagenesis was

performed at this loci to gain further detailed information on the structure-function relationship of L140 in MC4R.

Seventeen additional L140 mutants were generated (excluding L140H, which we failed to generate after several attempts), and their cell surface and total receptor expression were studied (Fig. II-6). Two mutants, L140G and L140V, had both normal cell surface and total receptor expression. Three mutants, L140C, L140I and L140K, had significantly decreased cell surface expression with normal total expression, suggesting that these mutants were intracellularly retained. Two mutants, L140P and L140R, had normal cell surface expression (Fig. II-6A), but with decreased total expression (Fig. II-6B). Ten L140 mutants, including mutations of L140 to D, E, F, M, N, Q, S, T, W and Y, had significantly decreased cell surface expression, most likely due to their significantly decreased total expression. In order to study whether the decreased total receptor expression were due to accelerated protein degradation, total receptor expressions of these ten L140 mutants were characterized with or without the treatment of proteasome inhibitor (25 μM MG132), or lysosome inhibitors (25 mM NH_4Cl or 100 μM chloroquine) as described in *Materials and Methods*. As shown in Fig. II-7, there was no significant increase of total expression of the mutant and WT hMC4R upon the treatment of proteasome or lysosome inhibitors.

The ligand binding properties of the seventeen L140 mutants were also studied. As shown in Fig. II-8 and Table 2.2, L140G and L140V, although with normal cell surface expression, had significantly decreased maximal binding. Twelve mutants, mutations of L140 to C, D, E, F, I, M, N, Q, S, T, W and Y, had decreased maximal binding (Fig. II-8 and Table 2.2), most likely due to their largely decreased cell surface expression (Fig. II-6A). Three mutants, mutations of L140 to K, P and R, had normal ligand binding.

The signaling properties of the seventeen L140 mutants are presented in Fig. II-9 and Table 2.2. Upon NDP-MSH stimulation, ten mutants, mutations of L140 to C, D, F, I, M, N, Q, S, T and Y, had decreased maximal response and/or increased EC_{50}s, which were consistent with their decreased cell surface expression levels. Mutant L140G, expressed normally on the cell surface, was impaired in signaling in terms of maximal response, most likely due to its defect in ligand binding. Mutant L140R also had significantly decreased maximal response, although it had normal cell surface expression and ligand binding. Other mutants could signal at least as well as WT MC4R in terms of maximal signaling. L140V had decreased EC_{50}s (Fig. II-9 and Table 2.2).

We also measured the basal signaling of the L140 mutants (Table 2.2). Ten mutants, mutations of L140 to C, D, E, K, N, P, Q, S, T and V, had increased basal cAMP activity. Four mutants, mutations of L140 to I, M, R and Y, had decreased basal cAMP production, and the other mutants had normal basal signaling activities.

2.3.3 Signaling properties of L140 mutants at the ERK1/2 pathway

Because the MC4R can signal through both cAMP and ERK1/2, and ERK1/2 signaling pathway has been suggested to be one cellular mechanism underlying the regulation of energy homeostasis by MC4R (Sutton et al. 2005), we asked whether there is biased signaling at the MC4R. The L140 mutants studied herein (Fig. II-9) revealed four signaling defective mutants (SDM, including mutations of L140 to G, I, M and R) and nine constitutively active mutants (CAM, including mutations of L140 to C, E, K, N, P, Q, S, T and V) in the cAMP pathway. These mutants are excellent tools to address two questions: 1. Are mutants constitutively active in the cAMP pathway also constitutively active in the ERK1/2 pathway? 2. Are mutants defective in ligand-induced signaling in the cAMP pathway also defective in responding to

ligand stimulation in the ERK1/2 pathway? Therefore the ability of these L140 mutants to activate ERK1/2 was studied either in the basal condition (for the CAMs) or in maximal ligand-stimulated condition (for the SDMs). A time-course experiment was performed to determine the optimal time for ERK1/2 activation in WT MC4R, and the level of pERK1/2 reached maximum in 5 min (data not shown). In these experiments, the WT or mutant MC4R were transfected into HEK293T cells and subsequently treated for 5 min with 1 μM NDP-MSH (for SDM only) or not. The levels of pERK1/2 were measured using Western blots as described in *Materials and Methods*.

As shown in Fig. II-10A-C, despite that the CAMs had high constitutive activities in the cAMP pathway (Fig. II-10A), their basal pERK1/2 activities were at similar levels to that of WT MC4R (Fig. II-10B-C), different from their basal cAMP signaling. None of the CAM mutants had increased basal ERK1/2 signaling. Data for the four SDMs are shown in Fig. II-11A-C. Two mutants (L140I and L140M) were defective in ERK1/2 signaling upon NDP-MSH stimulation, consistent with their deficiency in cAMP production. Two SDMs (L140G and L140R) had similar level of ERK1/2 phosphorylation as that of WT MC4R upon NDP-MSH stimulation, different from their cAMP signaling properties. It is interesting to note that although L140R had decreased basal cAMP activity (see also (Tao 2008)), it had significant constitutive pERK1/2 activity (Fig. II-11C). These results suggested that the nine CAMs and two SDMs (L140G and L140R) had divergent cAMP and ERK1/2 signaling properties.

2.4 Discussion

In the present study, we systematically studied 26 residues of TM3 in MC4R. We showed that one mutant D126A had no measurable binding, and six other mutants, D122A, I125A, L133A, L134A, S139A and L140A, had significantly decreased maximal binding or receptor occupancy (Fig. II-3 and Table 2.1). These 7 mutants had normal levels of cell surface expression, suggesting that these mutants were defective in ligand binding *per se*.

D122, I125 and D126 sit at the top of the third transmembrane domain. We showed that D122A, I125A and D126A were expressed normally at the cell surface but with significantly decreased ligand binding or cAMP signaling. These results are consistent with previous mutagenesis experiments demonstrating D122, I125 and D126 as being essential for binding to peptide and nonpeptide ligands (Fleck, et al. 2005; Hogan, et al. 2006; Yang et al. 2000), hypothesized to interact with the pharmacophore His-D-Phe-Arg-Trp of NDP-MSH (Hogan et al. 2006; Nargund, et al. 2006). We also showed that the naturally occurring D126Y mutation is expressed normally at the surface, but had no binding to NDP-MSH, defective in ligand binding *per se* (Wang and Tao 2011).

Four mutants, including L133A, L134A, S139A, and L140A, were expressed on the cell surface normally, but had significantly decreased maximal binding (Fig. II-2A and Table 2.1). L133 and L134 are located in the middle of TM3, and S139 and L140 are located near the intracellular surface. Because of the modest decreases in maximal binding without affecting the binding affinity (similar IC_{50} as the WT MC4R) for L133A and L134A, and the inherent inaccuracies in the binding assay used here for estimating maximal binding, we cannot conclude whether these two residues are directly involved in ligand binding. A previous study mutating L133 to methionine showed that binding to NDP-MSH is not affected (Yang, et al. 2002).

Mutations of S139 and L140, located close to the intracellular surface of TM3, into alanine, caused dramatic decreases in maximal binding despite normal cell surface expression. Similar observations were made before in other peptide receptors. For example, in the GnRH receptor, mutations in the cytoplasmic end of TMs were found to affect ligand binding (Lu, et al. 2005). In the MC3R, we showed that mutations of E92 located near the intracellular end of TM1 abolished ligand binding despite expression at the cell surface (Wang et al. 2008). These mutations likely affected the conformation of the receptor rather than directly disrupted ligand-receptor interaction.

Four mutants, D122A, D126A, L133A and L140A, had cAMP signaling deficiency, most likely due to their impaired binding properties as discussed before. Five mutants, S127A, V128A, S132A, L141A and I143A, had impaired signaling, but normal ligand binding, suggesting that these residues are important in signal transduction. Several previous studies (Hinney, et al. 2003; Valli-Jaakola, et al. 2004), including ours (Fan and Tao 2009), showed that one naturally occurring mutation, S127L, has significantly decreased maximal responses, and modest (less than threefold), but significant increase in basal cAMP signaling activity. In our experiments, mutating S127 to Ala resulted in decreased maximal response. However, its basal activity was similar to that of WT MC4R.

We previously reported that one naturally occurring mutation, S136F, is a loss-of-function mutation with normal total and cell surface expression, as well as normal ligand binding properties (Fan and Tao 2009), consistent with other studies (Tan, et al. 2009; Valli-Jaakola et al. 2004). Multiple mutagenesis experiments further showed that S136 is important for signaling. We showed that mutating S136 to Ala, Cys, and Arg, resulted in decreased maximal response, whereas mutations into Cys, Leu, Arg and Try, resulted in increased EC_{50}s (Fan and Tao 2009).

The DRY motif and surrounding residues has been known to be essential for receptor signaling in various GPCRs (Gether 2000; Govaerts et al. 2005; Yamano et al. 2004). Mutations in this motif are known to greatly affect the receptor functions by directly disrupting the G protein coupling, and disrupting the ionic lock formed between TM3 and TM6 (reviewed in (Gether 2000)). In the MC4R, mutations of DRY motif resulted in profoundly impaired ligand binding and receptor signaling properties (Yamano et al. 2004). Previous studies mutating D146 into Ala resulted in significantly increased basal cAMP activity. We also reported that one naturally occurring mutation, D146N, has increased basal cAMP activity with decreased cell surface expression, decreased ligand binding but normal maximal responses (Tao et al. 2010; Wang and Tao 2011). T150I in the DRYxxI motif has significantly decreased receptor signaling (Govaerts et al. 2005). We showed that I151 in the DRYxxI motif at the end of TM3 is also critical for ligand-induced cAMP signaling; mutation of this isoleucine to asparagine totally disrupts NDP-MSH-induced signaling despite normal ligand binding.

The remaining mutants could signal at least as well as WT MC4R in terms of maximal signaling upon NDP-MSH stimulation, consistent with several previous studies (Chen et al. 2007; Yang et al. 2000). Three mutants, S142A, A144G and V145A, even had significantly increased maximal response. Similar observations were made before in other mutant MC4Rs (Wang and Tao 2011).

We also showed that one mutant, I121A, had normal cell surface expression, ligand binding and cAMP signaling. Some previous studies demonstrated that one naturally occurring mutation, I121T, is impaired in cAMP signaling upon stimulation with α-MSH (Hinney et al. 2003). Recent studies, however, showed that I121T has decreased cell surface expression, decreased binding affinity with NDP-MSH, but normal potency upon α-MSH or NDP-MSH

stimulation (Xiang, et al. 2010). Therefore, the structure-function relationship of the residue I121 is still unclear.

In addition, our data showed that one mutant, I137A, normally expressed on cell surface, had normal binding and signaling properties. In previous studies, one naturally occurring mutation, I137T, has been shown to be expressed on the cell surface but defective in agonist binding (Gu, et al. 1999).

Basal signaling of the MC4R has been suggested to be important for maintaining energy homeostasis in humans (Srinivasan et al. 2004). We have also identified a number of naturally occurring *MC4R* mutations that have decreased basal activities (Fan and Tao 2009; Tao et al. 2010; Wang and Tao 2011). In this study, we also measured the basal signaling of the mutant MC4Rs. We showed that two mutants, $I137^{3.40}A$ and $L140^{3.43}A$, had significantly increased basal signaling. These results are consistent with the crystal structure of β_2 adrenoceptor, showing that $I121^{3.40}$ (Rasmussen et al. 2011) and $L124^{3.43}$ (Rosenbaum et al. 2007) are essential for stabilizing receptor in its inactive conformation, hypothesized to interact with other residues through hydrophobic interactions.

In order to obtain more detailed insight into the function of L140 in MC4R, we generated seventeen additional L140 mutants. We showed that ten L140 mutants were constitutively active in cAMP signaling, including substitutions of L140 to three hydrophobic residues (C, P and V), four hydrophilic neutral residues (N, Q, S and T), two hydrophilic negatively charged residues (D and E) and one hydrophilic positively charged residue (K). Four L140 mutants had normal or even decreased basal cAMP activity, including substitutions of L140 to one hydrophilic positively charged residue (R) and six hydrophobic residues (F, G, I, M, W and Y).

Correlation analyses demonstrated that the basal cAMP activity was significantly correlated with the hydrophobicity (Jones 1975) of the residues (Pearson correlation coefficients = -0.5523, p-value = 0.0142). This result was consistent with the previous finding in the crystal structure of β_2-adrenoceptor that a hydrophobic pocket was formed around the Leu124$^{3.43}$ (Rosenbaum et al. 2007). Four mutants (L140 to C, P, R and V), however, were exceptions that cannot be fully explained by the hypothesis that constitutive activation resulted from the disruption of hydrophobic interactions that stabilize the receptor in its inactive state. Rather, in these cases, a more likely hypothesis is that constitutive activity arises from the disruption of interhelical interactions stabilizing the inactive conformation, as well as formation of other interactions involving the newly introduced residue, which can stabilize the receptor in an active state (Tao et al. 2000).

With the recent report that ERK1/2 signaling activated through MC4R is involved in melanocortin-induced decreases in food intake (Sutton et al. 2005), herein, we studied the ERK1/2 signaling of nine CAMs and four SDMs at the L140 locus. We demonstrated that two SDMs (L140I and L140M) had similar ERK1/2 activities as their cAMP signaling properties. The other two SDMs (L140 to G and R) had normal ERK1/2 phosphorylation upon stimulation. L140R had decreased basal cAMP activity, but had significant constitutive activity in the basal ERK1/2 signaling pathway. All nine L140 CAMs (L140 to C, E, K, N, P, Q, S, T and V) had no constitutive activation of ERK1/2 signaling. Therefore, we concluded that one mutant, L140G, was intrinsically biased in ERK1/2 signaling pathway. One mutant, L140R, was intrinsically biased in basal cAMP signaling, and was intrinsically biased in ERK1/2 signaling upon NDP-MSH stimulation. Nine mutants, mutations L140 to C, E, K, N, P, Q, S, T and V, were intrinsically biased MC4R mutants in basal cAMP signaling.

Several intrinsically biased GPCR mutants have been reported. For example, a mutant angiotensin type 1A receptor (DRY/AAY) is intrinsically biased in ERK1/2 signaling upon agonist stimulation (Wei, et al. 2003). The transgenic mice with this mutant receptor develop more severe hypertrophy and bradycardia than the WT mice (Zhai, et al. 2005). One mutant β_2-adrenoceptor (T68F, Y132G and Y219A) is also intrinsically biased in ERK1/2 signaling pathway (Shenoy, et al. 2006). In the MC4R, Patten et al. (Patten, et al. 2007) reported the first MC4R mutant, D298N, which is intrinsically biased in cAMP signaling. It was shown that D298N has cAMP signaling, but does not have ERK1/2 signaling upon hormone stimulation. To our best knowledge, we are the first to report mutant MC4Rs (mutations of L140 to C, E, K, N, P, Q, S, T and V) that were intrinsically biased in basal cAMP signaling, as well as the first to report mutant MC4Rs (L140G and L140R) that were intrinsically biased in ERK1/2 signaling pathway upon agonist stimulation.

The studies of biased agonism in other GPCRs have suggested that divergent signaling pathways might contribute to different physiological functions (reviewed in (Whalen, et al. 2011)). The MC4R has multiple physiological roles in regulating energy homeostasis, cachexia, cardiovascular function, glucose and lipid homeostasis, reproduction and sexual function, etc. The dominant signaling pathways activated in different physiological functions of MC4R, however, has not been determined. The intrinsically biased MC4R mutant constructs found in present study, hopefully, could be useful for generating transgenic animals, and thus be helpful in investigating the potential role of cAMP-dependent or ERK1/2-dependent signaling pathway in the physiology of the MC4R.

GPCRs have been known to have multiple signaling states, and a mutant receptor can adopt specific conformation (reviewed in (Perez and Karnik 2005)). Our data suggested that the

nine L140 CAMs might stabilize the MC4R in a conformation capable of coupling to cAMP but not ERK1/2 signaling pathway. The two L140 SDMs (L140 to G and R) might stabilize the MC4R in a conformation capable of coupling to ERK1/2 but not cAMP signaling pathway upon NDP-MSH stimulation. The SDM L140R might stabilize the MC4R in a conformation that constitutively activate ERK1/2 but not cAMP signaling. Our results further confirmed the divergent structural requirement for the cAMP and ERK1/2 signaling pathways, and suggested that L140 might be a key residue in switching MC4R between several thermodynamically favorable conformations.

The flow cytometry results showed that three mutants, L140C, L140I, and L140K, were intracellularly retained with decreased cell surface expression, but normal total receptor expression, suggesting that these mutants are defective in forward trafficking. Defective trafficking of mutant receptors to the cell surface has been shown to be a predominant defect in MC4R, as well as in other GPCR mutations. Interestingly, however, one TM3 mutant (I129A) and twelve L140 mutants (mutating L140 to D, E, F, M, N, P, Q, R, S, T, W and Y) had dramatic decreases in total receptor expression.

Decreased total receptor expression has been reported previously for some missense GPCR mutations including the MC4R (Fan and Tao 2009; Wang and Tao 2011; Zhang, et al. 2012), even for some synonymous mutations (Bartoszewski, et al. 2010; Duan, et al. 2003). We therefore further studied that whether the decreased total receptor expression were due to accelerated protein degradation (Yang, et al. 2011). Ten L140 mutants (mutating L140 to D, E, F, M, N, Q, S, T, W and Y) had almost abolished total receptor expression. Their total receptor expressions were characterized with or without the treatment of proteasome inhibitor (25 μM MG132), or lysosome inhibitors (25 mM NH_4Cl or 100 μM chloroquine). The results (as shown

in Fig. II-7), however, showed that there was no significant increase of total receptor expression upon the treatment of proteasome or lysosome inhibitors. These results suggested that increased degradation by the proteasomes or lysosomes were not responsible for the diminished expression of these mutants. Decreased protein synthesis due to altered secondary structure of mRNA (Bartoszewski et al. 2010; Duan et al. 2003) of those MC4R mutants might be responsible for the decreased receptor expression. Further studies are needed to address the detailed causes of the observed decreased total MC4R expression.

In summary, the results presented here showed that TM3 was critical for MC4R ligand binding and/or signaling, consistent with previous studies. Of the 26 TM3 mutants we studied herein, 13 were significantly impaired in ligand binding and/or cAMP signaling. Moreover, L140 was critical for locking MC4R in inactive conformation and several mutants displayed biased signaling in cAMP and ERK1/2 signaling pathways.

Table 2.1 Ligand binding and NDP-MSH-stimulated signaling of WT and mutant hMC4Rs.

hMC4R	n	NDP-MSH binding		NDP-MSH-stimulated cAMP	
		IC_{50} (nM)	B_{max} (% WT)	EC_{50} (nM)	R_{max} (% WT)
WT	29	108.83±22.60	100	0.51±0.07	100
V119A	5	56.71±6.08	104.21±24.30	0.30±0.11	115.88±18.10
N120A	5	46.01±17.89	86.16±27.89	0.39±0.03[c]	118.02±22.03
I121A	5	26.08±5.94	81.73±13.73	0.12±0.01	104.73±10.26
D122A	5	50.78±26.24	55.83±13.71[b]	0.68±0.12[b]	87.76±25.20
N123A	5	48.42±8.75	77.29±11.53	0.19±0.04	79.79±20.53
V124A	4	33.90±10.33[b]	119.56±35.00	0.18±0.07	99.58±19.14
I125A	4	18.43±10.17[c]	42.78±19.04[a]	0.28±0.04	74.83±32.50
D126A	4	ND[a]	ND[a]	ND[a]	ND[a]
S127A	4	68.53±33.61	71.11±12.12	0.63±0.38	64.10±7.79[c]
V128A	4	51.08±25.94	46.42±19.98	4.71±2.02	55.99±17.84[b]
I129A	4	24.09±5.43	116.79±24.01	0.63±0.26	86.12±23.93
C130A	4	100.09±36.06	82.81±19.51	0.68±0.31	88.09±10.64
S131A	4	78.71±22.06	108.99±32.19	0.42±0.33	74.71±15.97
S132A	4	70.13±22.33	75.89±3.78	0.64±0.22	66.46±13.24[b]
L133A	4	43.92±15.01	61.77±4.02[d]	0.74±0.35	41.46±13.88[c]
L134A	3	38.43±14.27	80.15±5.98[b]	0.71±0.18	105.16±15.57
A135G	3	125.60±55.60	81.74±9.94	0.89±0.07	91.08±13.80
I137A	3	75.85±28.20	99.74±5.46	0.67±0.28	84.14±10.06
C138A	3	126.78±48.30	91.40±14.92	0.56±0.07	85.40±15.06
S139A	3	9.59±3.69	36.66±7.87[c]	0.26±0.07[c]	90.66±17.02
L140A	4	3.12±0.70[c]	33.35±2.36[d]	0.01±0.01[c]	29.96±2.71[d]
L141A	4	100.58±23.25	95.47±10.38	0.13±0.01[c]	77.93±8.63[b]
S142A	4	338.76±137.72	103.38±7.49	0.64±0.06	130.60±8.46[c]
I143A	4	35.83±8.34[b]	103.83±9.26	3.91±0.24[d]	22.20±3.35[d]
A144G	4	136.53±32.68	105.06±6.81	1.30±0.09[d]	178.09±13.46[c]
V145A	4	194.46±52.91	135.63±11.70[a]	1.50±0.11[d]	223.55±29.43[c]

The data were expressed as the mean ± SEM of 3-5 independent experiments for the mutant hMC4Rs. The maximal responses (R_{max}) were 2509.55 ± 160.14 pmol cAMP/10^6 cells for WT hMC4R.

[a] ND, could not be detected

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

[c] Significantly different from corresponding WT receptor, $p < 0.01$.

[d] Significantly different from corresponding WT receptor, $p < 0.001$.

Table 2.2 NDP-MSH-stimulated signaling and ligand binding properties of WT and L140 mutant hMC4Rs.

hMC4R	n	Basal cAMP (% WT)	NDP-MSH binding		NDP-MSH-stimulated cAMP	
			IC_{50} (nM)	B_{max} (% WT)	EC_{50} (nM)	R_{max} (% WT)
WT	29	100.00	108.83±22.60	100	0.51±0.07	100
L140C	3	1832.12±303.62[c]	7.82±0.67[b]	6.99±0.99[d]	21.05±6.85[b]	65.36±7.54[c]
L140D	3	208.35±17.77[c]	ND[a]	8.83±4.88[d]	1.15±0.86	40.03±3.88[d]
L140E	3	1576.81±37.80[d]	5.04±0.71[b]	15.76±3.17[d]	0.63±0.18	86.97±6.67
L140F	3	67.73±26.97	31.68±12.74	46.49±7.29[c]	14.12±1.70[c]	69.75±3.67[c]
L140G	3	84.11±7.40	13.70±2.84	8.05±5.90[d]	2.20±0.54[b]	20.82±0.27[d]
L140I	3	49.75±8.02[c]	ND[a]	ND[a]	ND[a]	ND[a]
L140K	3	643.25±15.17[d]	11.81±2.62	129.16±5.09[c]	3.45±2.19	95.94±2.06
L140M	3	21.21±3.63[d]	ND[a]	ND[a]	ND[a]	ND[a]
L140N	3	2693.38±566.11[c]	5.57±0.93[b]	18.27±4.54[d]	0.27±0.11	64.23±4.67[c]
L140P	3	1227.83±266.28[c]	19.38±10.35	136.90±42.12	1.65±0.98	113.44±9.47
L140Q	3	1997.89±800.82	10.19±3.61[b]	47.32±5.23[d]	1.28±0.07[c]	75.61±13.88
L140R	3	51.17±4.85[c]	161.35±40.70	97.04±5.04	1.23±0.11[b]	45.09±2.07[c]
L140S	3	1696.26±448.25[b]	22.45±14.33	10.53±4.95[d]	1.56±0.76	44.36±9.81[c]
L140T	3	2868.03±903.00[b]	8.83±0.72[b]	20.45±6.40[d]	40.74±5.43[c]	96.89±5.16
L140V	3	1830.42±440.04[b]	7.60±1.20[b]	45.98±13.27[b]	0.27±0.03[b]	103.55±9.33
L140W	3	62.82±16.90	15.62±1.31[b]	9.32±1.69[d]	8.87±3.24	103.03±2.45
L140Y	3	32.84±9.61[c]	24.45±5.20	54.91±10.05[c]	29.56±7.97[b]	88.28±5.19

The data were expressed as the mean ± SEM of three independent experiments for the mutant hMC4Rs. The maximal responses (R_{max}) were 2509.55 ± 160.14 pmol cAMP/10^6 cells for WT hMC4R.

[a] ND, could not be detected

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

[c] Significantly different from corresponding WT receptor, $p < 0.01$.

[d] Significantly different from corresponding WT receptor, $p < 0.001$.

Figure II-1. Schematic model of the hMC4R with the mutations characterized in this study highlighted with gray background.

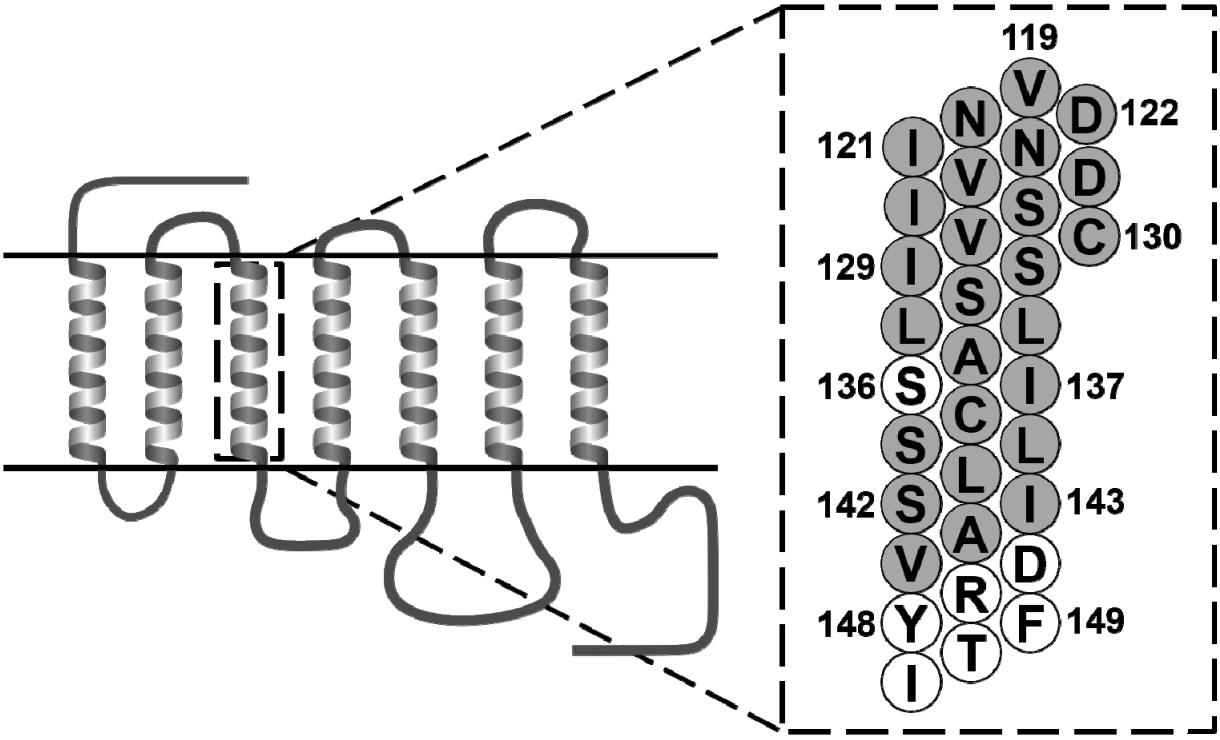

119
V
N
D
122
121
I
N
V
D
I
V
S
C
130
129
I
V
S
S
L
S
L
A
136
S
I
137
C
S
L
L
142
S
L
I
143
A
V
D
R
148
Y
T
F
149
I

Figure II-2. Cell surface expression (A) and total expression (B) of WT and TM3 mutant hMC4Rs measured by flow cytometry. The results are expressed as percentage of cell surface or total expression levels of the WT hMC4R after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and methods. Data were mean ± SEM of 3 experiments. The statistical significance are indicated as follows: a: significantly different from WT hMC4R, $p < 0.05$.

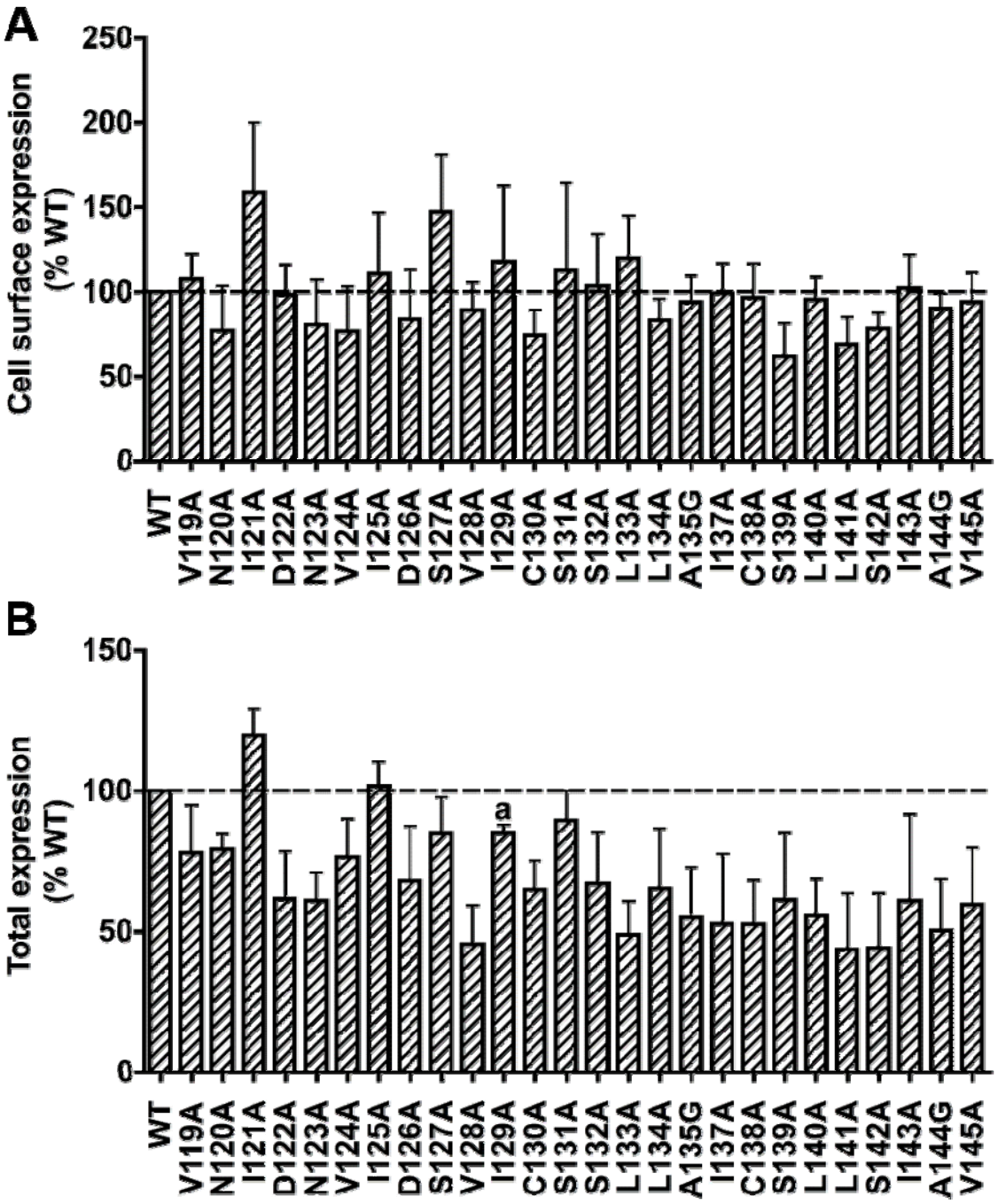
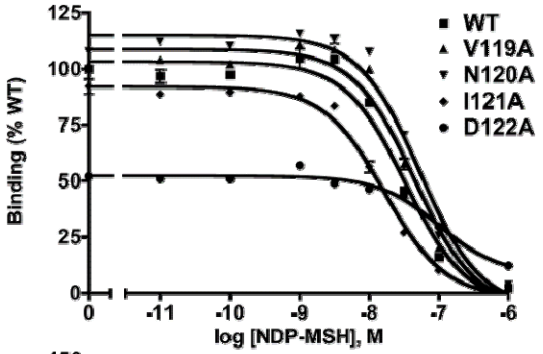
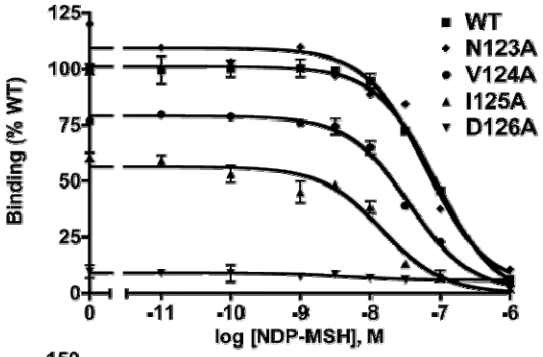
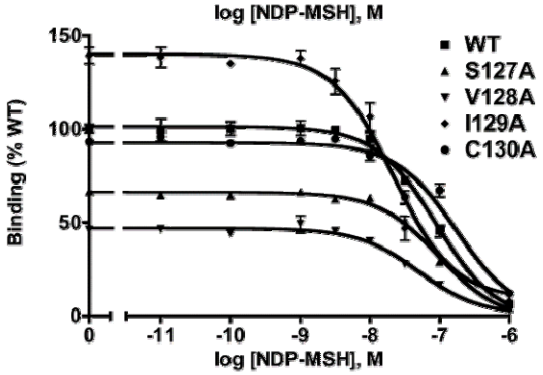
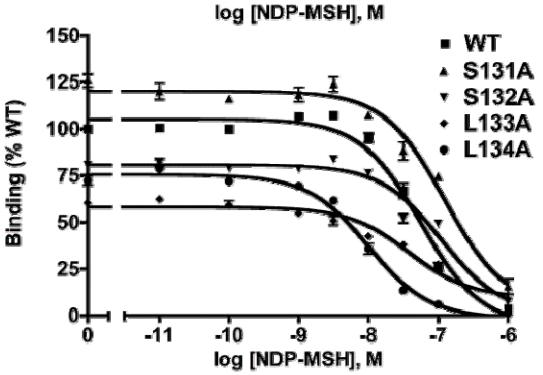

A
Cell surface expression (% WT)
250
200
150
100
50
0
WT
V119A
N120A
I121A
D122A
N123A
V124A
I125A
D126A
S127A
V128A
I129A
C130A
S131A
S132A
L133A
L134A
A135G
I137A
C138A
S139A
L140A
L141A
S142A
I143A
A144G
V145A
B
Total expression (% WT)
150
100
50
0
a
WT
V119A
N120A
I121A
D122A
N123A
V124A
I125A
D126A
S127A
V128A
I129A
C130A
S131A
S132A
L133A
L134A
A135G
I137A
C138A
S139A
L140A
L141A
S142A
I143A
A144G
V145A

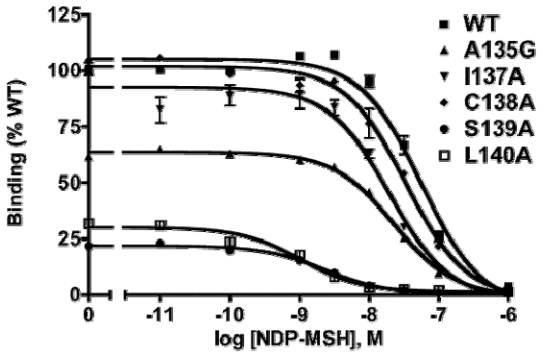
Figure II-3. Ligand binding properties of the WT and mutant hMC4Rs with NDP-MSH as the ligand. The binding of ^{125}I-NDP-MSH to hMC4R on intact cells were measured as described in Materials and methods. Results shown are expressed as percentage of WT binding ± range from duplicate determinations within one experiment. All experiments were performed 3-5 times.


Binding (% WT)
log [NDP-MSH], M
WT
V119A
N120A
I121A
D122A


Binding (% WT)
log [NDP-MSH], M
WT
N123A
V124A
I125A
D126A


Binding (% WT)
log [NDP-MSH], M
WT
S127A
V128A
I129A
C130A


Binding (% WT)
log [NDP-MSH], M
WT
S131A
S132A
L133A
L134A


Binding (% WT)
log [NDP-MSH], M
WT
A135G
I137A
C138A
S139A
L140A

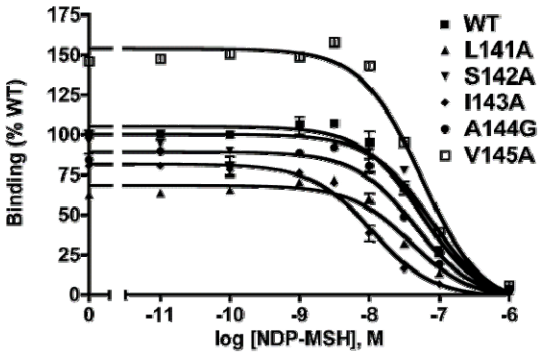
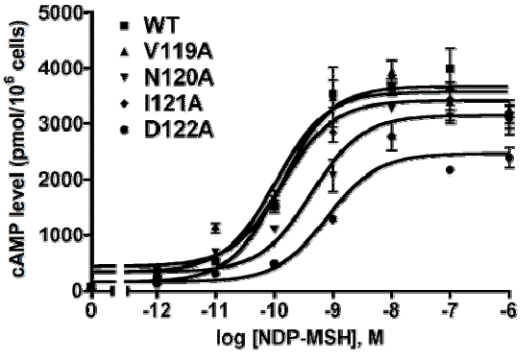
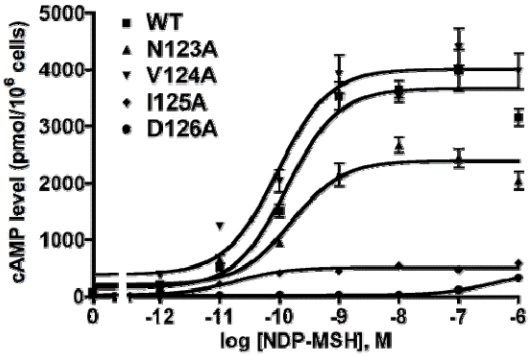
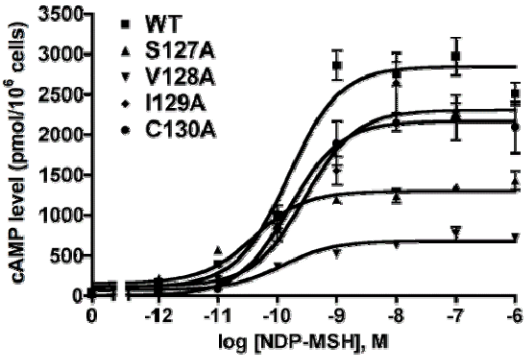
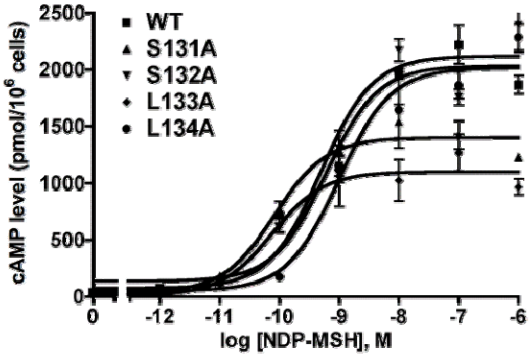

Binding (% WT)
log [NDP-MSH], M
WT
L141A
S142A
I143A
A144G
V145A

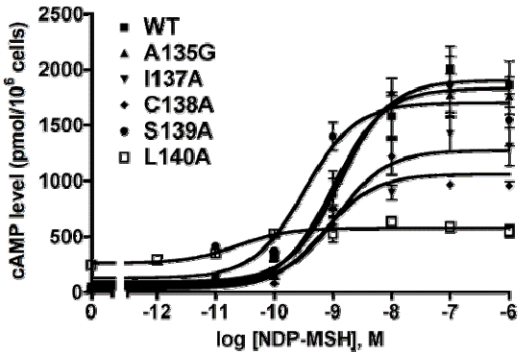
Figure II-4. Signaling properties of the WT and mutant hMC4Rs with NDP-MSH as the ligand. Intracellular cAMP levels were measured using RIA as described. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed 3-5 times.


WT
V119A
N120A
I121A
D122A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M


WT
N123A
V124A
I125A
D126A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M


WT
S127A
V128A
I129A
C130A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M


WT
S131A
S132A
L133A
L134A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M


WT
A135G
I137A
C138A
S139A
L140A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M

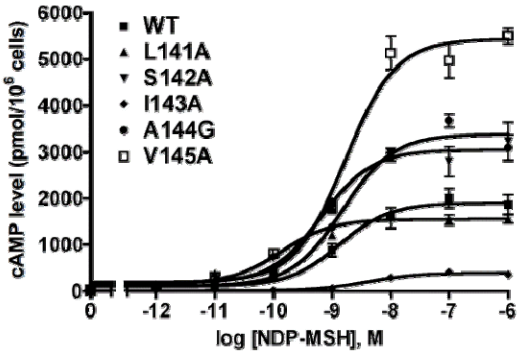

WT
L141A
S142A
I143A
A144G
V145A
cAMP level (pmol/10^6 cells)
log [NDP-MSH], M

Figure II-5. Constitutive activities of the WT and TM3 mutant hMC4Rs. Intracellular basal cAMP levels in cells transiently transfected with WT or mutant hMC4Rs were measured in the absence of any ligand. The results are expressed as % of WT basal activity. All experiments were performed 3-5 times. The basal cAMP levels were 47.25 ± 4.98 pmol/10^6 cells (mean ± SEM of 29 experiments). The statistical significance are indicated as follows: a: significantly different from WT hMC4R, $p < 0.01$; b: significantly different from WT hMC4R, $p < 0.001$.

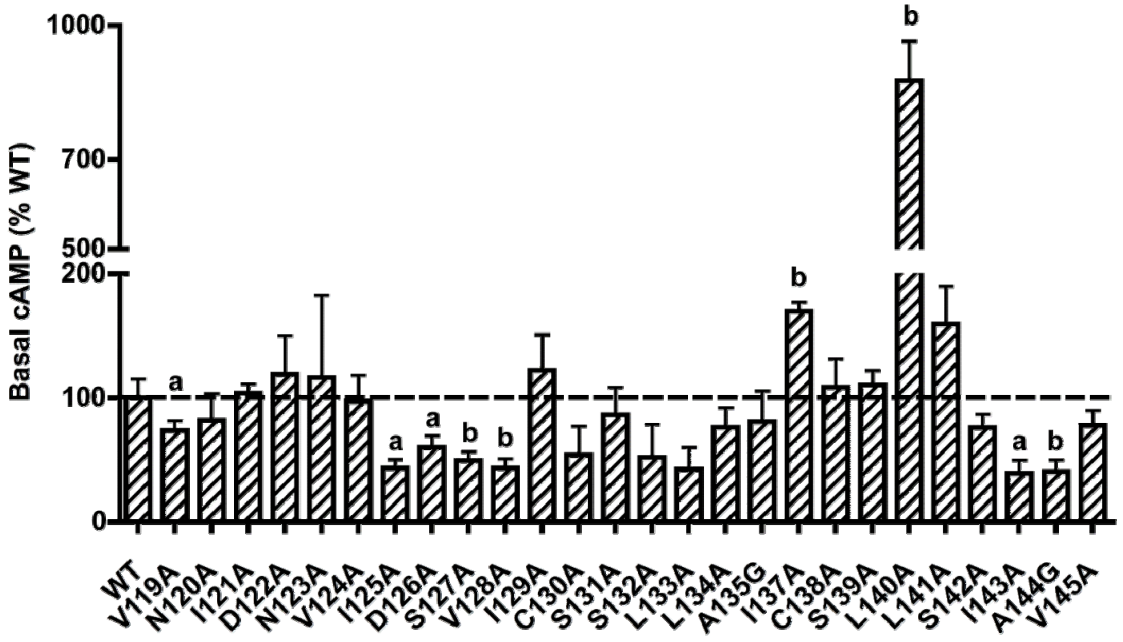

Basal cAMP (% WT)
1000
700
500
200
100
0
a
a
b
b
b
b
WT
V119A
N120A
I121A
D122A
N123A
V124A
I125A
D126A
S127A
V128A
I129A
C130A
S131A
S132A
L133A
L134A
A135G
I137A
C138A
S139A
L140A
L141A
S142A
I143A
A144G
V145A

Figure II-6. Cell surface expression (A) and total expression (B) of WT and L140 mutant hMC4Rs measured by flow cytometry. See the legend to Figure II-2 for details. The statistical significance are indicated as follows: a: significantly different from WT hMC4R, $p < 0.05$; b: significantly different from WT hMC4R, $p < 0.001$.

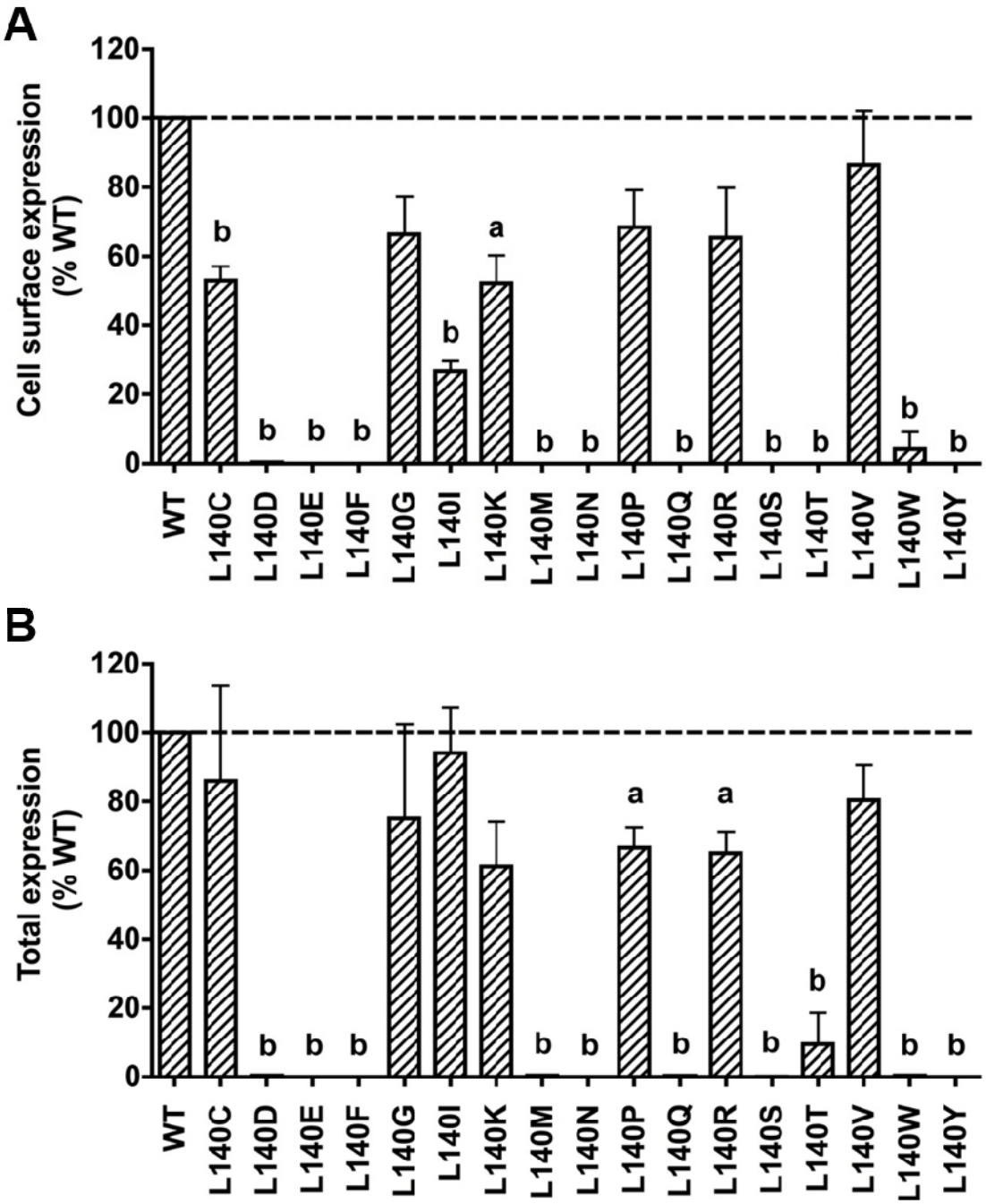

A
Cell surface expression (% WT)
120
100
80
60
40
20
0
b
b
b
b
b
b
a
b
b
b
b
b
b
b
b
WT
L140C
L140D
L140E
L140F
L140G
L140I
L140K
L140M
L140N
L140P
L140Q
L140R
L140S
L140T
L140V
L140W
L140Y
B
Total expression (% WT)
120
100
80
60
40
20
0
b
b
b
a
a
b
b
b
b
b
b
b
WT
L140C
L140D
L140E
L140F
L140G
L140I
L140K
L140M
L140N
L140P
L140Q
L140R
L140S
L140T
L140V
L140W
L140Y

Figure II-7. Total receptor expression of WT and ten L140 hMC4R mutants, that with totally abolished total receptor expression, measured by flow cytometry with or without the treatment of proteasome inhibitor (25 μM MG132), or lysosome inhibitors (25 mM NH_4Cl or 100 μM chloroquine). The results are expressed as percentage of cell total expression levels of the control of WT hMC4R after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and methods. Data were mean ± SEM of 3 experiments.

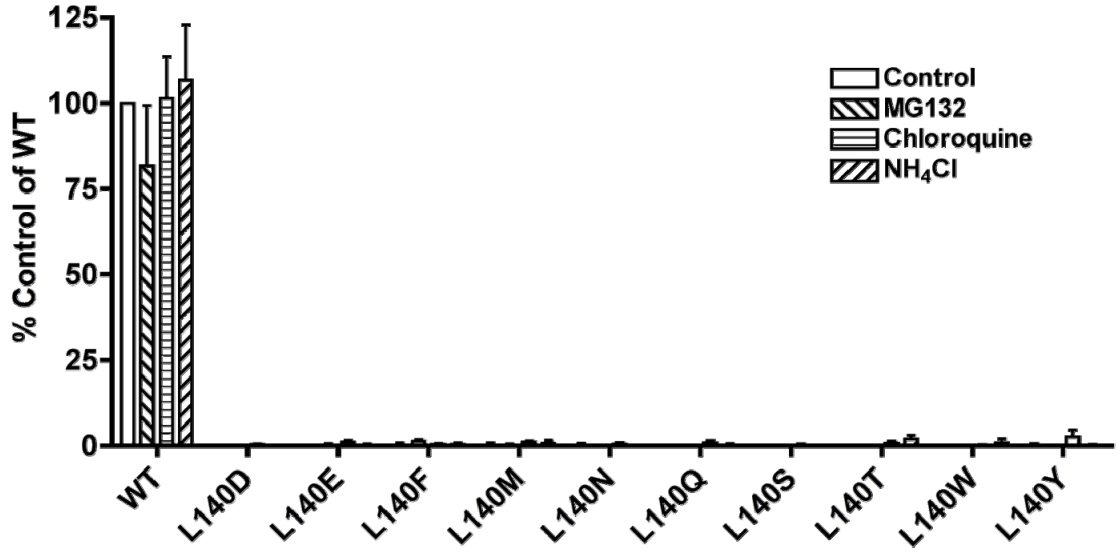
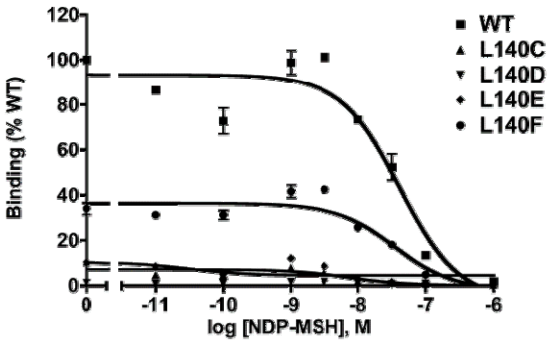
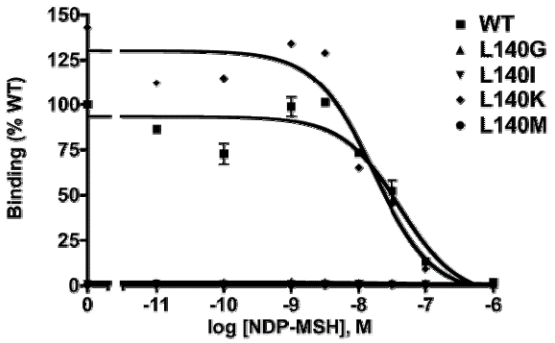
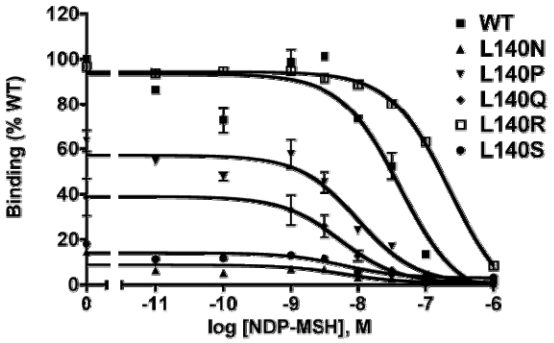

125
100
75
50
25
0
% Control of WT
Control
MG132
Chloroquine
NH4Cl
WT
L140D
L140E
L140F
L140M
L140N
L140Q
L140S
L140T
L140W
L140Y

Figure II-8. Ligand binding properties of the WT and L140 mutant hMC4Rs with NDP-MSH as the ligand. The binding of ^{125}I-NDP-MSH to hMC4R on intact cells were measured as described in Materials and methods. Results shown are expressed as percentage of WT binding ± range from duplicate determinations within one experiment. All experiments were performed 3-5 times.


WT
L140C
L140D
L140E
L140F
Binding (% WT)
log [NDP-MSH], M


WT
L140G
L140I
L140K
L140M
Binding (% WT)
log [NDP-MSH], M


WT
L140N
L140P
L140Q
L140R
L140S
Binding (% WT)
log [NDP-MSH], M

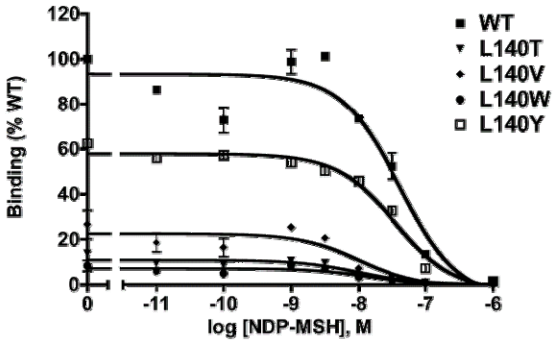
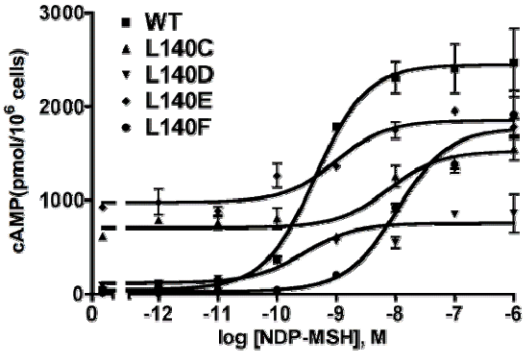
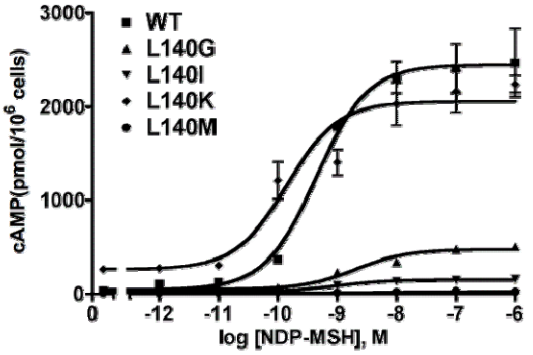
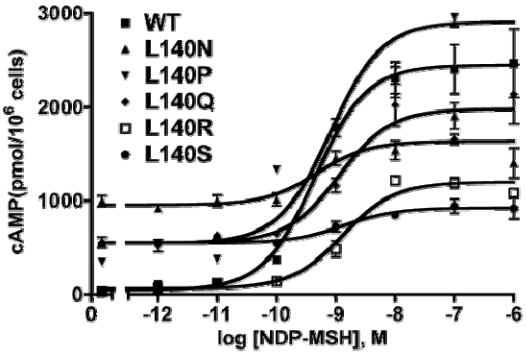

WT
L140T
L140V
L140W
L140Y
Binding (% WT)
log [NDP-MSH], M

Figure II-9. Signaling properties of the WT and L140 mutant hMC4Rs with NDP-MSH as the ligand. Intracellular cAMP levels were measured using RIA as described. Results are expressed as the mean ± SEM of triplicate determinations within one experiment. All experiments were performed 3-5 times.


WT
L140C
L140D
L140E
L140F
cAMP(pmol/10^6 cells)
3000
2000
1000
0
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M


WT
L140G
L140I
L140K
L140M
cAMP(pmol/10^6 cells)
3000
2000
1000
0
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M


WT
L140N
L140P
L140Q
L140R
L140S
cAMP(pmol/10^6 cells)
3000
2000
1000
0
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

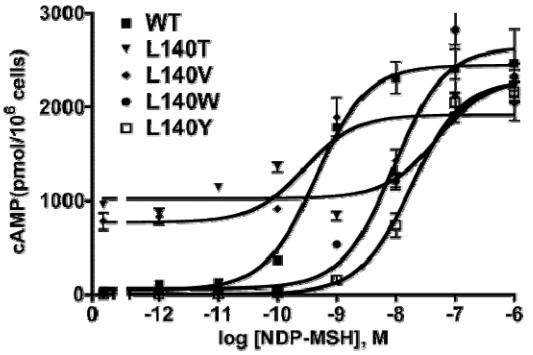

WT
L140T
L140V
L140W
L140Y
cAMP(pmol/10^6 cells)
3000
2000
1000
0
0
-12
-11
-10
-9
-8
-7
-6
log [NDP-MSH], M

Figure II-10. cAMP and ERK 1/2 signaling of WT and constitutively active L140 mutant hMC4Rs. cAMP accumulation (A) and ERK 1/2 phosphorylation (B-C) were assessed after a 1-h and a 5-min incubation as described in Materials and methods. Results are expressed as percentage of the value obtained in nonstimulated WT MC4R cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from WT hMC4R, $p < 0.05$; b: significantly different from WT hMC4R, $p < 0.01$; c: significantly different from WT hMC4R, $p < 0.001$.

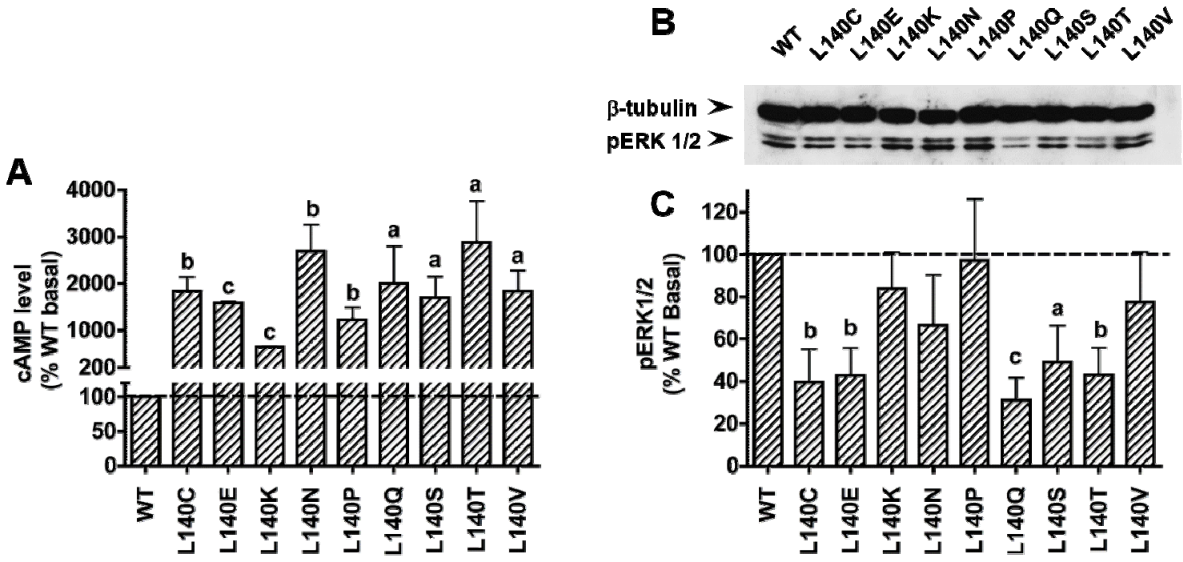
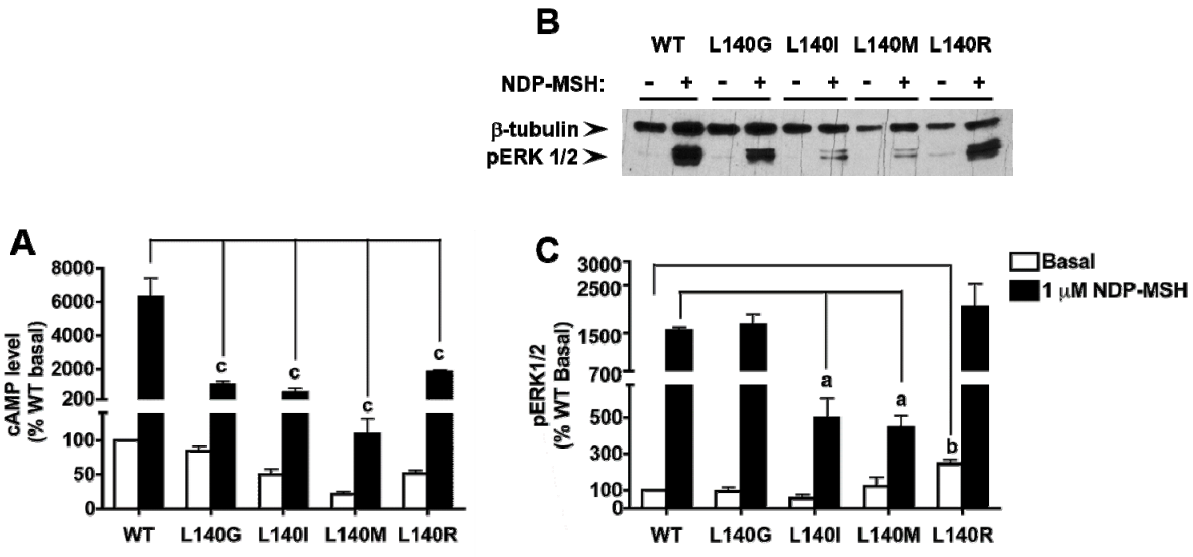

A
cAMP level
(% WT basal)
4000
3000
2000
1000
200
100
50
0
b
c
c
b
a
a
a
b
WT
L140C
L140E
L140K
L140N
L140P
L140Q
L140S
L140T
L140V
B
WT
L140C
L140E
L140K
L140N
L140P
L140Q
L140S
L140T
L140V
β-tubulin
pERK 1/2
C
pERK1/2
(% WT Basal)
120
100
80
60
40
20
0
b
b
c
a
b
WT
L140C
L140E
L140K
L140N
L140P
L140Q
L140S
L140T
L140V

Figure II-11. cAMP and ERK 1/2 signaling of WT and signal defective L140 mutants. See the legend to Figure II-10 for details. cAMP accumulation (A) and ERK 1/2 phosphorylation (B-C) were assessed after a 1-h and a 5-min incubation as described in Materials and methods. Results are expressed as percentage of the value obtained in nonstimulated WT MC4R cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from WT hMC4R, $p < 0.05$; b: significantly different from WT hMC4R, $p < 0.01$; c: significantly different from WT hMC4R, $p < 0.001$.


B
WT
L140G
L140I
L140M
L140R
NDP-MSH:
- + - + - + - + - +
β-tubulin
pERK 1/2
A
cAMP level
(% WT basal)
8000
6000
4000
2000
200
100
50
0
c
WT
L140G
L140I
L140M
L140R
C
pERK1/2
(% WT Basal)
3000
2500
1500
700
500
300
100
0
a
b
Basal
1 μM NDP-MSH

Chapter 3

3.1 Introduction

G protein-coupled receptors (GPCRs) are versatile signaling molecules that regulate almost all physiological processes including energy homeostasis (Tao, et al. 2013).The melanocortin-4 receptor (MC4R) is a member of family A GPCRs that has been shown to be a critical regulator of energy homeostasis, regulating both energy intake and expenditure (Balthasar et al. 2005; Huszar et al. 1997). Human genetic studies have identified that defects in *MC4R* are the most common monogenic form of obesity, characterized by its early-onset and severity (Farooqi et al. 2003). About 170 *MC4R* mutations, including nonsense, missense, frameshift, and inframe deletions, have been identified from obese patient cohorts of different ethnic origins (Tao 2005, 2010).

Since the cloning of the MC4R by Gantz et al. in 1993 (Gantz et al. 1993), it has been established that it primarily couples to the stimulatory G protein (G_s), which increases adenylyl cyclase activity, and subsequently leads to increased cyclic adenosine monophosphate (cAMP) production that then enhances the activity of protein kinase A (PKA). The activation of MC4R by the endogenous agonist, α-melanocyte stimulating hormone (α-MSH), results in decreased food intake and increased energy expenditure, while the inhibition of MC4R by the endogenous antagonist, agouti-related protein (AgRP), leads to increased food intake (Fan et al. 1997; Fong et al. 1997; Ollmann et al. 1997).

Previous functional studies of MC4R on both naturally occurring and laboratory-generated mutants have relied primarily on measurements of intracellular cAMP generation. For example, studies on the naturally occurring mutations identified some mutations with defects in intracellular cAMP signaling (reviewed in (Hinney et al. 2013; Tao 2005, 2009, 2010)). However, some mutants show no functional alterations on intracellular cAMP accumulation (Tao and Segaloff 2003; Wang and Tao 2011), and some mutants even constitutively activate cAMP production (Tao 2008; Vaisse, et al. 2000). Six naturally occurring mutations identified in obese patients, including H76R (Stutzmann, et al. 2008; Wang and Tao 2011), S127L (Fan and Tao 2009; Hinney et al. 2003), D146N (Stutzmann et al. 2008; Wang and Tao 2011), P230L (Fan and Tao 2009; Hinney et al. 2003), L250Q (Proneth, et al. 2006; Tao et al. 2010; Vaisse et al. 2000) and F280L (Beckers, et al. 2010; Wang and Tao 2011) (Fig. III-1), have been shown to cause constitutive activation. The obesity observed in vivo in these patients could not easily be explained by the in vitro cellular phenotype of these mutations. Indeed, gain of function mutation is expected to result in lean phenotype, even anorexia nervosa. This suggests that signaling pathways other than G_s might contribute to the physiological effect of the MC4R. Indeed, in addition to the conventional G_s-cAMP-PKA pathway, it has been demonstrated recently that MC4R also activates p44/42 mitogen-activated protein kinases (MAPK), also known as extracellular signal-regulated kinases 1 and 2 (ERK1/2), both in vitro and in vivo in rat hypothalamus (Chai et al. 2006; Daniels et al. 2003; Vongs et al. 2004). It has also been demonstrated that ERK1/2 is involved in melanocortin-induced decreases in food intake (Sutton et al. 2005). Thus, activation of ERK1/2 pathway is one cellular mechanism that may underlie the regulation of energy homeostasis mediated by the MC4R.

Recently, a few studies discovered the biased activation of ERK1/2 in MC4R. It has been shown that one mutant MC4R (D298N) retains cAMP generation, but abolishes ERK1/2 activation (Patten et al. 2007). We also reported that several artificially generated mutant MC4Rs have divergent cAMP and ERK1/2 signaling cascade upon agonist stimulation (Huang and Tao 2012; Mo, et al. 2012). However, little is known about the alteration of ERK1/2 pathway in naturally occurring MC4R mutants, and how MC4R ligands may regulate the ERK1/2 signaling cascade that is crucial to gain a better understanding of MC4R functions at the molecular basis. In the present study, we assessed the potential of MC4R ligands, particularly the antagonists, to regulate MAPK activation in WT and the six naturally occurring constitutively active mutant (CAM) MC4Rs.

3.2 Materials and methods

3.2.1 Reagents and supplies

[Nle^4, D-Phe^7]-α-melanocyte stimulating hormone (NDP-α-MSH) was purchased from Peptides International (Louisville, KY). AgRP(83-132) (Fig. III-2A) was purchased from Phoenix Pharmaceutical Inc. (Mountain View, CA). Ac-D-2-Nal-Arg-2-Nal-NH_2 (MCL0020) (Chaki, et al. 2003) (Fig. III-2B) was purchased from Tocris Bioscience (Ellisville, MO). Ipsen 5i (Poitout, et al. 2007) (Fig. III-2C) and ML00253764 (Vos, et al. 2004) (2-[2-[2-(5-bromo-2-methoxy-phenyl)-ethyl]-3-fluorophenyl]-4,5-dihydro-1H-imidazol) (Fig. III-2D) were custom synthesized by Enzo Life Science, Inc. (Plymouth Meeting, PA). [^{125}I]-cAMP was iodinated using chloramine T method. Cell culture plates and flasks were purchased from Corning (Corning, NY). Cell culture media, newborn calf serum, antibiotics and reagents were obtained from Invitrogen (Carlsbad, CA).

3.2.2 Cell culture and DNA transfection

HEK293T cells were obtained from American Type Culture Collection (Manassas, VA), and maintained at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10% newborn calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 50 μg/mL gentamicin. Cells were plated on gelatin-coated 35 mm 6-well clusters and transfected at 50-70% confluency using calcium phosphate transfection method (Chen and Okayama 1987). GT1-7 cells were generously provided by Dr. Pamela Mellon (Mellon, et al. 1990), and maintained at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 50 μg/mL gentamicin. For preparation of cellular lysates for immunoblotting, cells were plated on gelatin-coated 100 mm dishes, then cells were washed twice and incubated with Waymouth/BSA (Waymouth's MB752/1 media (Sigma-Aldrich, St. Louis, MO) containing 1 mg/ml bovine serum albumin (BSA)) 24 h after transfection (or 48h after plating GT1-7 cells) and starved overnight (18h for HEK293T or 24h for GT1-7) at 37 ^{o}C before ligand stimulation.

3.2.3 cAMP accumulation assay

HEK293T cells were washed twice, 48 hours after transfection, with warm Waymouth/BSA and then incubated in fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) at 37 ^{o}C for 15 min. Subsequently, either buffer alone or different ligands (1 μM NDP-MSH (Mo et al. 2012), 10 nM AgRP (Tao et al. 2010), 1 μM MCL0020 (Chaki et al. 2003), 1 μM ML00253764 (Tao 2010) or 1 μM Ipsen 5i (Tao et al. 2010)) were added and the incubation was continued for another hour. Cells were then

solubilized with cold 0.5 N percholoric acid containing 180 μg/ml theophylline (phosphodiesterase inhibitor). The supernatant was collected to measure the level of cAMP production using radioimmunoassay (Tao et al. 2010). All determinations were performed in triplicate, and each experiment was performed three times independently.

3.2.4 ERK1/2 phosphorylation assay

The phosphorylated ERK1/2 (pERK1/2) activity was measured as described previously (Mo et al. 2012). Briefly, 48 hours after transfection (or 72 hours after plating GT1-7 cells), cells were treated for 5 min (or various times for time-course study) with either buffer alone or different ligands (1 μM NDP-MSH, 10 nM AgRP, 1 μM MCL0020, 1 μM ML00253764 or 1 μM Ipsen 5i). The time points in the time-course experiments were chosen based on previous reports of MC4R-mediated ERK1/2 signaling (Chai et al. 2006; Damm, et al. 2012; Vongs et al. 2004). Cells were solubilized in lysis buffer and lysates were separated on 10% SDS-PAGE gel. Proteins were then transferred onto PVDF membrane. pERK1/2 and β-tubulin were detected by immunoblotting with rabbit anti-pERK1/2 antibody (1:1000~1:2000, Cell Signaling Technology, Beverly, MA) and mouse anti-β-tubulin antibody (1:5000~1:10000, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA), respectively. Blots were probed with horseradish peroxidase-conjugated secondary donkey anti-rabbit (1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA) and horseradish peroxidase-conjugated donkey anti-mouse (1:5000~1:10000, Jackson ImmunoResearch Laboratories) at room temperature for 2 h. The membranes were then visualized using enhanced chemiluminescence reagent (Pierce, Rockford, IL) and quantified using ImageJ 1.44 software (National Institute of Health, Bethesda, MD) after densitometric scanning of the films. ERK1/2 phosphorylation was normalized according to the loading of proteins by expressing the data as a ratio of pERK1/2 over β-tubulin.

3.2.5 Statistics

The significance of differences in cAMP accumulation and pERK1/2 activities were analyzed using paired two-tail Student's t-test with Prism 4.0 Software.

3.3 Results

3.3.1 cAMP and pERK1/2 signaling efficacies of MC4R agonist NDP-MSH

HEK293T cells lacking endogenous expression of the MC4R have been widely used as a mammalian expression system in MC4R studies. We used HEK293T cells transiently transfected with the WT or CAM hMC4R to study the effect of NDP-MSH on the conventional cAMP pathway. In accordance with our previous reports (Fan and Tao 2009; Tao et al. 2010; Wang and Tao 2011), the CAM hMC4Rs had significantly increased basal cAMP levels at 2-10 fold that of the WT (Fig. III-3A). NDP-MSH acted as an agonist for the WT and CAM hMC4R in the cAMP pathway, inducing significantly increased cAMP accumulation (Fig. III-3A).

To evaluate the efficacy of NDP-MSH on MAPK signaling, we first performed the time course study of NDP-MSH-induced ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT hMC4R. We found that NDP-MSH behaved as an agonist inducing a sustained activation of ERK1/2 with a 3-fold maximal increase at 5 min upon stimulation with 1 μM NDP-MSH (Fig. III-3B-C). The activation of ERK1/2 sustained for up to 30 min, and the level of pERK1/2 decreased to 59% of the maximal response at 60 min, but was still 1.8-fold higher compared to the basal pERK1/2 level (Fig. III-3B-C). No significant change of pERK1/2 level was observed upon NDP-MSH treatment in HEK293T cells transiently transfected with empty vector (data not shown).

To exclude artifact due to overexpression of the MC4R in a non-neuronal cell line, we also studied the NDP-α-MSH-induced ERK1/2 activation in a mouse hypothalamic cell line, GT1-7, that express murine (m) MC4R endogenously (Khong, et al. 2001; Shinyama, et al. 2003). Stimulation with 1 μM NDP-α-MSH induced a 2.3-fold transient activation of pERK1/2 with maximal response at 5 min after addition of 1 μM NDP-MSH (Fig. III-3B-C). The level of pERK1/2 quickly decreased to 60% of basal level after 20 min incubation.

Based on the kinetic profile of ERK1/2 activation in WT MC4R (Fig. III-3B-C), the pERK1/2 levels of the CAM MC4Rs were measured after 5 min stimulation with NDP-MSH in HEK293T cells transiently transfected with CAM MC4Rs. As shown in Fig. 3D-E, we found that NDP-MSH acted as an agonist for ERK1/2 signaling cascade in all CAM hMC4Rs, inducing significantly increased pERK1/2 level upon 5 min NDP-MSH stimulation. In addition, we found that all six CAM hMC4Rs displayed constitutively active basal pERK1/2 signaling (Fig. III-3F). Similar observations were made before in our previous report that some mutant hMC4Rs have increased basal cAMP production also have enhanced basal ERK1/2 phosphorylation (Huang and Tao 2012).

3.3.2 cAMP and pERK1/2 signaling efficacies of antagonist AgRP(83-132)

The melanocortin system is unique in having two endogenous antagonists, agouti and agouti-related protein (AgRP) (Tao et al. 2010). AgRP is an antagonist for the hMC4R. Previous studies have shown that a fragment of AgRP, AgRP(83-132), is indeed an inverse agonist that decreases basal cAMP signaling of WT or CAM hMC4Rs (Chai et al. 2003; Nijenhuis et al. 2001; Tao et al. 2010). In the present study, we showed that AgRP(83-132) indeed acted as inverse agonist, causing 50%-80% decrease of basal cAMP level of WT and six CAM hMC4Rs (Fig. III-4A) consistent with previous reports.

Although AgRP(83-132) has been demonstrated to be an inverse agonist in cAMP signaling pathway, little is known how it may regulate the ERK1/2 signaling. To study the effect of AgRP on ERK1/2 phosphorylation, we first studied the temporal dependence of pERK1/2 activity in HEK293T cells transiently transfected with WT hMC4R treated with 10 nM AgRP(83-132). We found that, interestingly, AgRP(83-132) acted as an agonist for MAPK cascade, inducing a transient phosphorylation of ERK1/2 with a 2.3-fold maximal increase at 5 min after stimulation with 10 nM AgRP(83-132) (Fig. III-4B-C). The activity of pERK1/2 decreased to 60% of the maximal response at 10 min and attenuated to almost the basal pERK1/2 level after 45 min. We did not observe significant change of pERK1/2 level in HEK293T cells transiently transfected with empty vector upon AgRP(83-132) treatment (data not shown).

A similar kinetic pattern of ERK1/2 phosphorylation was observed in GT1-7 cells endogenously expressing mMC4R. We found that AgRP(83-132) behaved as an agonist for ERK1/2 signaling in GT1-7 cells as that in HEK293T cells, inducing a transient maximal response at 5 min with 2.1-fold increase of pERK1/2 level upon AgRP(83-132) stimulation (Fig. III-4B-C). The level of pERK1/2 decreased to almost the basal pERK1/2 level after 10 min. The overall identical pattern of ERK1/2 phosphorylation for both exogenous (HEK293T cells) and endogenous (GT1-7 cells) receptor expression suggested that the observed ERK1/2 activation upon AgRP(83-132) stimulation was a physiological response for MC4R, but not an artifact of receptor overexpression in HEK293T cells.

Therefore, in subsequent experiments a stimulation of 5 min with 10 nM AgRP(83-132) was used to study the ERK1/2 signaling in other CAM hMC4Rs in HEK293T cells. In all six CAM hMC4Rs, we showed that AgRP(83-132) also acted as an agonist inducing significantly increased (1.6-2.8 fold increase) pERK1/2 level upon 5 min stimulation with AgRP(83-132) (Fig.

III-4D-E). These results demonstrated that AgRP(83-132), commonly classified as inverse agonist for the cAMP signaling pathway decreasing basal cAMP level, was shown to exert agonist properties on the MAPK signaling cascade activating the phosphorylation of ERK1/2 in WT and six CAM hMC4Rs.

3.3.3 cAMP and pERK1/2 signaling efficacies of peptidomimetic antagonist MCL0020

MCL0020, a tri-peptide mimetic MC4R antagonist, was first reported to exert its function as a neutral blocker exhibiting antidepressant, anxiolytic and anti-stress effect (Chaki et al. 2003). In the present study, however, we demonstrated that MCL0020 actually acted as inverse agonist inducing 46-85% decrease of the basal cAMP activities of WT or six CAM hMC4Rs (Fig. III-5A).

The effect of MCL0020 on the MAPK signaling was first assessed in HEK293T cells transiently transfected with WT hMC4R. We showed that MCL0020, behaving as an agonist, significantly activated a transient phosphorylation of ERK1/2, with 2.7-fold maximal increase at 5 min (Fig. III-5B-C). The pERK1/2 level decreased to 48% of the maximal response at 20 min and returned almost to the basal pERK1/2 level after 45 min (Fig. III-5B-C). No significant phosphorylation of ERK1/2 was observed in HEK293T cells transiently transfected with empty vector upon MCL0020 treatment (data not shown).

MCL0020 exhibited similar agonist activity for the MAPK signaling pathway in GT1-7 cells endogenously expressing mMC4R. We showed that 1 μM MCL0020 induced a transient activation of ERK1/2, with a maximal 2-fold phosphorylation at 5 min (Fig. III-5B-C). Subsequently, the pERK1/2 activity decreased rapidly to the basal level after 10 min incubation with MCL0020 (Fig. III-5B-C). These results demonstrated that the ERK1/2 phosphorylation

induced by MCL0020 was a physiological consequence of MC4R and was not due to the overexpression of receptor in HEK293T cells.

Therefore, 5 min incubation was used for subsequent study of the effect of MCL0020 in MAPK signaling in six CAM hMC4Rs. We showed that MCL0020 also exhibit agonist activity for the MAPK signaling in all six CAM hMC4Rs, inducing significantly increased pERK1/2 activity upon MCL0020 stimulation (Fig. III-5D-E). These results suggested that MCL0020, commonly classified as neutral antagonist, was shown to exhibit inverse agonist activity for the cAMP signaling pathway decreasing the basal cAMP level, but to behave as an agonist for the MAPK signaling cascade activating the phosphorylation of ERK1/2 in WT and six CAM hMC4Rs.

3.3.4 cAMP and pERK1/2 signaling efficacies of antagonist Ipsen 5i

Ipsen 5i, first synthesized by Poitout and his colleagues at Ipsen Research Laboratories, was reported to act as a selective MC4R antagonist with 2 nM binding affinity (Poitout et al. 2007). Here, we showed that Ipsen 5i was also an inverse agonist for both WT and CAM MC4Rs, with maximal inhibition of basal cAMP ranged from 37% to 80% (Fig. III-6A) consistent with our previous findings (Tao et al. 2010).

Next, we tested the potential of Ipsen 5i to affect the MAPK signaling in HEK293T cells transiently transfected with WT hMC4R and GT1-7 cells endogenously expressing the mMC4R. We found that the pERK1/2 level has no significant differences upon incubation with 1 μM Ipsen 5i within 1h, for both HEK293T and GT1-7 cells (Fig. III-6B-C). Even though we did not observe the change of MAPK signaling for WT MC4R, based on the results with the other ligands (NDP-MSH, AgRP and MCL0020), we still chose 5 min incubation to study the effect of Ipsen 5i for MAPK signaling of other six CAM hMC4Rs.

As shown in Fig. III-6D-E, Ipsen 5i induced significant increases of ERK1/2 phosphorylation for 3 CAM hMC4Rs (P230L, L250Q and F280L), acting as an agonist for MAPK cascade. No significant difference was observed for other 3 CAM hMC4Rs, including H76R, S127L and D146N, upon 5 min treatment with 1 μM Ipsen 5i. These results demonstrated that Ipsen 5i, normally identified as inverse agonist for cAMP signaling, exhibited agonist activity for MAPK signaling in some specific CAM hMC4Rs.

3.3.5 cAMP and pERK1/2 signaling efficacies of another small molecule antagonist ML00253764

ML00253764, first reported as MC4R antagonist, has been demonstrated to effectively reduce cancer cachexia (Nicholson, et al. 2006; Vos et al. 2004). Here, we showed that ML00253764 behaved as an inverse agonist for both WT and CAM hMC4Rs, inducing maximal decrease of basal cAMP level range from 47% to 82% (Fig. III-7A), consistent with our previous reports (Tao 2010; Tao et al. 2010).

The potential of ML00253764 to regulate MAPK signaling was first examined in HEK293T cells transiently transfected with WT hMC4R. As shown in Fig. III-7B-C, ML00253764, acting as an agonist for MAPK signaling, induced a significantly sustained activation of ERK1/2 with 2.9-fold maximal response at 5 min. The ML00253764-induced pERK1/2 level sustained for up to 1h, still with 2.0-fold increase over basal level (Fig. III-7B-C). We did not observe significant ERK1/2 activation upon ML00253764 treatment in HEK293T cells transiently transfected with empty vector (data not shown).

In GT1-7 cells, the ML00253764 also acted as an agonist for MAPK signaling, inducing a transient ERK1/2 phosphorylation with 2.0-fold maximal response at 5 min (Fig. III-7B-C). The level of ML00253764-induced pERK1/2 returned to the basal level after 20 min incubation

with ML00253764 (Fig. III-7B-C). Overall, the patterns of ERK1/2 phosphorylation in HEK293T and GT1-7 cells were similar in terms of the maximal activation at 5 min, suggesting that the observed ML00253764-induced ERK1/2 activation was physiological and not due to receptor overexpression in HEK293T cells. Therefore, 5 min incubation was used subsequently to study the effect of ML00253764 to regulate MAPK signaling in six CAM hMC4Rs.

As shown in Fig. III-7D-E, four CAM hMC4Rs (D146N, P230L, L250Q and F280L) had significantly elevated pERK1/2 level upon 5 min ML00253764 stimulation, with 2.2-3.6 fold increase of ERK1/2 activity. However, no significant change of pERK1/2 level was observed for the other two CAM hMC4Rs (H76R and S127L) upon 1 μM ML00253764 treatment (Fig. III-7D-E). These results demonstrated that ML00253764, which is normally classified as inverse agonist, was shown to behave as agonist inducing significantly increased pERK1/2 level in WT and some CAM hMC4Rs.

3.4 Discussion

In the present study, our results demonstrated that four ligands that were previously classified as antagonists or inverse agonists in terms of the G_s-cAMP cascade, including AgRP, MCL0020, Ipsen 5i, and ML00253764, exhibited agonist activity on the MAPK pathway. These findings are not consistent with the classical description of ligand efficacy, in which agonists and antagonists were purely defined in the scenario of the most common G protein-mediated signaling (Kenakin 2005). These results are more in accordance with the developing concept that ligands can exhibit dual efficacy for divergent cellular signaling pathways, and thus resulting in different physiological functions (reviewed in (Whalen et al. 2011)).

As summarized in Table 3.1, our results demonstrated that NDP-α-MSH acted as balanced agonist activating both cAMP and MAPK signaling pathways in the WT and all six CAM MC4Rs. Peptidic inverse agonist AgRP(83-132) and peptidomimetic inverse agonist MCL0020 behaved as biased agonists activating MAPK signaling, whereas decreasing cAMP production in WT and six CAM MC4Rs. Small molecule inverse agonist Ipsen 5i exhibited biased agonist efficacy for MAPK signaling in three CAM MC4Rs, including P230L, L250Q and F280L. Another small molecule inverse agonist ML00253764 acted as biased agonist inducing ERK1/2 phosphorylation in WT and four CAM MC4Rs, but not H76R or S127L.

NDP-α-MSH has been demonstrated to lead to sustained activation of ERK1/2 phosphorylation in dose- and time-dependent manner in CHO cells stably expressing WT hMC4R (Vongs et al. 2004) and in GT1-1 cells endogenously expressing mMC4R (Chai et al. 2006). In the present study, we showed that, in the HEK293T cells transiently transfected with WT hMC4R, NDP-α-MSH induced a sustained activation of ERK1/2 phosphorylation. However, we showed that in GT1-7 cells, NDP-α-MSH induced a more transient ERK1/2 activation, which is different from other cell lines (Fig. III-3B-C). A similar finding that α-MSH induced a transient ERK1/2 activation in GT1-7 cells, was recently reported (Damm et al. 2012). Therefore, our present study and those of others (Chai et al. 2006; Daniels et al. 2003; Vongs et al. 2004) suggested that MC4R mediated NDP-MSH-induced ERK1/2 activation could be cell-line dependent.

AgRP was initially identified as an orexigenic stimulus, through competitive binding of melanocortins to the MC4R (Ollmann et al. 1997). AgRP was later found to be an inverse agonist at the MC4R (Adan and Kas 2003; Haskell-Luevano and Monck 2001; Nijenhuis et al. 2001), suggesting that AgRP might exerts its function independently of melanocortins. Several

additional lines of evidence (Tolle and Low 2008; Wu et al. 2008) revealed that the effects of AgRP on energy homeostasis could be independent of melanocortin signaling. Our results demonstrated that AgRP(83-132) behaved as a biased agonist activating ERK1/2 signaling in WT and CAM MC4Rs in HEK293T cells. We also showed that AgRP(83-132) also behaved as an agonist activating ERK1/2 signaling in GT1-7 cells. The ERK1/2 upstream mediator has not been determined in the present study and need further investigation. Some alternative signaling pathways of AgRP have been identified recently, including $G_{i/o}$ or arrestin-dependent pathways (reviewed in (Breit, et al. 2011)). It has been shown that AgRP mediates a pertussis toxin-sensitive $G_{i/o}$-coupled signaling (Buch et al. 2009), and moreover $G_{i/o}$ proteins can activate the ERK1/2 signaling (Ferraguti, et al. 1999; Pace, et al. 1995; Shahabi, et al. 1999). In addition, it has been demonstrated that AgRP induces arrestin-mediated MC4R endocytosis in HEK293 or Cos-1 cells (Breit et al. 2006). Therefore, $G_{i/o}$ proteins or arrestins might be potential mediators of AgRP-induced ERK1/2 phosphorylation in WT and CAM MC4Rs.

In addition to AgRP, we further tested the potential of other MC4R inverse agonists, including MCL0020, Ipsen 5i and ML00253764, in activating ERK1/2 signaling. We found that all these inverse agonists exhibited agonist efficacy for the ERK1/2 signaling. In other GPCRs, several antagonists or inverse agonists have been identified to induce biased ERK1/2 signaling (Ahn, et al. 2012; Azzi, et al. 2003; Benned-Jensen, et al. 2011; Wisler, et al. 2007). For example, carvedilol, a nonsubtype-selective β-adrenergic receptor inverse agonist, has been shown to mediate a G-protein independent, β-arrestin2-dependent ERK1/2 activation (Wisler et al. 2007). These results suggested that the dual efficacy of inverse agonists might be a general concept applicable to other GPCRs.

MCL0020 was first described to be a neutral antagonist without affecting basal cAMP activity in COS-1 cells (Chaki et al. 2003). In the present study, however, we demonstrated that MCL0020 acted as a partial inverse agonist that decreasing basal cAMP accumulation by 46-85% of the in HEK293T cells transiently transfected with WT or CAM MC4Rs. Therefore, the effect of MCL0020 to MC4R might be cell-line dependent.

In addition to the biased ligands, some receptor mutants have been reported to be intrinsically biased even stimulated with traditional balanced ligands (Mo et al. 2012; Shenoy et al. 2006; Wei et al. 2003; Zhai et al. 2005). In the present study, we demonstrated that the patterns of biased ERK1/2 signaling in different MC4R mutants varied even in response to the same ligand. For example, upon Ipsen 5i stimulation three CAM MC4Rs (P230L, L250Q and F280L) had decreased cAMP, but increased ERK1/2 signaling, whereas WT and other three CAM MC4Rs (H76R, S127L and D146N) had only decreased cAMP signaling, but no significant change of ERK1/2 activity was observed. ML00253764 activated ERK1/2 signaling in WT and four CAM MC4Rs (D146N, P230L, L250Q and F280L) but no effect on ERK1/2 activity in other two CAM MC4Rs (H76R and S127L). These results suggested that there were some structural requirements of receptor for ligands to exert their dual efficacies. Indeed, recent crystal structures of β_1-adrenoceptor (Warne, et al. 2012) and serotonin receptor (Wacker, et al. 2013) proved that the conformation of receptor bound with biased ligand is different from that with balanced ligand. Further systematic identification of intrinsically biased receptor mutants might be an alternative way to help designing more potent biased ligands.

Conventionally, the constitutive activity of receptor was solely characterized using the G-protein dependent signaling. Numerous CAM GPCRs have been reported to associate with diseases (Tao 2008). Even some WT receptors also have considerable constitutive activity

(Seifert and Wenzel-Seifert 2002; Tao 2008). Basal signaling of MC4R might be important for maintaining energy homeostasis in humans (Srinivasan et al. 2004). Paradoxically, several constitutively active mutants were identified from obese patients (reviewed in (Tao 2010)). Increased constitutive activity is expected to result in decreased food intake and increased energy expenditure, therefore the lean phenotype. Herein, we showed that all six CAM MC4Rs had not only constitutive activity in basal cAMP signaling, but also constitutive activity in basal ERK1/2 signaling. Moreover, the inverse agonist, AgRP(83-132), exhibited agonist efficacy for ERK1/2 signaling in WT and CAM MC4Rs. This novel AgRP-mediated ERK1/2 signaling pathway provided a potential missing link towards a better understanding of the physiological function of AgRP at molecular level. Hopefully, further in vivo study on the ERK1/2 signaling of these naturally occurring CAM MC4Rs might reveal the pathophysiology of the constitutive activity in MC4R.

It has been demonstrated that MC4R mediates melanocortin-induced long-lasting effects on regulating food intake and energy expenditure in rat (Grill et al. 1998). ERK1/2 is known to activate transcription factors to alter gene expression (Cobb 1999; Pearson, et al. 2001). We suggest alternation in basal and/or stimulated ERK1/2 signaling might be mediating the long-lasting effect of the MCR and the pathogenesis of the CAM MC4Rs in human obesity. Much more studies need to be done to elucidate the exact mechanisms.

In summary, the results of the present study demonstrated that several MC4R inverse agonists in the cAMP pathway acted as agonists, activating ERK1/2 signaling, adding a new layer of complexity to the MC4R signaling. The study also confirms a multiple activation states of MC4R with ligand-specific and/or mutant-specific conformations capable of differentially coupling the MC4R to distinct signaling pathways.

Table 3.1 The effect of agonist and antagonists on cAMP and pERK signaling in WT and CAM hMC4Rs.

	NDP-MSH			AgRP(83-132)			MCL0020			Ipsen 5i			ML00253764		
	cAMP	**pERK**	**Bias**	**cAMP**	**pERK**	**Bias**	**cAMP**	**pERK**	**Bias**	**cAMP**	**pERK**	**Bias**	**cAMP**	**pERK**	**Bias**
WT	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	—	No	↓	↑	Yes
H76R	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	—	No	↓	—	No
S127L	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	—	No	↓	—	No
D146N	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	—	No	↓	↑	Yes
P230L	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes
L250Q	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes
F280L	↑	↑	No	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes	↓	↑	Yes

The column “Bias” is designated for whether the ligand-induced cAMP and pERK1/2 signaling pathways were divergent, in which “No” denotes balanced cAMP and ERK1/2 signaling, whereas “Yes” denotes biased activation of ERK1/2 signaling. “↑”: denotes increased activity. “↓”: denotes decreased activity. “—”: denotes that the activity had no significant change.

Figure III-1. Schematic model of the hMC4R with the naturally occurring constitutively active mutations characterized in this study highlighted with gray background.

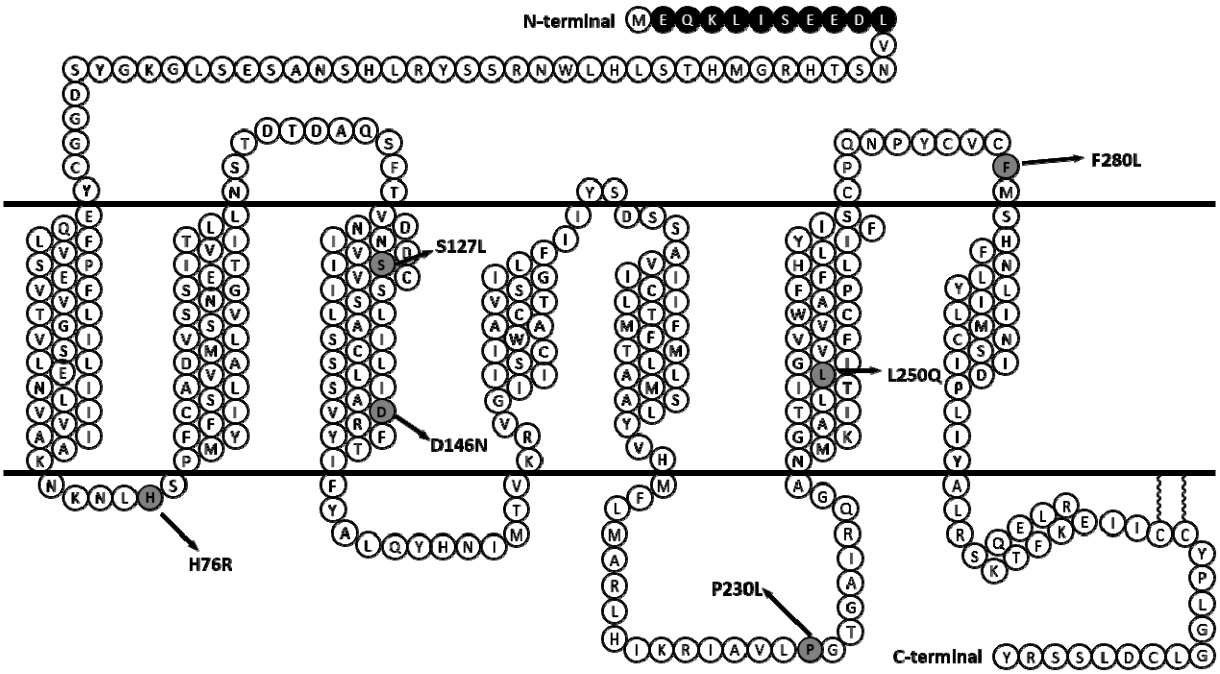
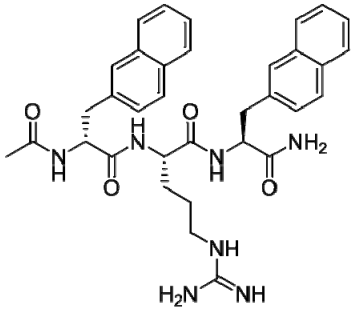

N-terminal
S127L
D146N
H76R
P230L
L250Q
F280L
C-terminal

Figure III-2. Amino acid sequence of AgRP (83-132) (A) and chemical structure of MCL0020 (B), Ipsen 5i (C) and ML00253764 (D).

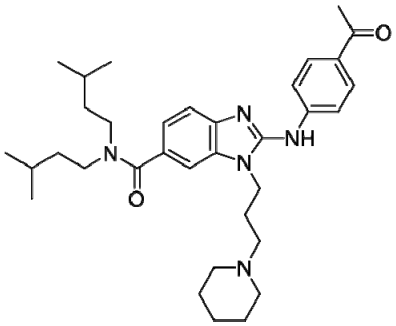
A: AgRP (83-132)

Ser - Ser - Arg - Arg - Cys - Val - Arg - Leu - His - Glu - Ser - Cys - Leu - Gly - Gln - Gln - Val - Pro - Cys - Cys - Asp - Pro - Cys - Ala - Thr - Cys - Tyr - Cys - Arg - Phe - Phe - Asn - Ala - Phe - Cys - Tyr - Cys - Arg - Lys - Leu - Gly - Thr - Ala - Met - Asn - Pro - Cys - Ser - Arg - Thr - NH_2

B: MCL0020



C: Ipsen 5i



D: ML00253764

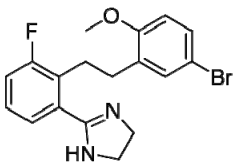


Figure III-3. cAMP and ERK1/2 signaling efficacy of MC4R superpotent agonist NDP-MSH. cAMP accumulation (A) and ERK1/2 phosphorylation (B-F) experiments were performed as described in Materials and Methods. Typical image of time-course study (B) and kinetics (C) of ERK1/2 phosphorylation carried out in HEK293T and GT1-7 cells. Representative image (D) and results (E) of ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT or six CAM MC4Rs. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean ± SEM of three independent experiments. (F) Basal pERK1/2 level of WT and CAM MC4Rs, and results are expressed as percentage of WT basal pERK1/2 activity. The statistical significance are indicated as follows: a: significantly different from basal cAMP or pERK activity, $p < 0.05$; b: significantly different from basal cAMP or pERK activity, $p < 0.01$; c: significantly different from basal cAMP or pERK activity, $p < 0.001$.

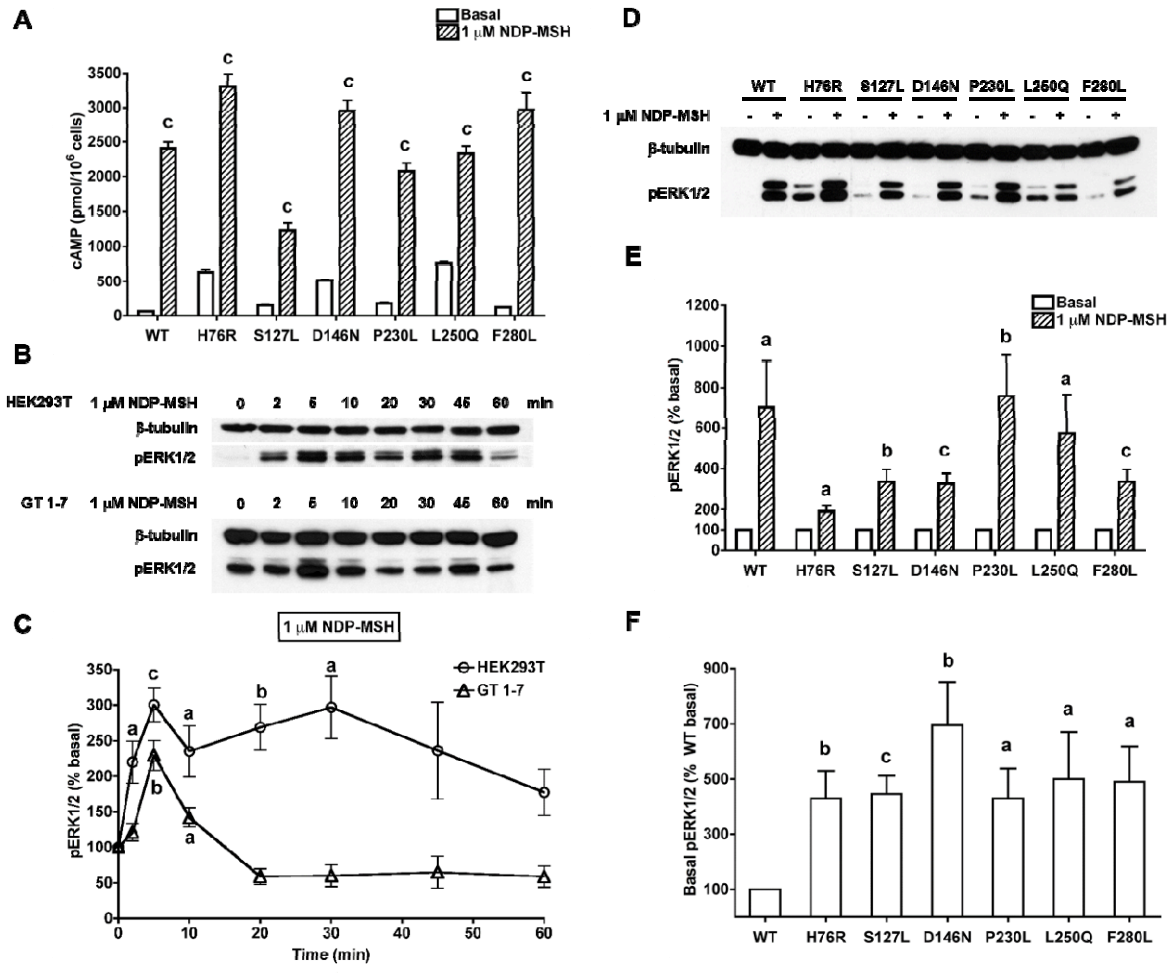

A
Basal
1 μM NDP-MSH
cAMP (pmol/10^6 cells)
WT H76R S127L D146N P230L L250Q F280L
B
HEK293T 1 μM NDP-MSH 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
GT 1-7 1 μM NDP-MSH 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
C
1 μM NDP-MSH
HEK293T
GT 1-7
pERK1/2 (% basal)
Time (min)
D
WT H76R S127L D146N P230L L250Q F280L
1 μM NDP-MSH
β-tubulin
pERK1/2
E
Basal
1 μM NDP-MSH
pERK1/2 (% basal)
WT H76R S127L D146N P230L L250Q F280L
F
Basal pERK1/2 (% WT basal)
WT H76R S127L D146N P230L L250Q F280L

Figure III-4. cAMP and ERK1/2 signaling efficacy of MC4R antagonist AgRP(83-132). cAMP accumulation (A) and ERK1/2 phosphorylation (B-E) was performed as described in Materials and Methods. Typical image of time-course study (B) and kinetics (C) of ERK1/2 phosphorylation carried out in HEK293T and GT1-7 cells. Representative image (D) and results (E) of ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT or six CAM MC4Rs. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from basal cAMP or pERK activity, $p < 0.05$; b: significantly different from basal cAMP or pERK activity, $p < 0.01$; c: significantly different from basal cAMP or pERK activity, $p < 0.001$.

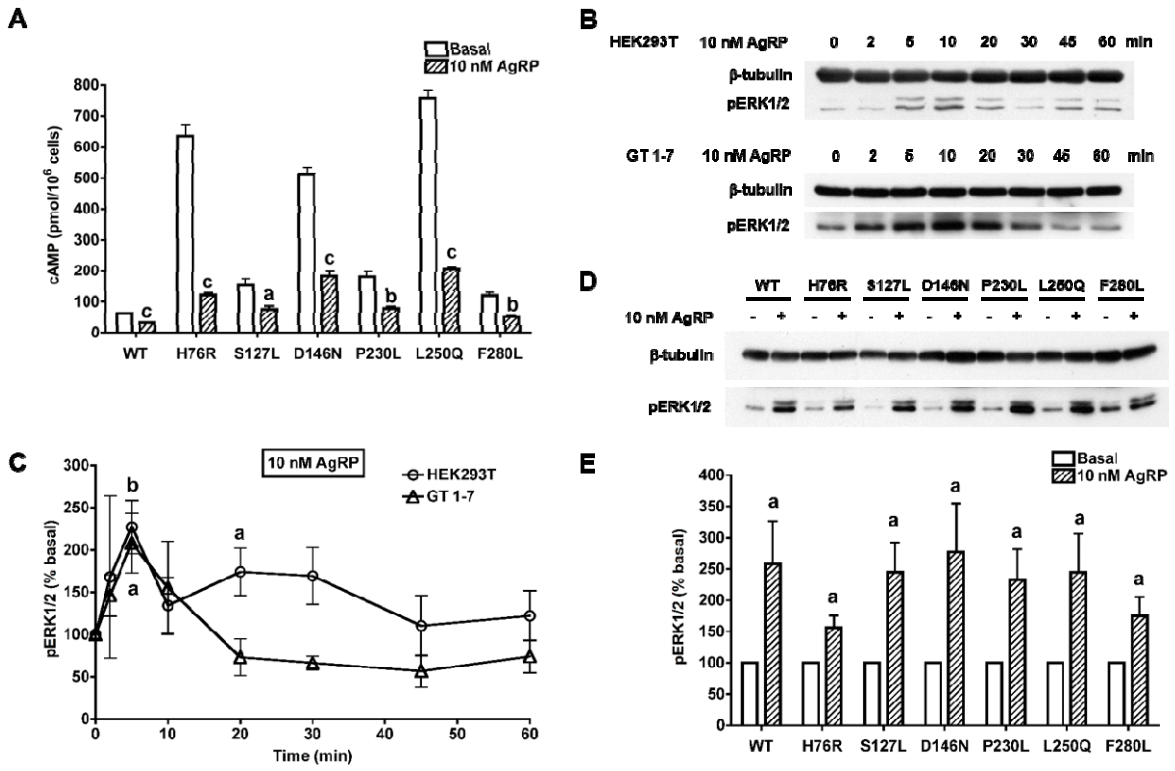

A
Basal
10 nM AgRP
cAMP (pmol/10^6 cells)
WT
H76R
S127L
D146N
P230L
L250Q
F280L
B
HEK293T
10 nM AgRP
0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
GT 1-7
10 nM AgRP
0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
C
10 nM AgRP
HEK293T
GT 1-7
pERK1/2 (% basal)
Time (min)
D
WT
H76R
S127L
D146N
P230L
L250Q
F280L
10 nM AgRP
β-tubulin
pERK1/2
E
Basal
10 nM AgRP
pERK1/2 (% basal)
WT
H76R
S127L
D146N
P230L
L250Q
F280L

Figure III-5. cAMP and ERK1/2 signaling efficacy of MC4R antagonist MCL0020. cAMP accumulation (A) and ERK1/2 phosphorylation (B-E) was performed as described in Materials and Methods. Typical image of time-course study (B) and kinetics (C) of ERK1/2 phosphorylation carried out in HEK293T and GT1-7 cells. Representative image (D) and results (E) of ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT or six CAM MC4Rs. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from basal cAMP or pERK activity, $p < 0.05$; b: significantly different from basal cAMP or pERK activity, $p < 0.01$; c: significantly different from basal cAMP or pERK activity, $p < 0.001$.

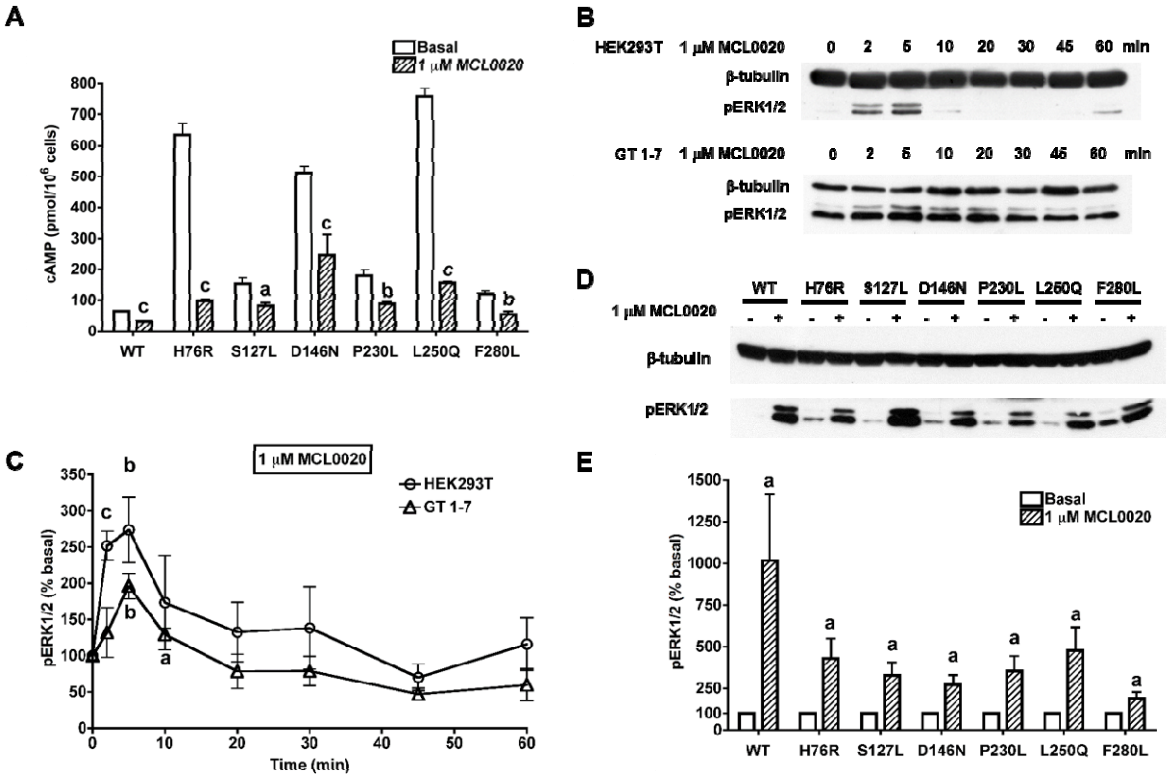

A
Basal
1 μM MCL0020
cAMP (pmol/1c^6 cells)
WT
H76R
S127L
D146N
P230L
L250Q
F280L
B
HEK293T 1 μM MCL0020 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
GT 1-7 1 μM MCL0020 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
C
1 μM MCL0020
HEK293T
GT 1-7
pERK1/2 (% basal)
Time (min)
D
WT
H76R
S127L
D146N
P230L
L250Q
F280L
1 μM MCL0020
β-tubulin
pERK1/2
E
Basal
1 μM MCL0020
pERK1/2 (% basal)
WT
H76R
S127L
D146N
P230L
L250Q
F280L

Figure III-6. cAMP and ERK1/2 signaling efficacy of MC4R antagonist Ipsen 5i. cAMP accumulation (A) and ERK1/2 phosphorylation (B-E) was performed as described in Materials and Methods. Typical image of time-course study (B) and kinetics (C) of ERK1/2 phosphorylation carried out in HEK293T and GT1-7 cells. Representative image (D) and results (E) of ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT or six CAM MC4Rs. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from basal cAMP or pERK activity, $p < 0.05$; b: significantly different from basal cAMP or pERK activity, $p < 0.01$; c: significantly different from basal cAMP or pERK activity, $p < 0.001$.

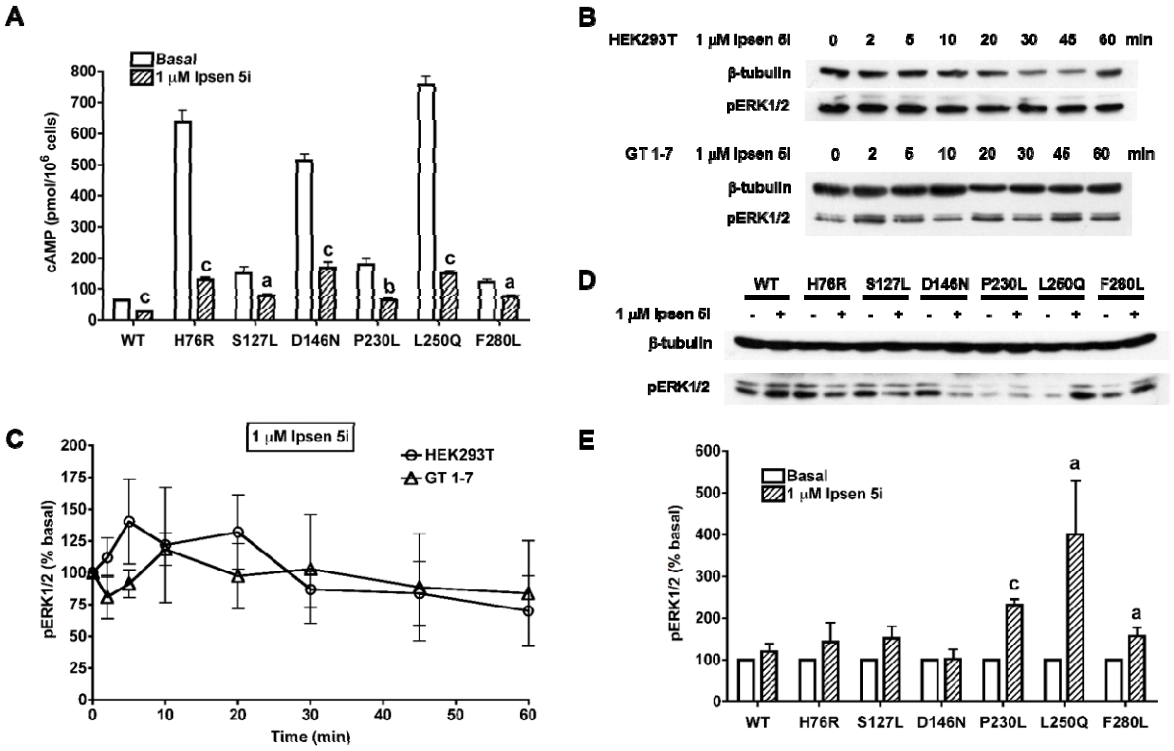
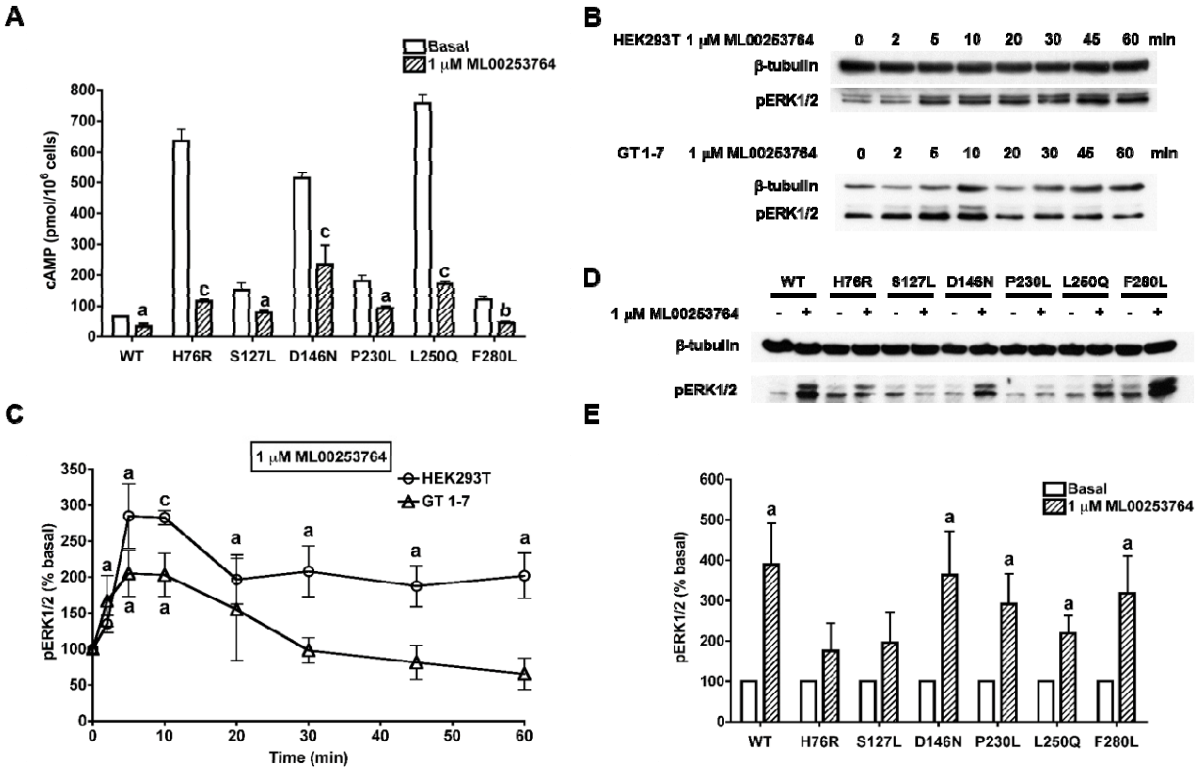

A
Basal
1 μM Ipsen 5i
cAMP (pmol/10^6 cells)
WT
H76R
S127L
D146N
P230L
L250Q
F280L
B
HEK293T 1 μM Ipsen 5i 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
GT 1-7 1 μM Ipsen 5i 0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
C
1 μM Ipsen 5i
HEK293T
GT 1-7
pERK1/2 (% basal)
Time (min)
D
WT H76R S127L D146N P230L L250Q F280L
1 μM Ipsen 5i
β-tubulin
pERK1/2
E
Basal
1 μM Ipsen 5i
pERK1/2 (% basal)
WT
H76R
S127L
D146N
P230L
L250Q
F280L

Figure III-7. cAMP and ERK1/2 signaling efficacy of MC4R antagonist ML00253764. cAMP accumulation (A) and ERK1/2 phosphorylation (B-E) was performed as described in Materials and Methods. Typical image of time-course study (B) and kinetics (C) of ERK1/2 phosphorylation carried out in HEK293T and GT1-7 cells. Representative image (D) and results (E) of ERK1/2 phosphorylation in HEK293T cells transiently transfected with WT or six CAM MC4Rs. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean ± SEM of three independent experiments. The statistical significance are indicated as follows: a: significantly different from basal cAMP or pERK activity, $p < 0.05$; b: significantly different from basal cAMP or pERK activity, $p < 0.01$; c: significantly different from basal cAMP or pERK activity, $p < 0.001$.


A
Basal
1 μM ML00253764
cAMP (pmol/10^6 cells)
WT
H76R
S127L
D146N
P230L
L250Q
F280L
B
HEK293T 1 μM ML00253784
0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
GT1-7 1 μM ML00253784
0 2 5 10 20 30 45 60 min
β-tubulin
pERK1/2
C
1 μM ML00253764
HEK293T
GT 1-7
pERK1/2 (% basal)
Time (min)
D
WT
H76R
S127L
D146N
P230L
L250Q
F280L
1 μM ML00253764
β-tubulin
pERK1/2
E
Basal
1 μM ML00253764
pERK1/2 (% basal)
WT
H76R
S127L
D146N
P230L
L250Q
F280L

Conclusions and Future Prospective

Obesity is a complex and heritable disease resulting from intricate interactions of behavior, environmental factor, and genetic disorders. Although several genetic variants have been identified to be associated with human obesity, the discovery of the MC4R has been considered as an important breakthrough in investigating human obesity.

Our systematic study of structure-function relationship of transmembrane domain 3 provided detailed information on the molecular basis of the MC4R function, and might be useful for future drug design and better pharmacotherapy of obesity. We found that TM3 of MC4R was critical for ligand binding and/or signaling, consistent with previous studies. Of the 26 TM3 mutants we studied herein, 13 were significantly impaired in ligand binding and/or cAMP signaling. Moreover, L140 was critical for locking MC4R in an inactive conformation and several mutants displayed biased signaling in cAMP and ERK1/2 signaling pathways.

In addition, the study presented in Chapter III demonstrated that several MC4R inverse agonists in the cAMP pathway acted as agonists, activating ERK1/2 signaling, in both WT and naturally occurring CAM MC4R. This novel finding adds a new layer of complexity to the MC4R signaling. The study also confirms the existence of multiple activation states of MC4R with ligand-specific and/or mutant-specific conformations capable of differentially coupling the MC4R to distinct signaling pathways.

Further studies to identify the ERK1/2 upstream signaling mediators will provide important missing links in understanding the molecular mechanism of the MC4R in regulating energy homeostasis.

References

Adan RA & Kas MJ 2003 Inverse agonism gains weight. *Trends Pharmacol. Sci.* **24** 315-321.

Ahn KH, Mahmoud MM & Kendall DA 2012 Allosteric modulator ORG27569 induces CB1 cannabinoid receptor high affinity agonist binding state, receptor internalization, and G_i protein-independent ERK1/2 kinase activation. *J. Biol. Chem.* **287** 12070-12082.

Allison DB, Downey M, Atkinson RL, Billington CJ, Bray GA, Eckel RH, Finkelstein EA, Jensen MD & Tremblay A 2008 Obesity as a disease: a white paper on evidence and arguments commissioned by the Council of the Obesity Society. *Obesity (Silver Spring)* **16** 1161-1177.

Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F & Ghigo E 2000 Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J. Endocrinol. Invest.* **23** 493-495.

Ashrafian H, Ahmed K, Rowland SP, Patel VM, Gooderham NJ, Holmes E, Darzi A & Athanasiou T 2011 Metabolic surgery and cancer: protective effects of bariatric procedures. *Cancer* **117** 1788-1799.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M & Pineyro G 2003 β-Arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc. Natl. Acad. Sci. U. S. A.* **100** 11406-11411.

Baessler A, Hasinoff MJ, Fischer M, Reinhard W, Sonnenberg GE, Olivier M, Erdmann J, Schunkert H, Doering A, Jacob HJ, et al. 2005 Genetic linkage and association of the growth hormone secretagogue receptor (ghrelin receptor) gene in human obesity. *Diabetes* **54** 259-267.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SGF, Shi L, Gether U & Javitch JA 2001 Activation of the β_2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J. Biol. Chem.* **276** 29171-29177.

Ballesteros JA & Weinstein H 1995 Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci.* **25** 366-428.

Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, et al. 2005 Divergence of melanocortin pathways in the control of food intake and energy expenditure. *Cell* **123** 493-505.

Banno R, Arima H, Hayashi M, Goto M, Watanabe M, Sato I, Ozaki N, Nagasaki H, Ozaki N & Oiso Y 2007 Central administration of melanocortin agonist increased insulin sensitivity in diet-induced obese rats. *FEBS Lett.* **581** 1131-1136.

Baranski TJ, Herzmark P, Lichtarge O, Gerber BO, Trueheart J, Meng EC, Iiri T, Sheikh SP & Bourne HR 1999 C5a receptor activation: genetic identification of critical residues in four transmembrane helices. *J. Biol. Chem.* **274** 15757-15765.

Bartoszewski RA, Jablonsky M, Bartoszewska S, Stevenson L, Dai Q, Kappes J, Collawn JF & Bebok Z 2010 A synonymous single nucleotide polymorphism in ΔF508 CFTR alters the secondary structure of the mRNA and the expression of the mutant protein. *J. Biol. Chem.* **285** 28741-28748.

Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, et al. 2002 Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* **418** 650-654.

Beckers S, Zegers D, Freitas F, Peeters AV, Verhulst SL, Massa G, Van Gaal LF, Timmermans JP, Desager KN & Van Hul W 2010 Identification and functional characterization of novel mutations in the melanocortin-4 receptor. *Obes. Facts* **3** 304-311.

Benned-Jensen T, Smethurst C, Holst PJ, Page KR, Sauls H, Sivertsen B, Schwartz TW, Blanchard A, Jepras R & Rosenkilde MM 2011 Ligand modulation of the Epstein-Barr virus-induced seven-transmembrane receptor EBI2: identification of a potent and efficacious inverse agonist. *J. Biol. Chem.* **286** 29292-29302.

Bertagna X 1994 Proopiomelanocortin-derived peptides. *Endocrinol. Metab. Clin. North Am.* **23** 467-485.

Bhargava SK, Sachdev HS, Fall CHD, Osmond C, Lakshmy R, Barker DJP, Biswas SKD, Ramji S, Prabhakaran D & Reddy KS 2004 Relation of serial changes in childhood body-mass index to impaired glucose tolerance in young adulthood. *N. Engl. J. Med.* **350** 865-875.

Blevins JE, Morton GJ, Williams DL, Caldwell DW, Bastian LS, Wisse BE, Schwartz MW & Baskin DG 2009 Forebrain melanocortin signaling enhances the hindbrain satiety response to CCK-8. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **296** R476-484.

Boston BA, Blaydon KM, Varnerin J & Cone RD 1997 Independent and additive effects of central POMC and leptin pathways on murine obesity. *Science* **278** 1641-1644.

Breit A, Buch TRH, Boekhoff I, Solinski HJ, Damm E & Gudermann T 2011 Alternative G protein coupling and biased agonism: new insights into melanocortin-4 receptor signalling. *Mol. Cell. Endocrinol.* **331** 232-240.

Breit A, Wolff K, Kalwa H, Jarry H, Buch T & Gudermann T 2006 The natural inverse agonist agouti-related protein induces arrestin-mediated endocytosis of melanocortin-3 and-4 receptors. *J. Biol. Chem.* **281** 37447-37456.

Bronstein DM, Schafer MK, Watson SJ & Akil H 1992 Evidence that beta-endorphin is synthesized in cells in the nucleus tractus solitarius: detection of POMC mRNA. *Brain Res.* **587** 269-275.

Brown KS, Gentry RM & Rowland NE 1998 Central injection in rats of alpha-melanocyte-stimulating hormone analog: effects on food intake and brain Fos. *Regul. Pept.* **78** 89-94.

Buch TR, Heling D, Damm E, Gudermann T & Breit A 2009 Pertussis toxin-sensitive signaling of melanocortin-4 receptors in hypothalamic GT1-7 cells defines agouti-related protein as a biased agonist. *J. Biol. Chem.* **284** 26411-26420.

Buchwald H, Avidor Y, Braunwald E, Jensen MD, Pories W, Fahrbach K & Schoelles K 2004 Bariatric surgery: a systematic review and meta-analysis. *JAMA* **292** 1724-1737.

Buffa R, Solcia E & Go VL 1976 Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. *Gastroenterology* **70** 528-532.

Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M & Cone RD 2000 A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* **141** 3518-3521.

Caro JF, Kolaczynski JW, Nyce MR, Ohannesian JP, Opentanova I, Goldman WH, Lynn RB, Zhang PL, Sinha MK & Considine RV 1996 Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. *Lancet* **348** 159-161.

Caruso C, Durand D, Schioth HB, Rey R, Seilicovich A & Lasaga M 2007 Activation of melanocortin 4 receptors reduces the inflammatory response and prevents apoptosis

induced by lipopolysaccharide and interferon-gamma in astrocytes. *Endocrinology* **148** 4918-4926.

Chai B, Li JY, Zhang W, Newman E, Ammori J & Mulholland MW 2006 Melanocortin-4 receptor-mediated inhibition of apoptosis in immortalized hypothalamic neurons via mitogen-activated protein kinase. *Peptides* **27** 2846-2857.

Chai B, Li JY, Zhang W, Wang H & Mulholland MW 2009 Melanocortin-4 receptor activation inhibits c-Jun N-terminal kinase activity and promotes insulin signaling. *Peptides* **30** 1098-1104.

Chai BX, Neubig RR, Millhauser GL, Thompson DA, Jackson PJ, Barsh GS, Dickinson CJ, Li JY, Lai YM & Gantz I 2003 Inverse agonist activity of agouti and agouti-related protein. *Peptides* **24** 603-609.

Chaki S, Ogawa S, Toda Y, Funakoshi T & Okuyama S 2003 Involvement of the melanocortin MC4 receptor in stress-related behavior in rodents. *Eur. J. Pharmacol.* **474** 95-101.

Challis BG, Coll AP, Yeo GS, Pinnock SB, Dickson SL, Thresher RR, Dixon J, Zahn D, Rochford JJ, White A, et al. 2004 Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36). *Proc. Natl. Acad. Sci. U. S. A.* **101** 4695-4700.

Chambers AP, Jessen L, Ryan KK, Sisley S, Wilson-Perez HE, Stefater MA, Gaitonde SG, Sorrell JE, Toure M, Berger J, et al. 2011 Weight-independent changes in blood glucose homeostasis after gastric bypass or vertical sleeve gastrectomy in rats. *Gastroenterology* **141** 950-958.

Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, et al. 2000 Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat. Genet.* **26** 97-102.

Chen C & Okayama H 1987 High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7** 2745-2752.

Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, et al. 1996 Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* **84** 491-495.

Chen M, Cai MY, Aprahamian CJ, Georgeson KE, Hruby V, Harmon CM & Yang YK 2007 Contribution of the conserved amino acids of the melanocortin-4 receptor in [Nle^4, D-Phe^7]-α-melanocyte-stimulating hormone binding and signaling. *J. Biol. Chem.* **282** 21712-21719.

Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ & Cone RD 1997 Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* **91** 789-798.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, et al. 2007 High-resolution crystal structure of an engineered human β_2-adrenergic G protein-coupled receptor. *Science* **318** 1258-1265.

Cheung CC, Clifton DK & Steiner RA 1997 Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* **138** 4489-4492.

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, et al. 1998 A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392** 398-401.

Cobb MH 1999 MAP kinase pathways. *Prog. Biophys. Mol. Biol.* **71** 479-500.

Cole SA, Butte NF, Voruganti VS, Cai G, Haack K, Kent JW, Jr., Blangero J, Comuzzie AG, McPherson JD & Gibbs RA 2010 Evidence that multiple genetic variants of MC4R play a functional role in the regulation of energy expenditure and appetite in Hispanic children. *Am. J. Clin. Nutr.* **91** 191-199.

Conde-Frieboes K, Thogersen H, Lau JF, Sensfuss U, Hansen TK, Christensen L, Spetzler J, Olsen HB, Nilsson C, Raun K, et al. 2012 Identification and in vivo and in vitro characterization of long acting and melanocortin 4 receptor (MC4-R) selective alpha-melanocyte-stimulating hormone (alpha-MSH) analogues. *J. Med. Chem.* **55** 1969-1977.

Cone RD 2005 Anatomy and regulation of the central melanocortin system. *Nat. Neurosci.* **8** 571-578.

Cone RD, Cowley MA, Butler AA, Fan W, Marks DL & Low MJ 2001 The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int. J. Obes. Relat. Metab. Disord.* **25 Suppl 5** S63-67.

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD & Low MJ 2001 Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411** 480-484.

Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, et al. 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* **37** 649-661.

Damm E, Buech TR, Gudermann T & Breit A 2012 Melanocortin-induced PKA activation inhibits AMPK activity via ERK-1/2 and LKB-1 in hypothalamic GT1-7 cells. *Mol. Endocrinol.* **26** 643-654.

Daniels D, Patten CS, Roth JD, Yee DK & Fluharty SJ 2003 Melanocortin receptor signaling kinase in vitro and through mitogen-activated protein in rat hypothalamus. *Brain Res.* **986** 1-11.

de Rijke CE, Jackson PJ, Garner KM, van Rozen RJ, Douglas NR, Kas MJ, Millhauser GL & Adan RA 2005 Functional analysis of the Ala67Thr polymorphism in agouti related protein associated with anorexia nervosa and leanness. *Biochem. Pharmacol.* **70** 308-316.

De Spiegeleer B, Vergote V, Pezeshki A, Peremans K & Burvenich C 2008 Impurity profiling quality control testing of synthetic peptides using liquid chromatography-photodiode array-fluorescence and liquid chromatography-electrospray ionization-mass spectrometry: the obestatin case. *Anal. Biochem.* **376** 229-234.

DeMaria EJ 2007 Bariatric surgery for morbid obesity. *N. Engl. J. Med.* **356** 2176-2183.

Desvergne B, Michalik L & Wahli W 2004 Be fit or be sick: peroxisome proliferator-activated receptors are down the road. *Mol. Endocrinol.* **18** 1321-1332.

Duan JB, Wainwright MS, Comeron JM, Saitou N, Sanders AR, Gelernter J & Gejman PV 2003 Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Hum. Mol. Genet.* **12** 205-216.

Ebihara K, Ogawa Y, Katsuura G, Numata Y, Masuzaki H, Satoh N, Tamaki M, Yoshioka T, Hayase M, Matsuoka N, et al. 1999 Involvement of agouti-related protein, an endogenous antagonist of hypothalamic melanocortin receptor, in leptin action. *Diabetes* **48** 2028-2033.

Eckel RH 2008 Clinical practice. Nonsurgical management of obesity in adults. *N. Engl. J. Med.* **358** 1941-1950.

Fan W, Boston BA, Kesterson RA, Hruby VJ & Cone RD 1997 Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* **385** 165-168.

Fan W, Ellacott KL, Halatchev IG, Takahashi K, Yu P & Cone RD 2004 Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nat. Neurosci.* **7** 335-336.

Fan ZC, Sartin JL & Tao YX 2008 Molecular cloning and pharmacological characterization of porcine melanocortin-3 receptor. *J. Endocrinol.* **196** 139-148.

Fan ZC & Tao YX 2009 Functional characterization and pharmacological rescue of melanocortin-4 receptor mutations identified from obese patients. *J. Cell. Mol. Med.* **13** 3268-3282.

Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T & O'Rahilly S 2003 Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N. Engl. J. Med.* **348** 1085-1095.

Farrens DL, Altenbach C, Yang K, Hubbell WL & Khorana HG 1996 Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science* **274** 768-770.

Ferraguti F, Baldani-Guerra B, Corsi M, Nakanishi S & Corti C 1999 Activation of the extracellular signal-regulated kinase 2 by metabotropic glutamate receptors. *Eur. J. Neurosci.* **11** 2073-2082.

Finkelstein EA, Fiebelkorn IC & Wang G 2004 State-level estimates of annual medical expenditures attributable to obesity. *Obes. Res.* **12** 18-24.

Finkelstein EA, Trogdon JG, Brown DS, Allaire BT, Dellea PS & Kamal-Bahl SJ 2008 The lifetime medical cost burden of overweight and obesity: implications for obesity prevention. *Obesity (Silver Spring)* **16** 1843-1848.

Fleck BA, Chen C, Yang WD, Huntley R, Markison S, Nickolls SA, Foster AC & Hoare SRJ 2005 Molecular interactions of nonpeptide agonists and antagonists with the melanocortin-4 receptor. *Biochemistry (Mosc.)* **44** 14494-14508.

Flegal KM, Carroll MD, Kit BK & Ogden CL 2012 Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *JAMA* **307** 491-497.

Flier JS & Maratos-Flier E 2010 Lasker lauds leptin. *Cell Metab.* **12** 317-320.

Flum DR, Belle SH, King WC, Wahed AS, Berk P, Chapman W, Pories W, Courcoulas A, McCloskey C, Mitchell J, et al. 2009 Perioperative safety in the longitudinal assessment of bariatric surgery. *N. Engl. J. Med.* **361** 445-454.

Fong TM, Mao C, MacNeil T, Kalyani R, Smith T, Weinberg D, Tota MR & VanderPloeg LHT 1997 ART (protein product of agouti-related transcript) as an antagonist of MC-3 and MC-4 receptors. *Biochem. Biophys. Res. Commun.* **237** 629-631.

Fontaine KR, Redden DT, Wang CX, Westfall AO & Allison DB 2003 Years of life lost due to obesity. *JAMA* **289** 187-193.

Friedman JM 2004 Modern science versus the stigma of obesity. *Nat. Med.* **10** 563-569.

Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J & Yamada T 1993 Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. *J. Biol. Chem.* **268** 15174-15179.

Gether U 2000 Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev.* **21** 90-113.

Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H & Kobilka BK 1997 Agonists induce conformational changes in transmembrane domains III and VI of the β_2-adrenoceptor. *EMBO J.* **16** 6737-6747.

Giraudo SQ, Billington CJ & Levine AS 1998 Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands. *Brain Res.* **809** 302-306.

Gjesing AP, Larsen LH, Torekov SS, Hainerova IA, Kapur R, Johansen A, Albrechtsen A, Boj S, Holst B, Harper A, et al. 2010 Family and population-based studies of variation within the ghrelin receptor locus in relation to measures of obesity. *PLoS ONE* **5** e10084.

Gourcerol G, Coskun T, Craft LS, Mayer JP, Heiman ML, Wang L, Million M, St-Pierre DH & Tache Y 2007a Preproghrelin-derived peptide, obestatin, fails to influence food intake in lean or obese rodents. *Obesity (Silver Spring)* **15** 2643-2652.

Gourcerol G, St-Pierre DH & Tache Y 2007b Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? *Regul. Pept.* **141** 1-7.

Govaerts C, Srinivasan S, Shapiro A, Zhang SM, Picard F, Clement K, Lubrano-Berthelier C & Vaisse C 2005 Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function. *Peptides* **26** 1909-1919.

Graham M, Shutter JR, Sarmiento U, Sarosi I & Stark KL 1997 Overexpression of Agrt leads to obesity in transgenic mice. *Nat. Genet.* **17** 273-274.

Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, Nano R, Annunziata M, Campiglia P, Arnoletti E, et al. 2008 Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. *Diabetes* **57** 967-979.

Grill HJ, Ginsberg AB, Seeley RJ & Kaplan JM 1998 Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. *J. Neurosci.* **18** 10128-10135.

Grill HJ & Hayes MR 2009 The nucleus tractus solitarius: a portal for visceral afferent signal processing, energy status assessment and integration of their combined effects on food intake. *Int. J. Obes.* **33 Suppl 1** S11-15.

Gu W, Tu Z, Kleyn PW, Kissebah A, Duprat L, Lee J, Chin W, Maruti S, Deng N, Fisher SL, et al. 1999 Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes* **48** 635-639.

Guyenet SJ & Schwartz MW 2012 Clinical review: Regulation of food intake, energy balance, and body fat mass: implications for the pathogenesis and treatment of obesity. *J. Clin. Endocrinol. Metab.* **97** 745-755.

Hagan MM, Rushing PA, Schwartz MW, Yagaloff KA, Burn P, Woods SC & Seeley RJ 1999 Role of the CNS melanocortin system in the response to overfeeding. *J. Neurosci.* **19** 2362-2367.

Hahn TM, Breininger JF, Baskin DG & Schwartz MW 1998 Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat. Neurosci.* **1** 271-272.

Haskell-Luevano C, Cone RD, Monck EK & Wan YP 2001 Structure activity studies of the melanocortin-4 receptor by in vitro mutagenesis: identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants. *Biochemistry (Mosc.)* **40** 6164-6179.

Haskell-Luevano C & Monck EK 2001 Agouti-related protein functions as an inverse agonist at a constitutively active brain melanocortin-4 receptor. *Regul. Pept.* **99** 1-7.

Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, et al. 1999 Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* **282** 1568-1575.

Hinney A, Hohmann S, Geller F, Vogel C, Hess C, Wermter AK, Brokamp B, Goldschmidt H, Siegfried W, Remschmidt H, et al. 2003 Melanocortin-4 receptor gene: Case-control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. *J. Clin. Endocrinol. Metab.* **88** 4258-4267.

Hinney A, Volckmar AL & Knoll N 2013 Melanocortin-4 receptor in energy homeostasis and obesity pathogenesis. *Prog Mol Biol Transl Sci* **114** 147-191.

Hogan K, Peluso S, Gould S, Parsons I, Ryan D, Wu LJ & Visiers I 2006 Mapping the binding site of melanocortin 4 receptor agonists: a hydrophobic pocket formed by I3.28(125), I3.32(129), and I7.42(291) is critical for receptor activation. *J. Med. Chem.* **49** 911-922.

Holder JL, Jr., Butte NF & Zinn AR 2000 Profound obesity associated with a balanced translocation that disrupts the SIM1 gene. *Hum. Mol. Genet.* **9** 101-108.

Holst B & Schwartz TW 2006 Ghrelin receptor mutations--too little height and too much hunger. *J. Clin. Invest.* **116** 637-641.

Huang H & Tao YX 2012 Pleiotropic functions of the transmembrane domain 6 of human melanocortin-4 receptor. *J. Mol. Endocrinol.* **49** 237-248.

Huszar D, Lynch CA, FairchildHuntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, et al. 1997 Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* **88** 131-141.

Ilnytska O & Argyropoulos G 2008 The role of the Agouti-Related Protein in energy balance regulation. *Cell. Mol. Life Sci.* **65** 2721-2731.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EYT, Lane JR, Ijzerman AP & Stevens RC 2008 The 2.6 angstrom crystal structure of a human A(2A) adenosine receptor bound to an antagonist. *Science* **322** 1211-1217.

Jackson RS, Creemers JW, Ohagi S, Raffin-Sanson ML, Sanders L, Montague CT, Hutton JC & O'Rahilly S 1997 Obesity and impaired prohormone processing associated with mutations in the human prohormone convertase 1 gene. *Nat. Genet.* **16** 303-306.

James WP, Caterson ID, Coutinho W, Finer N, Van Gaal LF, Maggioni AP, Torp-Pedersen C, Sharma AM, Shepherd GM, Rode RA, et al. 2010 Effect of sibutramine on cardiovascular outcomes in overweight and obese subjects. *N. Engl. J. Med.* **363** 905-917.

Jones DD 1975 Amino acid properties and side-chain orientation in proteins: a cross correlation approach. *J. Theor. Biol.* **50** 167-183.

Kask A, Mutulis F, Muceniece R, Pahkla R, Mutule I, Wikberg JE, Rago L & Schioth HB 1998a Discovery of a novel superpotent and selective melanocortin-4 receptor antagonist (HS024): evaluation in vitro and in vivo. *Endocrinology* **139** 5006-5014.

Kask A, Rago L, Mutulis F, Pahkla R, Wikberg JE & Schioth HB 1998b Selective antagonist for the melanocortin 4 receptor (HS014) increases food intake in free-feeding rats. *Biochem. Biophys. Res. Commun.* **245** 90-93.

Kask A, Rago L, Wikberg JE & Schioth HB 1998c Evidence for involvement of the melanocortin MC4 receptor in the effects of leptin on food intake and body weight. *Eur. J. Pharmacol.* **360** 15-19.

Kenakin T 2005 New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat. Rev. Drug Discov.* **4** 919-927.

Kennedy GC 1953 The role of depot fat in the hypothalamic control of food intake in the rat. *Proc. R. Soc. Lond. B Biol. Sci.* **140** 578-596.

Khong K, Kurtz SE, Sykes RL & Cone RD 2001 Expression of functional melanocortin-4 receptor in the hypothalamic GT1-1 cell line. *Neuroendocrinology* **74** 193-201.

Kim MS, Rossi M, Abusnana S, Sunter D, Morgan DG, Small CJ, Edwards CM, Heath MM, Stanley SA, Seal LJ, et al. 2000 Hypothalamic localization of the feeding effect of agouti-related peptide and alpha-melanocyte-stimulating hormone. *Diabetes* **49** 177-182.

Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB & Elmquist JK 2003 Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J. Comp. Neurol.* **457** 213-235.

Kobelt P, Wisser AS, Stengel A, Goebel M, Bannert N, Gourcerol G, Inhoff T, Noetzel S, Wiedenmann B, Klapp BF, et al. 2008 Peripheral obestatin has no effect on feeding behavior and brain Fos expression in rodents. *Peptides* **29** 1018-1027.

Kosugi S, Hai N, Okamoto H, Sugawa H & Mori T 2000 A novel activating mutation in the thyrotropin receptor gene in an autonomously functioning thyroid nodule developed by a Japanese patient. *Eur. J. Endocrinol.* **143** 471-477.

Krude H, Biebermann H, Luck W, Horn R, Brabant G & Gruters A 1998 Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat. Genet.* **19** 155-157.

Lagerstrom MC, Klovins J, Fredriksson R, Fridmanis D, Haitina T, Ling MK, Berglund MM & Schioth HB 2003 High affinity agonistic metal ion binding sites within the melanocortin

4 receptor illustrate conformational change of transmembrane region 3. *J. Biol. Chem.* **278** 51521-51526.

Larsson LI & Rehfeld JF 1978 Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Evidence for the occurrence of three distinct cell types storing COOH-terminal gastrin immunoreactivity. *Histochemistry* **58** 23-31.

Latronico AC, Abell AN, Arnhold IJP, Liu X, Lins TSS, Brito VN, Billerbeck AE, Segaloff DL & Mendonca BB 1998 A unique constitutively activating mutation in third transmembrane helix of luteinizing hormone receptor causes sporadic male gonadotropin-independent precocious puberty. *J. Clin. Endocrinol. Metab.* **83** 2435-2440.

Li Z, Li Y & Zhang W 2013 Ghrelin receptor in energy homeostasis and obesity pathogenesis. *Prog Mol Biol Transl Sci* **114** 45-87.

Lindgren CM, Loos RJ, Li SX, McCarthy MI, Wareham NJ, Barroso I & & COC 2008 Association studies involving over 90,000 samples demonstrate that common variants near to MC4R influence fat mass, weight and risk of obesity. *Diabetes* **57** A485-A486.

Lobstein T, Baur L, Uauy R & TaskForce IIO 2004 Obesity in children and young people: a crisis in public health. *Obes. Rev.* **5 Suppl 1** 4-104.

Loos RJF, Lindgren CM, Li SX, Wheeler E, Zhao JH, Prokopenko I, Inouye M, Freathy RM, Attwood AP, Beckmann JS, et al. 2008 Common variants near MC4R are associated with fat mass, weight and risk of obesity. *Nat. Genet.* **40** 768-775.

Lu ZL, Gallagher R, Sellar R, Coetsee M & Millar RP 2005 Mutations remote from the human gonadotropin-releasing hormone (GnRH) receptor-binding sites specifically increase binding affinity for GnRH II but not GnRH I - Evidence for ligand-selective, receptor-active conformations. *J. Biol. Chem.* **280** 29796-29803.

Lu ZL & Hulme EC 1999 The functional topography of transmembrane domain 3 of the M-1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. *J. Biol. Chem.* **274** 7309-7315.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al. 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.* **1** 1155-1161.

Magenis RE, Smith L, Nadeau JH, Johnson KR, Mountjoy KG & Cone RD 1994 Mapping of the ACTH, MSH, and neural (MC3 and MC4) melanocortin receptors in the mouse and human. *Mamm. Genome* **5** 503-508.

Mager U, Degenhardt T, Pulkkinen L, Kolehmainen M, Tolppanen AM, Lindstrom J, Eriksson JG, Carlberg C, Tuomilehto J, Uusitupa M, et al. 2008 Variations in the ghrelin receptor gene associate with obesity and glucose metabolism in individuals with impaired glucose tolerance. *PLoS ONE* **3** e2941.

Marks DL, Boucher N, Lanouette CM, Perusse L, Brookhart G, Comuzzie AG, Chagnon YC & Cone RD 2004 Ala67Thr polymorphism in the Agouti-related peptide gene is associated with inherited leanness in humans. *Am. J. Med. Genet. A.* **126A** 267-271.

Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL & Weiner RI 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* **5** 1-10.

Michaud JL, Boucher F, Melnyk A, Gauthier F, Goshu E, Levy E, Mitchell GA, Himms-Hagen J & Fan CM 2001 Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus. *Hum. Mol. Genet.* **10** 1465-1473.

Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T & Westfall T 1998 XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **50** 143-150.

Mirzadegan T, Benko G, Filipek S & Palczewski K 2003 Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry (Mosc.)* **42** 2759-2767.

Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA & Mobbs CV 1998 Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* **47** 294-297.

Mizuno TM & Mobbs CV 1999 Hypothalamic agouti-related protein messenger ribonucleic acid is inhibited by leptin and stimulated by fasting. *Endocrinology* **140** 814-817.

Mo XL, Yang R & Tao YX 2012 Functions of transmembrane domain 3 of human melanocortin-4 receptor. *J. Mol. Endocrinol.* **49** 221-235.

Moechars D, Depoortere I, Moreaux B, de Smet B, Goris I, Hoskens L, Daneels G, Kass S, Ver Donck L, Peeters T, et al. 2006 Altered gastrointestinal and metabolic function in the GPR39-obestatin receptor-knockout mouse. *Gastroenterology* **131** 1131-1141.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, et al. 1997 Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **387** 903-908.

Morley JE 1987 Neuropeptide regulation of appetite and weight. *Endocr. Rev.* **8** 256-287.

Morton GJ, Cummings DE, Baskin DG, Barsh GS & Schwartz MW 2006 Central nervous system control of food intake and body weight. *Nature* **443** 289-295.

Mountjoy KG, Mortrud MT, Low MJ, Simerly RB & Cone RD 1994 Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.* **8** 1298-1308.

Myers MG, Cowley MA & Munzberg H 2008 Mechanisms of leptin action and leptin resistance. *Annu. Rev. Physiol.* **70** 537-556.

Myers MG, Jr., Leibel RL, Seeley RJ & Schwartz MW 2010 Obesity and leptin resistance: distinguishing cause from effect. *Trends Endocrinol. Metab.* **21** 643-651.

Myers MG, Jr., Munzberg H, Leinninger GM & Leshan RL 2009 The geometry of leptin action in the brain: more complicated than a simple ARC. *Cell Metab.* **9** 117-123.

Naggert JK, Fricker LD, Varlamov O, Nishina PM, Rouille Y, Steiner DF, Carroll RJ, Paigen BJ & Leiter EH 1995 Hyperproinsulinaemia in obese *fat/fat* mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nat. Genet.* **10** 135-142.

Nakamura K & Morrison SF 2008 A thermosensory pathway that controls body temperature. *Nat. Neurosci.* **11** 62-71.

Nargund RP, Strack AM & Fong TM 2006 Melanocortin-4 receptor (MC4R) agonists for the treatment of obesity. *J. Med. Chem.* **49** 4035-4043.

Newman EA, Chai BX, Zhang W, Li JY, Ammori JB & Mulholland MW 2006 Activation of the melanocortin-4 receptor mobilizes intracellular free calcium in immortalized hypothalamic neurons. *J. Surg. Res.* **132** 201-207.

Nguyen N, Champion JK, Ponce J, Quebbemann B, Patterson E, Pham B, Raum W, Buchwald JN, Segato G & Favretti F 2012 A review of unmet needs in obesity management. *Obes. Surg.* **22** 956-966.

Nicholson JR, Kohler G, Schaerer F, Senn C, Weyermann P & Hofbauer KG 2006 Peripheral administration of a melanocortin 4-receptor inverse agonist prevents loss of lean body mass in tumor-bearing mice. *J. Pharmacol. Exp. Ther.* **317** 771-777.

Nickolls SA, Fleck B, Hoare SR & Maki RA 2005 Functional selectivity of melanocortin 4 receptor peptide and nonpeptide agonists: evidence for ligand-specific conformational states. *J. Pharmacol. Exp. Ther.* **313** 1281-1288.

Nijenhuis WAJ, Oosterom J & Adan RAH 2001 AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Mol. Endocrinol.* **15** 164-171.

Niswender KD & Schwartz MW 2003 Insulin and leptin revisited: adiposity signals with overlapping physiological and intracellular signaling capabilities. *Front. Neuroendocrinol.* **24** 1-10.

Nogueiras R, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, Perez-Tilve D, Vazquez MJ, Wiedmer P, Castaneda TR, et al. 2007 Effects of obestatin on energy balance and growth hormone secretion in rodents. *Endocrinology* **148** 21-26.

Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I & Barsh GS 1997 Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* **278** 135-138.

Pace AM, Faure M & Bourne HR 1995 G_{i2}-mediated activation of the MAP kinase cascade. *Mol. Biol. Cell* **6** 1685-1695.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. 2000 Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289** 739-745.

Palkovits M, Mezey E & Eskay RL 1987 Pro-opiomelanocortin-derived peptides (ACTH/beta-endorphin/alpha-MSH) in brainstem baroreceptor areas of the rat. *Brain Res.* **436** 323-338.

Patten CS, Daniels D, Suzuki A, Fluharty SJ & Yee DK 2007 Structural and signaling requirements of the human melanocortin 4 receptor for MAP kinase activation. *Regul. Pept.* **142** 111-122.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K & Cobb MH 2001 Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22** 153-183.

Perez DM & Karnik SS 2005 Multiple signaling states of G-protein-coupled receptors. *Pharmacol. Rev.* **57** 147-161.

Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ & Hess JF 1996 Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* **13** 18-19.

Poggioli R, Vergoni AV & Bertolini A 1986 ACTH-(1-24) and alpha-MSH antagonize feeding behavior stimulated by kappa opiate agonists. *Peptides* **7** 843-848.

Pogozheva ID, Chai BX, Lomize AL, Fong TM, Weinberg DH, Nargund RP, Mulholland MW, Gantz I & Mosberg HI 2005 Interactions of human melanocortin 4 receptor with nonpeptide and peptide agonists. *Biochemistry (Mosc.)* **44** 11329-11341.

Poitout L, Brault V, Sackur C, Bernetiere S, Camara J, Plas P & Roubert P 2007 Identification of a novel series of benzimidazoles as potent and selective antagonists of the human melanocortin-4 receptor. *Bioorg. Med. Chem. Lett.* **17** 4464-4470.

Polak JM, Bloom SR, Rayford PL, Pearse AG, Buchan AM & Thompson JC 1975 Identification of cholecystokinin-secreting cells. *Lancet* **2** 1016-1018.

Pontiroli AE & Morabito A 2011 Long-term prevention of mortality in morbid obesity through bariatric surgery. a systematic review and meta-analysis of trials performed with gastric banding and gastric bypass. *Ann. Surg.* **253** 484-487.

Proneth B, Xiang ZM, Pogozheva ID, Litherland SA, Gorbatyuk OS, Shaw AM, Millard WJ, Mosberg HI & Haskell-Luevano C 2006 Molecular mechanism of the constitutive activation of the L250Q human melanocortin-4 receptor polymorphism. *Chem. Biol. Drug Des.* **67** 215-229.

Qian S, Chen H, Weingarth D, Trumbauer ME, Novi DE, Guan X, Yu H, Shen Z, Feng Y, Frazier E, et al. 2002 Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. *Mol. Cell. Biol.* **22** 5027-5035.

Rasmussen SGF, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, DeVree BT, Rosenbaum DM, Thian FS, Kobilka TS, et al. 2011 Structure of a nanobody-stabilized active state of the β_2 adrenoceptor. *Nature* **469** 175-180.

Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, et al. 2007 Crystal structure of the human β_2-adrenergic G protein-coupled receptor. *Nature* **450** 383-U384.

Rasmussen SGF, Jensen AD, Liapakis G, Ghanouni P, Javitch JA & Gether U 1999 Mutation of a highly conserved aspartic acid in the β_2-adrenergic receptor: Constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol. Pharmacol.* **56** 175-184.

Redmann SM, Jr. & Argyropoulos G 2006 AgRP-deficiency could lead to increased lifespan. *Biochem. Biophys. Res. Commun.* **351** 860-864.

Rohner-Jeanrenaud F & Jeanrenaud B 1996 Obesity, leptin, and the brain. *N. Engl. J. Med.* **334** 324-325.

Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, et al. 2007 GPCR engineering yields high-resolution structural insights into β_2-adrenergic receptor function. *Science* **318** 1266-1273.

Rosenbaum DM, Zhang C, Lyons JA, Holl R, Aragao D, Arlow DH, Rasmussen SGF, Choi HJ, DeVree BT, Sunahara RK, et al. 2011 Structure and function of an irreversible agonist-β_2 adrenoceptor complex. *Nature* **469** 236-240.

Rossi M, Kim MS, Morgan DG, Small CJ, Edwards CM, Sunter D, Abusnana S, Goldstone AP, Russell SH, Stanley SA, et al. 1998 A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo. *Endocrinology* **139** 4428-4431.

Roth CB, Hanson MA & Stevens RC 2008 Stabilization of the human β_2-adrenergic receptor TM4-TM3-TM5 helix interface by mutagenesis of Glu122(3.41), a critical residue in GPCR structure. *J. Mol. Biol.* **376** 1305-1319.

Sawyer TK, Sanfilippo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB & Hadley ME 1980 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: A highly potent α-melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. U. S. A.* **77** 5754-5758.

Sayegh AI 2013 The role of cholecystokinin receptors in the short-term control of food intake. *Prog Mol Biol Transl Sci* **114** 277-316.

Schwartz GJ 2006 Integrative capacity of the caudal brainstem in the control of food intake. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **361** 1275-1280.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P & Baskin DG 1997 Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* **46** 2119-2123.

Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ & Baskin DG 2000 Central nervous system control of food intake. *Nature* **404** 661-671.

Seeley RJ, Yagaloff KA, Fisher SL, Burn P, Thiele TE, van Dijk G, Baskin DG & Schwartz MW 1997 Melanocortin receptors in leptin effects. *Nature* **390** 349.

Seifert R & Wenzel-Seifert K 2002 Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn. Schmiedebergs Arch. Pharmacol.* **366** 381-416.

Selkirk JV, Nottebaum LM, Lee J, Yang W, Foster AC & Lechner SM 2007 Identification of differential melanocortin 4 receptor agonist profiles at natively expressed receptors in rat cortical astrocytes and recombinantly expressed receptors in human embryonic kidney cells. *Neuropharmacology* **52** 459-466.

Seoane LM, Al-Massadi O, Pazos Y, Pagotto U & Casanueva FF 2006 Central obestatin administration does not modify either spontaneous or ghrelin-induced food intake in rats. *J. Endocrinol. Invest.* **29** RC13-15.

Shahabi NA, Daaka Y, McAllen K & Sharp BM 1999 Delta opioid receptors expressed by stably transfected jurkat cells signal through the map kinase pathway in a ras-independent manner. *J. Neuroimmunol.* **94** 48-57.

Sheikh SP, Zvyaga TA, Lichtarge O, Sakmar TP & Bourne HR 1996 Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature* **383** 347-350.

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O & Lefkowitz RJ 2006 β-Arrestin-dependent, G protein-independent ERK1/2 activation by the β2 adrenergic receptor. *J. Biol. Chem.* **281** 1261-1273.

Shinyama H, Masuzaki H, Fang H & Flier JS 2003 Regulation of melanocortin-4 receptor signaling: agonist-mediated desensitization and internalization. *Endocrinology* **144** 1301-1314.

Skibicka KP & Grill HJ 2008 Energetic responses are triggered by caudal brainstem melanocortin receptor stimulation and mediated by local sympathetic effector circuits. *Endocrinology* **149** 3605-3616.

Skibicka KP & Grill HJ 2009 Hindbrain leptin stimulation induces anorexia and hyperthermia mediated by hindbrain melanocortin receptors. *Endocrinology* **150** 1705-1711.

Skuladottir GV, Jonsson L, Skarphedinsson JO, Mutulis F, Muceniece R, Raine A, Mutule I, Helgason J, Prusis P, Wikberg JE, et al. 1999 Long term orexigenic effect of a novel melanocortin 4 receptor selective antagonist. *Br. J. Pharmacol.* **126** 27-34.

Smith AI & Funder JW 1988 Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr. Rev.* **9** 159-179.

Srinivasan S, Lubrano-Berthelier C, Govaerts C, Picard F, Santiago P, Conklin BR & Vaisse C 2004 Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *J. Clin. Investig.* **114** 1158-1164.

St-Pierre J & Tremblay ML 2012 Modulation of leptin resistance by protein tyrosine phosphatases. *Cell Metab.* **15** 292-297.

Stefater MA, Wilson-Perez HE, Chambers AP, Sandoval DA & Seeley RJ 2012 All bariatric surgeries are not created equal: insights from mechanistic comparisons. *Endocr. Rev.* **33** 595-622.

Strobel A, Issad T, Camoin L, Ozata M & Strosberg AD 1998 A leptin missense mutation associated with hypogonadism and morbid obesity. *Nat. Genet.* **18** 213-215.

Stutzmann F, Tan K, Vatin V, Dina C, Jouret B, Tichet J, Balkau B, Potoczna N, Horber F, O'Rahilly S, et al. 2008 Prevalence of melanocortin-4 receptor deficiency in Europeans and their age-dependent penetrance in multigenerational pedigrees. *Diabetes* **57** 2511-2518.

Sutton GM, Duos B, Patterson LM & Berthoud HR 2005 Melanocortinergic modulation of cholecystokinin-induced suppression of feeding through extracellular signal-regulated kinase signaling in rat solitary nucleus. *Endocrinology* **146** 3739-3747.

Tan K, Pogozheva ID, Yeo GS, Hadaschik D, Keogh JM, Haskell-Leuvano C, O'Rahilly S, Mosberg HI & Farooqi IS 2009 Functional characterization and structural modeling of obesity associated mutations in the melanocortin 4 receptor. *Endocrinology* **150** 114-125.

Tansky MF, Pothoulakis C & Leeman SE 2007 Functional consequences of alteration of N-linked glycosylation sites on the neurokinin 1 receptor. *Proc. Natl. Acad. Sci. U. S. A.* **104** 10691-10696.

Tao YX 2005 Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity. *Mol. Cell. Endocrinol.* **239** 1-14.

Tao YX 2008 Constitutive activation of G protein-coupled receptors and diseases: insights into mechanisms of activation and therapeutics. *Pharmacol. Ther.* **120** 129-148.

Tao YX 2009 Mutations in melanocortin-4 receptor and human obesity. *Prog Mol Biol Transl Sci* **88** 173-204.

Tao YX 2010 The melanocortin-4 receptor: physiology, pharmacology, and pathophysiology. *Endocr. Rev.* **31** 506-543.

Tao YX, Abell AN, Liu XB, Nakamura K & Segaloff DL 2000 Constitutive activation of G protein-coupled receptors as a result of selective substitution of a conserved leucine residue in transmembrane helix III. *Mol. Endocrinol.* **14** 1272-1282.

Tao YX, Huang H, Wang ZQ, Yang F, Williams JN & Nikiforovich GV 2010 Constitutive activity of neural melanocortin receptors. *Methods Enzymol.* **484** 267-279.

Tao YX & Segaloff DL 2003 Functional characterization of melanocortin-4 receptor mutations associated with childhood obesity. *Endocrinology* **144** 4544-4551.

Tao YX, Yuan ZH & Xie J 2013 G protein-coupled receptors as regulators of energy homeostasis. *Prog Mol Biol Transl Sci* **114** 1-43.

Tarnow P, Schoneberg T, Krude H, Gruters A & Biebermann H 2003 Mutationally induced disulfide bond formation within the third extracellular loop causes melanocortin 4 receptor inactivation in patients with obesity. *J. Biol. Chem.* **278** 48666-48673.

Terranova L, Busetto L, Vestri A & Zappa MA 2012 Bariatric surgery: cost-effectiveness and budget impact. *Obes. Surg.* **22** 646-653.

Thornton JE, Cheung CC, Clifton DK & Steiner RA 1997 Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. *Endocrinology* **138** 5063-5066.

Tolle V & Low MJ 2008 In vivo evidence for inverse agonism of agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. *Diabetes* **57** 86-94.

Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan JL, Wang QP, Funahashi H, Sakurai T, et al. 2003 Ghrelin-induced food intake is mediated via the orexin pathway. *Endocrinology* **144** 1506-1512.

Trevaskis JL & Butler AA 2005 Double leptin and melanocortin-4 receptor gene mutations have an additive effect on fat mass and are associated with reduced effects of leptin on weight loss and food intake. *Endocrinology* **146** 4257-4265.

Trevaskis JL, Meyer EA, Galgani JE & Butler AA 2008 Counterintuitive effects of double-heterozygous null melanocortin-4 receptor and leptin genes on diet-induced obesity and insulin resistance in C57BL/6J mice. *Endocrinology* **149** 174-184.

Unniappan S, Speck M & Kieffer TJ 2008 Metabolic effects of chronic obestatin infusion in rats. *Peptides* **29** 1354-1361.

Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B & Froguel P 2000 Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J. Clin. Invest.* **106** 253-262.

Vaisse C, Clement K, Guy-Grand B & Froguel P 1998 A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat. Genet.* **20** 113-114.

Valli-Jaakola K, Lipsanen-Nyman M, Oksanen L, Hollenberg AN, Kontula K, Bjorbaek C & Schalin-Jantti C 2004 Identification and characterization of melanocortin-4 receptor gene mutations in morbidly obese finnish children and adults. *J. Clin. Endocrinol. Metab.* **89** 940-945.

van der Kraan M, Tatro JB, Entwistle ML, Brakkee JH, Burbach JP, Adan RA & Gispen WH 1999 Expression of melanocortin receptors and pro-opiomelanocortin in the rat spinal

cord in relation to neurotrophic effects of melanocortins. *Brain Res. Mol. Brain Res.* **63** 276-286.

Vergoni AV, Poggioli R & Bertolini A 1986 Corticotropin inhibits food intake in rats. *Neuropeptides* **7** 153-158.

Vink T, Hinney A, van Elburg AA, van Goozen SH, Sandkuijl LA, Sinke RJ, Herpertz-Dahlmann BM, Hebebrand J, Remschmidt H, van Engeland H, et al. 2001 Association between an agouti-related protein gene polymorphism and anorexia nervosa. *Mol. Psychiatry* **6** 325-328.

Violin JD & Lefkowitz RJ 2007 Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol. Sci.* **28** 416-422.

Vongs A, Lynn NM & Rosenblum CI 2004 Activation of MAP kinase by MC4-R through PI3 kinase. *Regul. Pept.* **120** 113-118.

Vos TJ, Caracoti A, Che JL, Dai M, Farrer CA, Forsyth NE, Drabic SV, Horlick RA, Lamppu D, Yowe DL, et al. 2004 Identification of 2-{2-[2-(5-bromo-2-methoxyphenyl)-ethyl]-3-fluoro-phenyl}-4,5-dihydro-1H-imidazole (ML00253764), a small molecule melanocortin 4 receptor antagonist that effectively reduces tumor-induced weight loss in a mouse model. *J. Med. Chem.* **47** 1602-1604.

Wacker D, Wang C, Katritch V, Han GW, Huang XP, Vardy E, McCorvy JD, Jiang Y, Chu M, Siu FY, et al. 2013 Structural features for functional selectivity at serotonin receptors. *Science*.

Wang HJ, Geller F, Dempfle A, Schauble N, Friedel S, Lichtner P, Fontenla-Horro F, Wudy S, Hagemann S, Gortner L, et al. 2004 Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight

and underweight students, and children with short normal stature. *J. Clin. Endocrinol. Metab.* **89** 157-162.

Wang SX, Fan ZC & Tao YX 2008 Functions of acidic transmembrane residues in human melanocortin-3 receptor binding and activation. *Biochem. Pharmacol.* **76** 520-530.

Wang ZQ & Tao YX 2011 Functional studies on twenty novel naturally occurring melanocortin-4 receptor mutations. *Biochim. Biophys. Acta* **1812** 1190-1199.

Warne T, Edwards PC, Leslie AG & Tate CG 2012 Crystal structures of a stabilized β1-adrenoceptor bound to the biased agonists bucindolol and carvedilol. *Structure* **20** 841-849.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM & Lefkowitz RJ 2003 Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc. Natl. Acad. Sci. U. S. A.* **100** 10782-10787.

Whalen EJ, Rajagopal S & Lefkowitz RJ 2011 Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends Mol. Med.* **17** 126-139.

Willesen MG, Kristensen P & Romer J 1999 Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* **70** 306-316.

Williams DL, Kaplan JM & Grill HJ 2000 The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. *Endocrinology* **141** 1332-1337.

Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK & Lefkowitz RJ 2007 A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **104** 16657-16662.

Woods SC, Schwartz MW, Baskin DG & Seeley RJ 2000 Food intake and the regulation of body weight. *Annu. Rev. Psychol.* **51** 255-277.

Wortley KE, Anderson KD, Yasenchak J, Murphy A, Valenzuela D, Diano S, Yancopoulos GD, Wiegand SJ & Sleeman MW 2005 Agouti-related protein-deficient mice display an age-related lean phenotype. *Cell Metab.* **2** 421-427.

Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, et al. 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* **141** 4325-4328.

Wu Q, Howell MP, Cowley MA & Palmiter RD 2008 Starvation after AgRP neuron ablation is independent of melanocortin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **105** 2687-2692.

Xi B, Liang Y, He T, Reilly KH, Hu Y, Wang Q, Yan Y & Mi J 2012 Secular trends in the prevalence of general and abdominal obesity among Chinese adults, 1993-2009. *Obes. Rev.* **13** 287-296.

Xiang Z, Litherland SA, Sorensen NB, Proneth B, Wood MS, Shaw AM, Millard WJ & Haskell-Luevano C 2006 Pharmacological characterization of 40 human melanocortin-4 receptor polymorphisms with the endogenous proopiomelanocortin-derived agonists and the Agouti-related protein (AGRP) antagonist. *Biochemistry (Mosc.)* **45** 7277-7288.

Xiang Z, Proneth B, Dirain ML, Litherland SA & Haskell-Luevano C 2010 Pharmacological characterization of 30 human melanocortin-4 receptor polymorphisms with the

endogenous proopiomelanocortin-derived agonists, synthetic agonists, and the endogenous agouti-related protein antagonist. *Biochemistry (Mosc.)* **49** 4583-4600.

Yamano Y, Kamon R, Yoshimizu T, Toda Y, Oshida Y, Chaki S, Yoshioka M & Morishima I 2004 The role of the DRY motif of human MC4R for receptor activation. *Biosci. Biotechnol. Biochem.* **68** 1369-1371.

Yang CZ, Asthagiri AR, Iyer RR, Lu J, Xu DS, Ksendzovsky A, Brady RO, Zhuang ZP & Lonser RR 2011 Missense mutations in the NF2 gene result in the quantitative loss of merlin protein and minimally affect protein intrinsic function. *Proc. Natl. Acad. Sci. U. S. A.* **108** 4980-4985.

Yang Y, Chen M, Lai Y, Gantz I, Georgeson KE & Harmon CM 2002 Molecular determinants of human melanocortin-4 receptor responsible for antagonist SHU9119 selective activity. *J. Biol. Chem.* **277** 20328-20335.

Yang Y, Fong TM, Dickinson CJ, Mao C, Li JY, Tota MR, Mosley R, Van der Ploeg LHT & Gantz I 2000 Molecular determinants of ligand binding to the human melanocortin-4 receptor. *Biochemistry (Mosc.)* **39** 14900-14911.

Yaswen L, Diehl N, Brennan MB & Hochgeschwender U 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat. Med.* **5** 1066-1070.

Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG & O'Rahilly S 1998 A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat. Genet.* **20** 111-112.

Zac-Varghese S, De Silva A & Bloom SR 2011 Translational studies on PYY as a novel target in obesity. *Curr. Opin. Pharmacol.* **11** 582-585.

Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupershmidt S, et al. 2005 Cardiac-specific overexpression of AT1 receptor mutant lacking $G_{\alpha}q/G_{\alpha}i$ coupling causes hypertrophy and bradycardia in transgenic mice. *J. Clin. Invest.* **115** 3045-3056.

Zhang JV, Li L, Huang Q & Ren PG 2013 Obestatin receptor in energy homeostasis and obesity pathogenesis. *Prog Mol Biol Transl Sci* **114** 89-107.

Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C & Hsueh AJ 2005 Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310** 996-999.

Zhang ML, Guan RB & Segaloff DL 2012 Revisiting and questioning functional rescue between dimerized LH receptor mutants. *Mol. Endocrinol.* **26** 655-668.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425-432.

Zhang Y, Wu X, He Y, Kastin AJ, Hsuchou H, Rosenblum CI & Pan W 2009 Melanocortin potentiates leptin-induced STAT3 signaling via MAPK pathway. *J. Neurochem.* **110** 390-399.

Zheng H, Patterson LM, Morrison C, Banfield BW, Randall JA, Browning KN, Travagli RA & Berthoud HR 2005a Melanin concentrating hormone innervation of caudal brainstem areas involved in gastrointestinal functions and energy balance. *Neuroscience* **135** 611-625.

Zheng H, Patterson LM, Phifer CB & Berthoud HR 2005b Brain stem melanocortinergic modulation of meal size and identification of hypothalamic POMC projections. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289** R247-258.