

**Human melanocortin-3 receptor: structure-function relationship of DPLIY motif
and helix 8 and biased signaling**

by

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Abstract

The melanocortin-3 receptor (MC3R) is a member of family A G protein-coupled receptors (GPCRs). The MC3R remains the most enigmatic of the melanocortin receptors with regard to its physiological functions, especially the role in energy homeostasis. The N/DPxxY motif and the eighth helix (helix 8) in the carboxyl terminus of GPCRs have been identified to be important for receptor functions. To gain a better understanding of the structure-function relationship of MC3R, we performed systematic study of all the 20 residues in this domain using alanine-scanning mutagenesis. We showed that eleven residues were important for ligand binding and one was indispensable for downstream cAMP generation. F347A was identified to be constitutively active in cAMP signaling while all the other mutants had normal basal activities. We also studied the signaling capacity of nine mutants in the ERK1/2 signaling pathway. All of these mutants showed normal basal ERK1/2 phosphorylation levels. The pERK1/2 levels of six binding- or signaling-defective mutants were enhanced upon agonist stimulation. The unbalanced cAMP and pERK1/2 signaling pathways suggested the existence of biased signaling in MC3R mutants.

Not only mutant receptors can be biased, different ligands of the same receptor have been shown to induce distinct receptor conformations that lead to the activation of diverse signaling pathways, resulting in biased physiological responses. The orexigenic agouti-related peptide (AgRP), which was initially identified as an endogenous antagonist

for both neural MCRs, has been suggested to be a biased agonist of MC4R independently of its antagonizing effects. However, little is known about the underlying mechanism or whether the biased agonism of AgRP also exists in MC3R.

Herein, we investigated the potential of AgRP to regulate the activation of intracellular kinases through both neural MCRs. We showed that AgRP acted as a biased agonist in MC3R, decreasing the basal cAMP activity of constitutively active mutant (F347A) but stimulating ERK1/2 activation in both wide type and F347A hMC3Rs. AgRP-stimulated ERK1/2 phosphorylation through MC3R was abolished by protein kinase A (PKA) inhibitor H-89, whereas AgRP-initiated ERK1/2 activation through MC4R was inhibited by phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002. Both NDP-MSH and AgRP treatment induced significant AKT phosphorylation in GT1-7 cells but not in either MC3R- or MC4R-transfected HEK293T cells. The pAMPK levels in both GT1-7 cells and HEK293T cells transfected with neural MCRs were significantly decreased upon stimulation with NDP-MSH but not AgRP.

In summary, we showed that the DPLIY motif and Helix 8 are important for MC3R activation and signal transduction. We also provided novel data for the biased agonism of AgRP in neural MCRs. Our result established a theoretical basis for the structure-function relationship of MC3R and AgRP-initiated multiple intracellular signaling pathways, leading to a better understanding of neural MCR pharmacology.

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List of Abbreviations

AC	Adenylyl cyclase
ACTH	Adrenocorticotrophic hormone
AgRP	Agouti-related peptide
AMPK	5' AMP-activated protein kinase
ARC	Arcuate nucleus
B _{max}	Apparent maximal binding
BMI	Body mass index
BSA	Bovine serum albumin
CAM	Constitutively active mutant
CART	Cocaine- and amphetamine-regulated transcript
CNS	Central nervous system
DDP	Dipeptidyl peptidase
DMEM	Dulbecco's modified eagle medium
DMN	Dorsomedial nucleus
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GABA	γ -Aminobutyric acid
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal

GLP-1	Glucagon-like peptide-1
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HEK	Human embryonic kidney
ICV	Intracerebroventricular
IR	Insulin receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knockout
LHA	Lateral hypothalamic area
MAPK	Mitogen-activated protein kinase
MC3R	Melanocortin-3 receptor
MC4R	Melanocortin-4 receptor
MCR	Melanocortin receptor
MSH	Melanocyte stimulating hormone
MTII	Melanotan II
NPY	Neuropeptide Y
NTS	nucleus tractus solitarius
ObR	Leptin receptor
PBS-IH	PBS for immunohistochemistry
PC	Prohormone convertase
PI3K	Phosphoinositide 3-kinase

PKA	Protein kinase A
PKC	Protein kinase C
POMC	Proopiomelanocortin
PTX	Pertussis toxin
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
R _{max}	Maximal response
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TM	Transmembrane
VMN	Ventromedial nucleus
VTA	Ventral tegmental area
WHO	World Health Organization
WT	Wild type

Chapter 1

1.1 Introduction

Obesity is a multifactorial epidemic with enormous health, economic and social burdens. Therapeutic options for obesity available now, such as pharmacotherapy and bariatric surgery, are far from ideal and novel avenues and targets are urgently sought. Obesity is a metabolic disorder caused by the imbalance of energy intake and expenditure and there is mounting evidence demonstrating that genetic factors contribute to the pathogenesis of obesity. Therefore, there is an urgent need for understanding the regulation of energy homeostasis from genetic perspective. To date, a number of genes, such as *leptin*, *leptin receptor*, *proopimelanocortin* and *melanocortin-4 receptor (MC4R)* have been identified to be associated with human energy balance.

The melanocortin-3 receptor (MC3R), which is referred as neural melanocortin receptor together with MC4R, has been increasingly considered to be involved in the regulation of energy homeostasis, mediating both feed efficiency and circadian rhythm. Although twenty-seven *MC3R* mutations and two common polymorphic variants have been identified so far in different cohorts, the pathogenic role of MC3R in human obesity remains controversial. Recent studies of MC3R signaling pathways other than cAMP-protein kinase A pathway have shed light for future research of MC3R, and more detailed

functional studies would be needed to unravel the role of MC3R in human body weight regulation and obesity pathogenesis.

This chapter presents an overview of obesity trends and current treatment. The regulation of energy homeostasis by central nervous system and gastrointestinal hormones are discussed. The pathogenic roles and signaling pathways of neural melanocortin receptors, especially MC3R, are highlighted.

1.2 Obesity

1.2.1 The prevalence of obesity

Obesity is characterized as a medical condition in which excessive fat is accumulated in adipose tissue and other organs. The National Heart, Lung, and Blood Institute and the World Health Organization (WHO) define obesity as a body mass index (BMI, body weight in kilograms divided by height in meters squared) equal to or greater than 30 kg/m² and overweight as a BMI of 25 to 29.9 kg/m² (Flegal et al. 2010).

Obesity has become a global epidemic. According to WHO, 1.46 billion adults are overweight and over 200 million men and 300 million women are obese globally (Finucane et al. 2011). In the United States, the most recent National Health and Nutrition Examination Survey indicates that 68.5% of the adults are presently classified as overweight or obese with 34.9% as obese (Ogden et al. 2014). The obesity epidemic is not restricted to developed countries. Sharp increase of obesity has also been observed in developing countries.

Obesity in children and adolescents is also increasing at an alarming rate. About 170 million children globally are classified as overweight or obese, a quarter of which are under 5 years of age (Lobstein et al. 2004). Although the prevalence of obesity in certain age groups of childhood from some developed countries appears to be flattening, the overall prevalence is still high (Ogden et al. 2012; Ogden et al. 2014). Obese children are more likely to be afflicted by obesity-related diseases such as type 2 diabetes mellitus and heart diseases before or during adulthood (Baker et al. 2007; Lobstein et al. 2004).

Obesity is becoming a significant public health burden since it is associated with a number of comorbidities including type 2 diabetes mellitus, cardiovascular diseases, hypertension, arteriosclerosis, sleep apnea and certain types of cancer (Desvergne et al. 2004; Tao et al. 2013). According to the new guidelines released in November 2013 by the American Heart Association, American College of Cardiology and The Obesity Society, obesity itself is currently recognized as a disease (Jensen et al. 2014).

In addition to the health crisis, obesity is also associated with enormous economic costs. The annual medical expenditures attributed to obesity and obesity-related diseases in the United States increased from \$75 billion in 2003 to \$147 billion in 2008, and the number was estimated to reach \$344 billion by 2018 (Trogon et al. 2012). Obesity is now accounting for up to 6% of total healthcare cost in the United States while this number is estimated to rise to roughly 21% by 2018 (Finkelstein et al. 2004). Indeed, obesity has now overtaken tobacco as the largest preventable cause of disease burden in some countries. Moreover, the negative psychological effect of obesity, such as discrimination and exclusion, cannot be ignored since these negative emotions can lead to social problems (Puhl et al. 2013).

1.2.2 Current treatments for obesity

Although considerable attention has been paid to obesity research during the past few decades, there are only very limited effective therapeutic options currently available. Current treatments for obesity include lifestyle intervention, pharmacological therapy and bariatric surgery.

Lifestyle interventions or modifications, such as exercise and dieting, have conventionally been used for treating obesity and can achieve short-term success for the majority of obese patients (Bray 2008; Wadden et al. 2012). However, strong physiological and behavioral responses to caloric restriction together with the “obesogenic environment” make the lifestyle modification extremely difficult for obese individuals to maintain the weight loss. The human body’s defense of body weight prevents the obese patients from long-term success in weight loss (Guyenet and Schwartz 2012; Woods et al. 2000).

Pharmacotherapy will be considered if lifestyle interventions are ineffective for obese individuals. Many anti-obesity drugs have been developed to manage obesity over the past several decades (Hofbauer et al. 2007). However, most of the drugs, such as phentermine and rimonabant, have now been withdrawn due to serious side effects (Christensen et al. 2007; Connolly et al. 1997). The most recently withdrawn drug for obesity control is sibutramine, a selective noradrenaline/serotonin re-uptake inhibitor (Nisoli and Carruba 2000), which has a strong association with increased cardiovascular events and stroke (James et al. 2010). The only currently available drug for the long-term management of obesity is orlistat, a potent and reversible gastrointestinal lipase inhibitor that prevents dietary fat absorption by inhibiting pancreatic and gastric lipases. Although

the efficacy of orlistat has been proven in clinic (Davidson et al. 1999; Torgerson et al. 2004), it is associated with side effects such as diarrhea, flatulence and dyspepsia (Filippatos et al. 2008).

Bariatric surgery, such as laparoscopic-adjustable gastric banding, Roux-en-Y gastric bypass and sleeve gastrectomy, can restrict the flow of food through the gastrointestinal (GI) tract and is currently considered the most effective treatment for morbid obesity. The average percentage of excess weight loss by bariatric surgery is 61.2% (Buchwald et al. 2004) and it can also reduce obesity-related comorbidities including diabetes, hypertension, heart diseases and certain types of cancers (Ashrafian et al. 2011; Buchwald et al. 2004; Pontiroli and Morabito 2011; Stefater et al. 2012). Moreover, both the short- and long-term efficacies of bariatric surgery have been established (Sjostrom et al. 2004). However, the significant cost, risks and complication rates prevent the majority of obese patients from undergoing the surgeries (DeMaria 2007; Terranova et al. 2012).

1.2.3 Regulation of energy homeostasis

Energy homeostasis refers to the matching of energy intake with energy expenditure, and is achieved when anabolic and catabolic influences are in balance over long intervals. The energy intake is derived from food and drinks while the energy expenditure includes physical activity that refers to all voluntary movement, and basal metabolism which represents the myriad biochemical processes necessary to sustain minimal daily functions (Spiegelman and Flier 2001).

The participation of central nervous system (CNS) in the regulation of energy homeostasis was first suggested by lesioning studies in rodents more than 50 years ago (Kennedy 1953; Mayer and Thomas 1967). Since then, mounting experimental evidence from both rodent and human studies have confirmed the hypothalamus to be a fundamental nexus in the neuronal hierarchy controlling whole-body energy homeostasis. The hypothalamus senses multiple metabolic signals, which are generated by peripheral systems and conveying information about the energy status, and in turn regulates the energy balance through the coordination of feeding behavior and energy expenditure (Schneeberger et al. 2014). Several hypothalamic nuclei anatomically localized around the third ventricle, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN), and the ventromedial nucleus (VMN), have been identified to be functionally crucial in responding to numerous peripheral metabolic signals.

Among these hypothalamic nuclei, the ARC is the most extensively studied and has been recognized to be the core region of hypothalamic regulation of energy homeostasis. The ARC is located on both sides of the third ventricle and is closely adjacent to the median eminence. The semi-permeable blood-brain barrier in this area enables the ARC neurons to directly sense a wide array of hormonal and nutrient fluctuations in the circulating bloodstream (Broadwell and Brightman 1976). There are two physiologically distinct populations of neuron in the ARC, the proopiomelanocortin (POMC) neurons and the agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons. The POMC neurons are located laterally to the third ventricle and coexpress the anorexigenic neuropeptides cocaine- and amphetamine-regulated transcript (CART) and

α -melanocyte stimulating hormone (α -MSH), whereas the AgRP/NPY neurons are located more medio-centrally and coexpress the orexigenic AgRP and NPY (Cowley et al. 1999; Jeong et al. 2014). These two populations of neurons, together with downstream neurons expressing melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R), constitute the neural melanocortin system that is crucial for the precise regulation of food intake and energy expenditure (see Section 1.3.1).

The functional importance of other hypothalamic nuclei in the regulation of energy balance has also been demonstrated. The PVN receives projection of POMC and AgRP neurons from the ARC and has been shown to mediate the energy balance through two subsets of anorectic-related neurons expressing thyrotropin-releasing hormone and corticotropin-releasing hormone, respectively (Fekete et al. 2000; Richard and Baraboi 2004). In contrast, the LHA plays an important role in the mediation of orexigenic responses through two neuropeptides, orexin and melanin-concentrating hormone, the expression of which leads to promoted food intake (Ludwig et al. 2001; Sakurai et al. 1998). The DMN functions as a transmitter by receiving projection from the ARC and sending projections into the PVN and LHA. It plays a significant role in the regulation of thermogenesis and the development of diet-induced obesity (Chao et al. 2011; Suzuki et al. 2012). The VMN is featured with abundant expression of brain-derived neurotrophic factor, the central or peripheral administration of which results in the decrease of food intake and loss of body weight through MC4R signaling (Xu et al. 2003).

In addition to hypothalamus, central regulation of energy homeostasis has also been attributed to midbrain and brainstem. Dopaminergic neurons within the midbrain ventral tegmental area (VTA) that innervate the ventral striatum have been implicated in

the rewarding aspects of food (Volkow and Wise 2005). Although the activity of the mesolimbic dopaminergic system was initially considered to be regulated by direct or indirect projections from the ARC to the VTA or ventral striatum (Berthoud 2002), emerging evidence have revealed that the activity of dopaminergic neurons within the VTA could be modulated directly by peripheral metabolic signals (Gao and Horvath 2008). Within the brainstem, the dorsal vagal complex, which comprises the dorsal motor nucleus of vagus, the area postrema, and the nucleus tractus solitarius (NTS), has been demonstrated to play a key role in relaying peripheral signals to hypothalamus through vagal afferents. The brainstem, especially the NST, is involved in the control of meal size via vagus signaling from the GI tract (Grill and Hayes 2009). Besides its role in receiving descending projection from hypothalamus, the NTS is also featured with abundant expression of POMC and MC4R that regulate food intake and energy expenditure similarly to those in the hypothalamus (Grill et al. 1998; Skibicka and Grill 2009).

The peripheral metabolic signals circulating in bloodstream include long-term and short-term regulatory signals. The two major adiposity signals for long-term regulation are leptin and insulin. The involvement of leptin in the regulation of energy homeostasis was first observed in mice in 1994 (Zhang et al. 1994) and in humans in 1997 (Montague et al. 1997). Leptin is a 16 kDa peptide hormone secreted from adipocytes in proportion to the degree of adiposity. It can be transported across the blood-brain barrier and act directly in the hypothalamus, especially the ARC, by stimulating the leptin receptor (ObR). Multiple ObR isoforms have been identified, with the long form ObRb being fully functional and essential for the effects of leptin (Bjorbaek et al. 1997). The ObRb is a member of Class I cytokine receptor family with a single transmembrane (TM) domain. Activation of

ObR initiates a cascade of signal transduction pathways, including Janus kinase/signal transducers and activators of transcription (JAK/STAT) and phosphoinositide 3-kinase (PI3K) pathways, which consequently activate the POMC neurons but inhibit the AgRP neurons (Ghilardi and Skoda 1997; Niswender et al. 2001). Deficiency of leptin or ObRb in both rodents and humans has been shown to cause severe obesity characterized by hyperphagia and reduced energy expenditure (Chen et al. 1996; Clement et al. 1998; Halaas et al. 1995). However, most obese individuals have very high circulating leptin levels in the absence of central anorexigenic actions, exhibiting a state of leptin resistance (Maffei et al. 1995; Rohner-Jeanrenaud and Jeanrenaud 1996), and the mechanism still remains unclear.

Insulin, which is produced by pancreatic β -cells and traditionally considered to be a regulator of glucose metabolism, has been reported to act as an anorectic signal within the CNS. Intracerebroventricular (ICV) administration of insulin in primates and rodents results in a dose-dependent suppression of food intake and body weight gain (Air et al. 2002; McGowan et al. 1993). Insulin enters the CNS through a saturable and receptor-mediated transport process (Baura et al. 1993). The insulin receptor (IR) belongs to the large class of tyrosine kinase receptors. Insulin binding to IR leads to the autophosphorylation of the receptor, the recruitment of insulin receptor substrate protein (IRS), especially IRS-2 in the CNS (Vogt and Bruning 2013), and the consequent converge with the leptin-initiated signaling at PI3K pathway, resulting in increased expression of POMC and reduced expression of AgRP and NPY (Sadagurski et al. 2014; Xu et al. 2005).

In addition to adiposity signals, the GI tract also sends hormonal and nutritional signals to the CNS representing a short-term regulatory mechanism of energy homeostasis. Important gut hormones that have been extensively studied include ghrelin, glucagon-like peptide-1 (GLP-1), and peptide tyrosine tyrosine (PYY).

Ghrelin is a 28-amino acid hormone produced mainly by X/A-like cells within gastric oxyntic glands (Date et al. 2000). There are two major forms of mature ghrelin, acylated and desacylated ghrelin, the former of which is found to be the predominant active form (Kojima et al. 1999). Ghrelin is unique to be the only known orexigenic gut hormone and the levels of circulating ghrelin rise preprandially and fall rapidly in the postprandial period (Cummings et al. 2001). Both central and peripheral administration of ghrelin in rodents have been shown to increase food intake and body weight with a reduction of fat utilization (Nakazato et al. 2001; Tschop et al. 2000). Negative correlations between ghrelin levels and BMI are also found in normal weight human but not obese subjects, indicating ghrelin resistance (le Roux et al. 2005; Otto et al. 2001). Ghrelin regulates energy homeostasis through the growth hormone secretagogue receptor (GHSR), which is a member of G protein-coupled receptor (GPCR) superfamily and abundantly expressed on almost all AgRP/NPY neurons (Davenport et al. 2005; Willesen et al. 1999). Ghrelin binding to GHSR leads to direct stimulation and activation of AgRP/NPY neurons within the ARC and also indirect inhibition of POMC neurons by inducing γ -aminobutyric acid (GABA) release from AgRP/NPY neurons (Cowley et al. 2003; Nakazato et al. 2001).

GLP-1 is secreted from intestine L-cells in response to nutrient ingestion. GLP-1 has two biologically active forms, GLP-1₇₋₃₇ and GLP-1₇₋₃₆ amide, the latter of which is the

major circulating form in human but has equivalent potency with the other form (Orskov et al. 1994). Intravenous administration of GLP-1 could induce dose-dependent reduction of food intake in both normal weight and obese individuals (Verdich et al. 2001). Moreover, GLP-1 has been reported to stimulate insulin secretion, suppress glucagon production and delay gastric emptying (Cummings and Overduin 2007; Kreymann et al. 1987). GLP-1 exerts anorexigenic effects by activating GLP-1 receptor (GLP1R), which is also a member of GPCRs and widely distributed in CNS, GI tract and pancreas (Holst 2007). Although GLP-1 has the potential for obesity treatment, its application has been greatly limited by its short plasma half-life, which is partly due to the enzymatic degradation by dipeptidyl peptidase IV (DPP-IV) (Deacon 2004; Mentlein et al. 1993). The DPP-IV-resistant analogs of GLP-1 have been actively studied and developed, but more research still needs to be carried out to translate the GLP-1-based therapies into clinical practice.

PYY is another appetite suppressing hormone secreted from the L-cells of the intestinal epithelium (Tatemoto and Mutt 1980). PYY has two circulating forms, PYY₁₋₃₆ and PYY₃₋₃₆, with the latter being produced by cleavage of the N-terminal tyrosine-proline residues from the PYY₁₋₃₆ by DPP-IV and acting as the predominant biologically active form (Eberlein et al. 1989). PYY release is increased in proportion to calorie intake and peripheral PYY administration in rodents induces a reduction in food intake and body weight gain (Adrian et al. 1985; Batterham et al. 2002). In both lean and obese humans, intravenous administration of PYY leads to appetite suppression and food intake reduction, suggesting the significant role of PYY in the regulation of energy homeostasis (Batterham and Bloom 2003; Batterham et al. 2002). Moreover, PYY has also been reported to regulate energy expenditure and inhibit GI functions by delaying gastric

emptying, reducing gastric acid production, and limiting pancreatic exocrine secretion (Ashby and Bloom 2007; Sloth et al. 2007). PYY signals through binding to the G protein-coupled NPY receptors (Y1, Y2, Y4, Y5, and Y6). PYY₃₋₃₆ selectively binds to Y2 receptors in the ARC to inhibit NPY release and decrease food intake (Batterham et al. 2003). In contrast, PYY₁₋₃₆ was suggested to initiate orexigenic signal through Y1 and Y5 receptor, but the detailed mechanism remains unclear (Ballantyne 2006).

These long-term and short-term circulating metabolic signals act in concert with specific neuronal circuits in the hypothalamus and brainstem to establish a dynamic and integrated system that tightly regulates the whole-body energy homeostasis.

1.3 The central melanocortin system

1.3.1 The melanocortin system

The melanocortin system is comprised of six major known endogenous ligands (four agonists and two antagonists) and 5 receptors that were named according to the order of their cloning (Cone 2005; Cone et al. 1996; Gantz and Fong 2003).

The four endogenous agonists, α -MSH, β -MSH, γ -MSH, and adrenocorticotropin (ACTH), are produced from the prohormone POMC, which is post-translationally processed by prohormone convertases (PC1/PC2). POMC neurons are mainly located in the ARC of the hypothalamus with a small population found in the NTS in the brainstem. The melanocortin system is unique to have two endogenous antagonists, Agouti and AgRP.

The diverse actions of melanocortins are mediated by five melanocortin receptors (MCRs). The melanocortin-1 receptor (MC1R) regulates skin and hair pigmentation while MC2R is the regulator of adrenal steroidogenesis (Mountjoy et al. 1992; Valverde et al. 1995). Two neural MCRs, MC3R and MC4R, have been shown to play important roles in energy homeostasis (Cone 2006). MC5R is mainly involved in the regulation of exocrine gland secretion (Chen et al. 1997).

The endogenous ligands exhibit variable degrees of specificity for different MCRs. α -MSH and β -MSH display agonist activities on MC1R, MC3R, MC4R and MC5R. γ -MSH exhibits modest selectivity for MC3R while MC2R only binds to ACTH (Abdel-Malek 2001). Agouti acts as a selective antagonist on MC1R and MC4R (Fan et al. 1997; Lu et al. 1994), whereas AgRP functions specifically on MC3R and MC4R (Fong et al. 1997; Ollmann et al. 1997). Recent studies also indicated that AgRP could inhibit the basal activity of MC3R and MC4R, suggesting inverse agonist property (Haskell-Luevano and Monck 2001; Nijenhuis et al. 2001; Tao et al. 2010).

Among the five MCRs, MC3R and MC4R are primarily expressed in the CNS and are involved in the physiological regulation of body weight and metabolic activity. The central melanocortin system serves as the intersection connecting the neural pathways governing satiety and metabolism with signals of metabolic status to maintain energy homeostasis mainly through the leptin-melanocortin circuit. As mentioned previously, binding of circulating leptin to widely-distributed ObRs on the POMC and AgRP neurons, which are referred as “first order” neurons since they are the primary targets of metabolic signals, leads to the activation of POMC neurons but inhibition of AgRP neurons. The released α -MSH or AgRP further affects the “second order” neurons, where “first order”

neurons project their axon terminals and MC3R or MC4R are highly expressed, and finally leads to the maintenance of energy homeostasis.

The importance of this leptin-melanocortin circuit has been highlighted in both rodent and human since mutations of any component in this circuit can lead to obesity and other metabolic disorders. Typical models in rodent, such as *ob/ob* mice, *db/db* mice, and *POMC* knockout (KO) mice, and mutations in human including *leptin*, *ObR*, *POMC* and *MC4R* mutations, can all result in severe obesity (Chen et al. 1996; Clement et al. 1998; Graham et al. 1997; Krude et al. 1998; Montague et al. 1997; Vaisse et al. 1998; Yaswen et al. 1999; Yeo et al. 1998; Zhang et al. 1994).

1.3.2 The melanocortin-4 receptor

The human MC4R (hMC4R), first cloned in 1990s, is a protein of 332 amino acids encoded by an intronless *hMC4R* gene located at chromosome 18q21.3 (Gantz et al. 1993a; Mountjoy et al. 1994). The MC4R has been shown to be extensively expressed in the CNS including the cortex, thalamus, hippocampus, hypothalamus, brain stem, and spinal cord (Mountjoy et al. 1994). In human hypothalamus, the MC4R is primarily expressed in the PVN, the supraoptic nucleus, and the nucleus basalis of Meynert, which is highly in agreement with that observed in rats and mice (Siljee et al. 2013). The expression of MC4R mRNA has also been detected in several peripheral tissues, such as heart, lung, muscle, kidney, and testis during the fetal period (Mountjoy et al. 2003). However, the functions of the MC4R in these tissues remain to be further elucidated.

The important role of MC4R in regulating energy homeostasis has been established by numerous studies. Fan *et al.* reported that ICV administration of melanotan

II (MTII), a superpotent analogue of MCRs agonist α -MSH, inhibits the hyperphagia in four different obese mouse models, and the MTII-induced inhibition is blocked by co-administration of SHU9119, which is a high-affinity antagonist of MC3R and MC4R (Fan et al. 1997). Moreover, direct administration of MTII and SHU9119 into the PVN of rats where MC4R is highly expressed results in extremely potent effects on food intake, suggesting the PVN to be the primary sites of action in melanocortin-induced feeding behavior regulation (Giraudo et al. 1998). In order to exclude the interference of MC3R, several agonists and antagonists selectively targeting MC4R have been developed and the results of animal studies using these selective ligands have further confirmed the indisputable role of MC4R in the regulation of energy homeostasis (Benoit et al. 2000; Kask et al. 1998a; Kask et al. 1998b; Palucki et al. 2005; Vergoni et al. 2000).

The critical importance of MC4R in the mediation of both food intake and energy expenditure has been substantially verified by the development of *Mc4r* KO mouse model. The *Mc4r*^{-/-} mice are demonstrated to be hyperphagic, hyperglycemic, and hyperinsulinemic with maturity-onset obesity and increased linear growth (Huszar et al. 1997). In addition to hyperphagia, the *Mc4r*^{-/-} mice are also featured with decreased energy expenditure. Intraperitoneal administration of the MT-II was reported to increase the metabolic rate in wide type (WT) mice but not in *Mc4r*^{-/-} mice (Chen et al. 2000b). Moreover, unlike the WT mice that respond to an increase in the fat content of the diet by rapidly increasing diet-induced thermogenesis and by increasing physical activity, the *Mc4r*^{-/-} mice fail to elicit these responses (Butler et al. 2001).

In addition to its role in the regulation of energy homeostasis, MC4R has also been reported to be involved in multiple physiological functions such as glucose and lipid

homeostasis, reproductive and sexual behaviors, cardiovascular functions, brain inflammation, and bone metabolism (Tao 2010a).

The first frameshift mutations in *Mc4r* gene associated with severe early-onset obesity were reported in 1998 (Vaisse et al. 1998; Yeo et al. 1998). Since then, more than 170 distinct *MC4R* mutations, including nonsense, frameshift, deletion and nonsynonymous mutations, have been identified from cohorts of different ethnic origins (Hinney et al. 2013; Tao 2009). *MC4R* mutations are detected in 2-6% of (extremely) obese individuals with most mutations carriers being heterozygous, making it the most prevalent form of human monogenic obesity (Farooqi et al. 2003; Hinney et al. 2013).

MC4R is a typical member of rhodopsin-like Family A of the GPCR superfamily, consisting of 7 TM helices with an extracellular N-terminus and an intracellular C-terminus. Activation of MC4R results in the GDP/GTP exchange in G_s protein, activating adenylyl cyclase to increase the production of cAMP, the second messenger in the system. Based on this life cycle of receptors from the biosynthesis to intracellular signaling, Tao proposed a classification scheme for MC4R mutants (Tao and Segaloff 2003). Class I mutants have decreased receptor levels either due to defective protein synthesis and/or increased protein degradation. Class II mutants are defective in trafficking onto the cell surface despite normal synthesis of the mutant receptors. Class III mutants have normal cell surface expression but defective ligand binding due to either decreased binding capacity and/or affinity. Class IV mutants have normal cell surface expression and ligand binding but impaired signaling with either decreased efficacy and/or potency. Class V Mutants have unknown defects with normal cell surface expression, ligand binding and cAMP production. Most of MC4R mutants reported to date belong to Class II, which are

misfolded and intracellularly retained. Therefore, approaches leading to increased cell surface expression of mutant MC4R, such as pharmacoperones, could potentially be of therapeutic value and have been actively studied (Huang and Tao 2014b; Tao and Huang 2014).

1.3.3 The melanocortin-3 receptor

1.3.3.1 Molecular cloning and tissue distribution of MC3R

The human *MC3R* was first cloned by Gantz and colleagues and predicted to encode a 360 amino acid protein as a single exon gene located on chromosome 20q13.2 (Gantz et al. 1993b; Magenis et al. 1994). Recent studies suggested that the translational starting site of human *MC3R* is at the evolutionary conserved second ATG codon instead of the originally assumed non-conserved first ATG, resulting in a 37 amino acid shorter protein (Park et al. 2014b; Tarnow et al. 2012). Park *et al.* further indicated that human *MC3R* has an additional upstream exon that directs the utilization of the second ATG codon as the translational start site (Park et al. 2014b).

The MC3R is primarily expressed in hypothalamus, especially in the ARC, the VMN, and the posterior hypothalamic region (Jegou et al. 2000). Although MC3R has a much narrower distribution compared with MC4R, it has been shown to be the only MCR expressed by both POMC and AgRP neurons in the ARC (Jegou et al. 2000; Mounien et al. 2005). In addition to its distribution in CNS, MC3R is also expressed in several peripheral tissues, including the placenta, gut, heart, kidney, and peritoneal macrophages (Chhajlani 1996; Gantz et al. 1993b; Getting et al. 2003; Ni et al. 2006). Based on its wide distribution, the MC3R has been shown to be involved in the regulation of hypothalamic–

pituitary–thyroid axis (Kim et al. 2002), cardiovascular function (Mioni et al. 2003; Versteeg et al. 1998), and natriuresis (Chandramohan et al. 2009; Ni et al. 2006).

1.3.3.2 MC3R in energy homeostasis and inflammation

The functional role of MC3R in energy homeostasis is less clear compared with that of MC4R that has been shown to be crucial for both food intake and energy expenditure. However, the more severe obesity phenotype observed in mice lacking both *Mc3r* and *Mc4r* suggested that these two receptors serve non-redundant roles in energy homeostasis (Chen et al. 2000a). This non-redundancy has been further confirmed by the study of the anorectic response of mice to the mixed MC3/4R agonist MT-II, showing that the double KO mice are completely unresponsive while both *Mc3r*^{-/-} and *Mc4r*^{-/-} mice show partial anorectic response (Rowland et al. 2010).

Unlike the hyperphagic and obese *Mc4r*^{-/-} mice, *Mc3r*^{-/-} mice display a moderate obesity syndrome characterized with no hyperphagia but increased fat mass and reduced lean mass (Butler et al. 2000; Chen et al. 2000a; Zhang et al. 2005). The increase in adiposity is exacerbated by feeding a high-fat diet (Trevaskis et al. 2007). Reduced energy expenditure is also observed in *Mc3r*^{-/-} mice with decreasing locomotor behavior (Chen et al. 2000a; Sutton et al. 2008). Therefore, MC3R is primarily involved in the mediation of feed efficiency and nutrient partitioning instead of direct regulation of food intake.

One possible mechanism for the regulation of feed efficiency by MC3R is suggested to be the subtle imbalance between fat intake and oxidation, since the fatty acid oxidation was significantly reduced in the skeletal muscle of *Mc3r*^{-/-} mice (Sutton et

al. 2006). Renquist *et al.* suggested that loss of MC3R in mice might impair the metabolic adaptation to fasting with defective white adipose tissue lipolysis, reduced liver triglyceride accumulation and blunted hypothalamic-pituitary-adrenal axis activation (Renquist et al. 2012).

Recently, studies from Butler's laboratory have revealed that MC3R is also involved in the regulation of inputs of feeding-related signals into systems expressing rhythms of food anticipatory activity. Their studies showed that *Mc3r*^{-/-} mice under restricted feeding exhibit reduced wakefulness before food presentation indicating impaired behavioral adaptation (Begrache et al. 2012; Sutton et al. 2008). Abnormal rhythmic expression of clock genes such as *Bmal1* and *Rev-erba* in *Mc3r*^{-/-} mice is also observed (Girardet and Butler 2014). In addition to the impaired behavioral adaptation, *Mc3r*^{-/-} mice also display abnormal metabolic adaptation to the restricted feeding protocol. Although previous studies suggested that *Mc3r*^{-/-} mice are protected from the development of fatty liver disease, severe insulin resistance and obesity-induced inflammation in white adipose tissue, different from the *Mc4r*^{-/-} mice (Albarado et al. 2004; Ellacott et al. 2007; Sutton et al. 2006), *Mc3r*^{-/-} mice under restricted feeding was found to show a sophisticated phenotype with decreased body weight but deteriorating metabolism including hyperinsulinemia, glucose intolerance, enhanced expression of lipogenic genes, and increased ketogenesis (Sutton et al. 2010).

Several recent intriguing studies revisited the potential direct participation of MC3R in food intake regulation. It is reported that both peripheral and central administration of selective MC3R agonist stimulates feeding in rats (Lee et al. 2008; Marks et al. 2006), and another study demonstrated that treatment of a mixed MC3R antagonist and MC4R

agonist tetrapeptide reduces food intake of *Mc4r*^{-/-} rats (Irani et al. 2011). These studies suggested that MC3R might act as an inhibitory autoreceptor on POMC neurons, but the conclusion could not be easily drawn since there was also some inconsistency within these studies. For example, the same researchers of the *Mc4r*^{-/-} rat model study also reported that treatment of a mixed MC3R and MC4R full agonist terapeptide results in reduced food intake in both WT and *Mc4r*^{-/-} rats (Irani et al. 2011).

In addition to its functions in regulating energy homeostasis, the MC3R is also found to be expressed in rodent and human macrophages and it has been shown to play an important modulatory role in host inflammatory responses (Getting et al. 1999; Taherzadeh et al. 1999). Getting *et al.* reported that treatment with MC3R selective agonist γ_2 -MSH, but not MC1R selective agonist MS05, inhibits the accumulation of CXC chemokine KC, interleukin-1 beta, and polymorphonuclear leukocytes elicited by monosodium urate crystal-induced peritonitis in both WT and *Mc1r*-defective mice (Getting et al. 2001; Getting et al. 2003). The similar result is obtained by using another MC3R selective agonist D-Trp⁸- γ -MSH, but the anti-inflammatory effects are absent in *Mc3r*^{-/-} mice (Getting et al. 2006). Moreover, the mixed MC3/4R antagonist SHU9119 could prevent the inhibitory actions induced by either γ_2 -MSH or D-Trp⁸- γ -MSH, whereas the selective MC4R antagonist HS024 has no effect, suggesting the pivotal and independent role of MC3R in inflammatory regulation.

The MC3R has so far been shown to be functionally important in the mediation of inflammatory responses in multiple organs and tissues, such as rheumatoid arthritis (Patel et al. 2010), vascular inflammation (Leoni et al. 2008), lung inflammation (Getting et al. 2008; Land 2012), and periodontal disease (Montero-Melendez et al. 2014). MC3R

activation by melanocortins leads to the inhibition of pro-inflammatory cytokines, chemokine or nitric oxide and the enhancement of anti-inflammatory mediators such as heme oxygenase-1 (Patel et al. 2011). In addition to its role in the regulation of inflammatory mediators, MC3R is also found to be associated with the phagocytosis of apoptotic neutrophils by macrophages, suggesting inflammation-resolving property that further makes it a potential target for innovative anti-inflammatory therapeutics (Montero-Melendez et al. 2011).

1.3.3.3 MC3R mutations in obesity pathogenesis

Prior to the revelation of the role of MC3R in energy homeostasis using *Mc3r*^{-/-} animal models, human genetic studies have demonstrated a positive linkage between obesity and the chromosomal region 20q13 that harbors *MC3R*, making the *MC3R* a plausible candidate gene for human obesity and type 2 diabetes (Bowden et al. 1997; Ghosh et al. 1999; Ji et al. 1997; Lembergas et al. 1997; Zouali et al. 1997). Several groups conducted large-scale screening studies in obese and type 2 diabetic individuals in order to identify potential *MC3R* mutation. The first two variants identified in *MC3R* are T6K and V81I, which are polymorphic variants in complete linkage disequilibrium (Hani et al. 2001; Li et al. 2000). Since then, 27 *MC3R* mutations have been identified so far in different cohorts from Asia, Europe and North America (Calton et al. 2009; Lee et al. 2007; Lee et al. 2002; Mencarelli et al. 2011; Mencarelli et al. 2008; Valli-Jaakola 2007; Zegers et al. 2011; Zegers et al. 2013).

Genotype analysis is usually categorized based on the combination of the two *MC3R* common non-synonymous variants, T6K and V81I. However, large-scale case control studies indicate that the frequencies of these two variants have always been

similar in obese and non-obese cohorts (Calton et al. 2009; Lee et al. 2007; Schalin-Jantti et al. 2003). In the United States, these two variants are more prevalent in African American than in Caucasian obese individuals (Feng et al. 2005). The prevalence of these two variants in Asians is intermediate (Lee et al. 2007) and the lowest prevalence has been identified in Italy and Chile (Obregon et al. 2012; Santoro et al. 2007). Except for these two common variants, the prevalence of the other *MC3R* mutations is generally low. A large-scale study of 839 obese and 967 control subjects performed in Italy revealed that the frequency of rare *MC3R* mutations is 1.55% in obese group compared with 0.83% in non-obese group (Mencarelli et al. 2011). Although there is no significant difference between the frequencies of rare *MC3R* mutations in obese and non-obese subjects in the above study, the prevalence of *MC3R* mutations with functional alterations is actually significantly higher in the obese group.

In the past decade, a number of epidemiological studies have focused on the effects of *MC3R* mutations on obesity and obesity-related phenotypes. The original studies of the two common variants (T6K and V81I) did not identify an association between the 6K/81I haplotype and obesity or diabetes, and these two variants were characterized as benign polymorphisms (Schalin-Jantti et al. 2003; Wong et al. 2002). However, subsequent studies revealed that homozygosity for the two variants is associated with higher BMI, more body fat (both fat mass and percentage fat mass), and increased insulin resistance compared with WT or heterozygous subjects (Feng et al. 2005), suggesting the contribution of common *MC3R* variants in human obesity. Of the 27 naturally occurring mutations of *MC3R*, 16 mutations (S17T, A70T, N128S, M134I, D158Y, V177I, I183N, V211I, L249V, A260V, M275T, T280S, A293T, L297V, L299V, and

I335S) were identified only from obese individuals and 5 mutations (F82S, I87T, L249F, R257S, and X361S) were identified from both obese and non-obese subjects, and 6 mutations (S69C, V124L, V255I, L285V, E342K, and G353D) were found only in individuals with normal body weight. Therefore, *MC3R* mutations are not associated with obesity in all cases.

For the *MC3R* mutations identified only from obese individuals, I183N and I335S are regarded as potential pathogenic mutations or at least predisposing genetic factors conferring susceptibility to excessive weight gain since the cosegregation of these two mutations with obesity in family has been identified (Cieslak et al. 2013; Lee et al. 2007; Lee et al. 2002; Mencarelli et al. 2008). The epidemiologic data of other *MC3R* mutations is limited. Probands of A70T, M134I, and I183N have higher leptin levels and body fat mass but lower hunger score, reminiscent of the *Mc3r*^{-/-} mice (Lee et al. 2007). Probands of N128S, V211I, and L299V have higher BMI compared with the control group (Zegers et al. 2011).

The receptor life cycle-based classification system (see Section 1.3.2) could also be applied in *MC3R* mutants. Similar as that in *MC4R*, Class II comprises the largest set of *MC3R* mutants. I335S is a typical Class II mutant with complete loss of ligand binding and signal transduction due to intracellular retention (Mencarelli et al. 2008; Tao 2007). I335 resides in the signature motif N/DPxxY (DPLIY in *MC3R*) at the cytoplasmic end of the TM7. This highly conserved motif, together with the additional eighth helix (helix 8) has been characterized as a versatile regulator in GPCR expression, trafficking, ligand binding, and signal transduction (Barak et al. 1995; Hunyady et al. 1995). A substitution of the corresponding residue I301 to Thr in *MC4R* has been described as a loss-of-

function mutation (Vaisse et al. 2000), suggesting the critical role of this residue for maintaining the normal functions of neural MCRs. Systematic study of I335 in MC3R demonstrated that mutations of I335 into charged residues such as Asp and Arg lead to intracellular retention while mutations into other residues, including Ala, Leu, and Asn, do not affect cell surface expression but result in severely impaired ligand binding. These results suggested that I335 might interact with the residues in Helix 8 by hydrophobic interactions to maintain the receptor conformation necessary for normal trafficking, binding and signaling properties (Tao 2007). However, the potential hydrophobic interaction as well as the detailed functions of the whole DPLIY motif and helix 8 remain to be further elucidated.

Collectively, *MC3R* mutations have been intensively analyzed for obesity-related traits and the pathogenic roles of some mutations, such as I183N and I335S, have been highlighted. Nonetheless, in contrast to MC4R that has been characterized as the most common monogenic form of obesity in human, the role of MC3R in obesity pathogenesis remains controversial. More detailed studies, such as *in vivo* studies applying transgenic animals, would be helpful. Moreover, fundamental functional studies are also urgently needed to obtain a better understanding of the structure-function relationship of MC3R, which would be important for future decipherment of this enigmatic receptor.

It is worth noting that recent studies of *MC3R* promoter sequences indicated that two single nucleotide polymorphisms (SNPs) in the promoter region of *MC3R*, rs11575886 and rs6127698, are significantly associated with pulmonary tuberculosis susceptibility in different cohorts (Adams et al. 2011; Hashemi et al. 2013; Park et al. 2014a), although these two SNPs are not related to obesity (Santos et al. 2011; Zegers

et al. 2010). These results suggested that the genetic effect of *MC3R* mutations might not be confined to obesity pathogenesis and provided new rationale into *MC3R* investigation.

1.3.4 Multiple signaling pathways of neural MCRs

It is important to note that several neural MCRs mutations, such as C40R (Rong et al. 2006) and V50M (Dubern et al. 2001) of *MC4R* as well as V177I (Mencarelli et al. 2011) and A293T (Mencarelli et al. 2008) of *MC3R* that were identified from obese individuals and were not present in non-obese controls, belong to Class V since they behave similarly as the WT receptors in functional studies (Tao 2007; Yang et al. 2015a). In addition, some mutations that were only identified from lean controls, such as F82S *MC3R* (Calton et al. 2009), have impaired responses to ligand stimulation (Yang et al. 2015a). Therefore, current indices for obesity identification might not comprehensively characterize the real status of energy homeostasis. Considerable attention has been paid to signaling pathways activated by neural MCRs other than the classical G_s -cAMP-PKA signaling.

In addition to coupling to G_s , both *MC3R* and *MC4R* are found to signal through G_q protein. In both *MC3R*- and *MC4R*-transfected HEK293 cells, α -MSH is reported to stimulate dose-dependent increase of intracellular calcium, which is shown to be cholera toxin sensitive and pertussis toxin insensitive (Mountjoy et al. 2001). The *MC3R*-mediated calcium mobilization has been further demonstrated to be IP_3 -dependent, indicating G_q protein activation (Konda et al. 1994). In GT1-1 cells, which is a murine hypothalamic cell line endogenously expressing *MC4R*, *MC4R* activation leads to intracellular calcium increase through G_q /phospholipase C-dependent signaling pathway (Newman et al. 2006). However, another study using a homologous cell line GT1-7 suggested that *MC4R*

activation is not associated with intracellular calcium accumulation (Buch et al. 2009). In contrast, the authors found that MC4R activation stimulates pertussis toxin-sensitive GTP γ S binding, indicating the coupling and interaction between the MC4R and G $_{i/o}$ protein.

Both MC3R and MC4R have been reported to activate mitogen-activated protein kinases (MAPKs), especially extracellular signal-regulated kinase 1 and 2 (ERK1/2). Although MC4R-mediated ERK1/2 activation has been observed both *in vitro* and *in vivo* (Chai et al. 2006; Daniels et al. 2003), the detailed mechanism remains obscure since discrepant results have been reported. The NDP-MSH-initiated ERK1/2 phosphorylation has been attributed to G $_i$ protein regulation in HEK293 cells expressing MC4R (Chai et al. 2006), or to Ca $^{2+}$ - and PKC-dependent pathway in GT1-1 cells that endogenously express MC4R (Chai et al. 2006), or to PI3K-involved signaling in CHO cells stably transfected with MC4R (Vongs et al. 2004), or to the activation of PKA in GT1-7 cells (Damm et al. 2012). Unlike MC4R, the MC3R-mediated ERK1/2 signaling is less studied. One report of NDP-MSH-induced ERK1/2 activation in HEK293 cells stably expressing hMC3R suggested it to be PI3K- and G $_i$ protein-dependent (Chai et al. 2007).

The ERK1/2 signaling mediated by GPCRs has been demonstrated to play a pivotal role in multiple essential cellular processes such as development, differentiation and proliferation (Aouadi et al. 2006; Johnson and Lapadat 2002; Nishimoto and Nishida 2006). In neural MCRs, in addition to its function in the mediation of cell proliferation (Chai et al. 2007; Chai et al. 2006), the ERK1/2 signaling cascade has also been reported to be involved in the regulation of energy homeostasis. MT II-induced ERK1/2 activation in the NTS is found to be necessary for food intake suppression (Sutton et al. 2005), whereas the melanocortin-stimulated ERK1/2 phosphorylation in the PVN might be associated with

the regulation of long-term feeding behaviors (Daniels et al. 2003). Recent studies from our lab suggested that the defects in basal or ligand-stimulated ERK1/2 signaling might contribute to obesity pathogenesis induced by mutations in both *MC3R* and *MC4R* genes (He and Tao 2014; Yang et al. 2015a). Therefore, the neural MCRs-mediated ERK1/2 signaling deserves further detailed investigation.

In addition to the signaling pathways discussed above, it is also reported that MC3R-regulated AKT activation is associated with cellular proliferation in neuronal CAD cells (Nyan et al. 2008). MC4R-mediated AKT phosphorylation has also been observed in GT1-7 cells (Perino et al. 2014) and murine osteoblasts (Guo et al. 2016), but not in MC4R-transfected HEK293 cells (Chai et al. 2009) or MC4R-expressing murine astrocytes (Ramirez et al. 2015). Moreover, MC4R activation is suggested to interact with insulin signaling through inhibiting the c-Jun N-terminal kinase (JNK) (Chai et al. 2009), indicating more complicated signaling network.

The classic paradigm of GPCR activation is based on the theory that the agonist binding to the receptor induces a conformational switch from inactive state to active state. However, it has been appreciated in the past decade that agonists could induce distinct receptor conformations that lead to the activation of different signaling pathway, referred as biased agonism, resulting in biased cellular and physiological responses (Galandrin and Bouvier 2006; Wisler et al. 2014). Biased signaling has already been established in many GPCRs, and studies of biased signaling would undoubtedly lead to a better understanding of GPCR pharmacology. Moreover, it is also with important therapeutic implications. Novel pharmaceuticals, designed based on biased signaling of GPCR with

enhanced therapeutic potential but diminished undesired effects, are in various stages of clinical trials (Boerrigter et al. 2011; Soergel et al. 2014; Valant et al. 2014).

AgRP, the endogenous antagonist shared by MC3R and MC4R, was initially identified as an orexigenic stimulus through competitively inhibiting the binding of melanocortins to the neural MCRs. In addition to its antagonizing effect, several lines of *in vivo* evidence indicated that AgRP could induce long-term orexigenic signals in a melanocortin-independent manner by an unknown mechanism (Hagan et al. 2000; Tolle and Low 2008). Recent *in vitro* studies also demonstrated that AgRP acts as a biased agonist that mediates MC4R activity through G_i protein activation and potassium channels regulation, independently of its inhibition of α -MSH binding, suggesting agonistic property (Buch et al. 2009; Ghamari-Langroudi et al. 2015). It is also reported that AgRP could induce endocytosis of MC3R and MC4R through β -arrestin to reduce the amount of MCRs molecules accessible to melanocortins (Breit et al. 2006). Therefore, the role of AgRP in the central melanocortin system needs to be re-evaluated and it would be of interest to study the biased signaling in neural MCRs using AgRP as an entry point from the orexigenic perspective to gain a better understanding of neural MCRs pharmacology.

Chapter 2

2.1 Introduction

The melanocortin-3 receptor (MC3R), a member of family A G protein-coupled receptors (GPCRs) (Gantz et al. 1993b; Roselli-Rehfuss et al. 1993), has received increasing attention with regard to its multiple physiological functions (reviewed in (Renquist et al. 2011)). The MC3R is primarily expressed in hypothalamus, especially in the arcuate nucleus, the ventromedial nucleus and the posterior hypothalamic region (Jegou et al. 2000). It is also expressed in several peripheral tissues, including the placenta, gut, heart, kidney, and peritoneal macrophages (Chhajlani 1996; Gantz et al. 1993b; Getting et al. 2003; Ni et al. 2006). Based on its wide distribution, the MC3R has been shown to be involved in regulating cardiovascular function (Mioni et al. 2003; Versteeg et al. 1998), natriuresis (Chandramohan et al. 2009; Ni et al. 2006), and inflammation (Catania et al. 2004; Getting et al. 2006; Getting et al. 2008).

The MC3R, together with melanocortin-4 receptor (MC4R), another member of melanocortin receptor family expressed in the central nervous system, has been considered as a potential regulator of energy homeostasis. But unlike the MC4R, which is a well-known mediator of leptin action (Cone 1999) and is crucial for both food intake and energy expenditure regulation (Huszar et al. 1997) (reviewed in (Tao 2010a)), the MC3R is shown to be primarily involved in affecting feed efficiency rather than mediating

food intake or energy expenditure (Butler et al. 2000; Chen et al. 2000a). The MC4R plays an undisputed role in human obesity pathogenesis since mutations in *MC4R* have been characterized as the most common monogenic form of obesity in human (Farooqi et al. 2003; Hinney et al. 2013; Tao 2009). However, the role of *MC3R* in human obesity pathogenesis is more controversial (reviewed in (Tao 2010b)), although some *MC3R* mutations (such as I183N and I335S) have been recognized as possible genetic contributors for morbid obesity (Lee et al. 2007; Lee et al. 2002; Mencarelli et al. 2008; Rached et al. 2004; Tao 2007; Tao and Segaloff 2004; Yang et al. 2015a; Yang and Tao 2012).

The MC3R is a typical GPCR consisting of seven transmembrane helices (TMs) with an extracellular N-terminus and intracellular C-terminus. The currently known crystal structures of typical family A GPCRs reveal the existence of an eighth helix (Helix 8) (Mustafi and Palczewski 2009; Rosenbaum et al. 2009), which initiates just after the highly conserved N/DPxxY motif (Asn/Asp-Pro-Xaa-Xaa-Tyr) in TM7 (DPLIY in the MC3R) and terminates either with the anchorage into the plasma membrane by acylation of cysteine residues, or with the kinks produced by proline residues. There are only a few GPCRs that do not have this helix in the crystal structures (Zhang et al. 2015). To date, the functional importance of the N/DPxxY motif and helix 8 has been emerging in GPCR expression, conformational switch upon GPCR activation, G protein coupling, and GPCR internalization (Barak et al. 1995; Delos Santos et al. 2006; Fritze et al. 2003; Prioleau et al. 2002; Swift et al. 2006; Tetsuka et al. 2004; Wess et al. 1993).

However, no systematic study of the DPLIY motif and the helix 8 of MC3R has been reported. In order to gain a better understanding of the structure-function

relationship of the human MC3R (hMC3R), we investigated the function of each residue in these two domains of the receptor using alanine-scanning mutagenesis. We generated 20 mutants and studied the cell surface expression, ligand binding and signaling properties of the mutant receptors. MC3R activation has also been reported to stimulate ERK1/2 phosphorylation (Begrache et al. 2012; Chai et al. 2007) (one report suggested that the MC3R does not activate ERK1/2 (Daniels et al. 2003)). Furthermore, we and others recently reported biased cAMP and ERK1/2 signaling in the MC3R (Huang and Tao 2014a; Montero-Melendez et al. 2015; Yang et al. 2015a). Therefore, the ERK1/2 signaling pathway of the hMC3R was also investigated in the present study.

2.2 Materials and methods

2.2.1 Materials

[Nle⁴, D-Phe⁷]- α -melanocyte stimulation hormone (NDP-MSH) was purchased from Bachem (King of Prussia, PA, USA). ¹²⁵I-NDP-MSH was iodinated as previously described (Mo et al. 2012). Radiolabeled cAMP was iodinated in our lab with chloramine T method (Tao et al. 2010).

2.2.2 Site-directed mutagenesis

The WT hMC3R tagged at the N-terminus with 3×HA tag was obtained from Missouri S&T cDNA Resource Center (<http://www.cDNA.org>). Mutations were generated from the WT receptor by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using primers listed in Table 2.1. Plasmid DNAs were purified by IsoPure Maxi Prep Kit (Denville Scientific, Metuchen, NJ, USA). DNA sequencing was performed by

the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA) to confirm the presence of intended mutations and nonexistence of unintended mutations.

2.2.3 Cell culture and DNA transfection

Human embryonic kidney (HEK) 293T cells, purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum. The cells were plated into 6-well clusters (or 100mm dishes for western blot) pre-coated with 0.1% gelatin and transfected with purified plasmids at 50-70% confluence using calcium phosphate precipitation method (Chen and Okayama 1987). Flow cytometry assay, ligand binding, and signaling studies were performed approximately 48h after transient transfection.

2.2.4 Quantification of MC3R cell surface expression by flow cytometry

Forty-eight hours after transfection, HEK 293T cells were washed with filtered PBS for immunohistochemistry (PBS-IH) (Tao and Segaloff 2003) and fixed by 4% paraformaldehyde in PBS-IH for 30 min. After blocking with PBS-IH containing 5% BSA for 1h, the cells were then incubated with the primary antibody anti-HA.11 (Covance, Princeton, NJ, USA), which was diluted 1:100 in PBS-IH containing 5% BSA, for another 1 h. The cells were washed once with 0.5% BSA in PBS-IH and then incubated with the secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) for 1 h. The cell surface expression of WT and mutant hMC3Rs was analyzed by Accuri flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). The expression levels of the mutant hMC3Rs were calculated as the percentage of WT hMC3R expression using the formula:

(mutant - pcDNA3.1) / (WT - pcDNA3.1) ×100%, where the pcDNA3.1 were used as control for background staining (Wang et al. 2008; Yang and Tao 2012).

2.2.5 Ligand binding assays

Forty-eight hours after transfection, HEK 293T cells were washed twice with warm Waymouth's MB752/1 media (Sigma-Aldrich) containing 1mg/ml bovine serum albumin (Waymouth/BSA). The cells were incubated with Waymouth/BSA containing 100,000 cpm of ¹²⁵I-NDP-MSH with or without different concentrations of unlabeled NDP-MSH (from 10⁻¹² to 10⁻⁶ M) at 37°C for 1 h. The reaction was terminated by washing twice with cold Hank's balanced salt solution on ice. The cells were lysed by 100 µl 0.5 N NaOH, collected using cotton swabs, and detected in gamma counter.

2.2.6 Ligand stimulated cAMP production

Forty-eight hours after the transfection, HEK 293T cells were washed twice with warm Waymouth/BSA and then incubated with fresh Waymouth/BSA containing 0.5mM isobutylmethylxanthine (Sigma-Aldrich) at 37°C for 15 min. Then different concentrations of NDP-MSH were added into each well to make the final volume to be 1 ml and final concentration ranging from 10⁻¹² to 10⁻⁶ M. After 1 h incubation at 37°C, the reaction was terminated on ice and the intracellular cAMP was extracted by adding 0.5 N perchloric acid containing 180 µg/ml theophylline and 0.72 M KOH/0.6 M KHCO₃ into each well. Cyclic AMP concentrations were determined by radioimmunoassay as described in detail before (Tao 2007; Yang and Tao 2012).

2.2.7 Protein preparation and western blot

Twenty-four hours after transfection, HEK 293T cells were starved in Waymouth/BSA at 37°C for 24 h. After being treated with or without 1 μ M NDP-MSH for 5 min at 37°C on the second day, the cells were transferred directly on ice, washed twice using cold OG (150 mM NaCl and 20 mM Hepes, pH 7.4), and then scraped into lysis buffer (OG containing 0.5% NP-40, 2 mM EDTA, 1 mM Na₃VO₄, and 1 mM NaF). Total protein concentrations were determined by Bradford protein assay and 35 μ g protein samples were separated on 10% SDA-PAGE gel, and then blotted onto PVDF membranes. After blocked in 10% nonfat dry milk (containing 0.2% Tween-20) for at least 4 h at room temperature, the membranes were then immunoblotted with rabbit anti-pERK1/2 antibody (Cell signaling, Beverly, MA, USA) 1:2000 and mouse anti- β -tubulin antibody (Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA, USA) 1:5000 diluted in Tris-buffered saline containing Tween 20 (TBST) with 5% BSA overnight at 4 °C. The membranes were then probed with HRP-conjugated secondary antibody, donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) 1:1500 and donkey anti-mouse IgG (Jackson ImmunoResearch) 1:5000 diluted in 10% nonfat dry milk for 2 h at room temperature. Specific bands were visualized using ECL reagent (Thermo Scientific, Rockford, IL, USA) and then analyzed using Image J 1.49 software (NIH, Bethesda, MD, USA).

2.2.8 Statistical analysis

GraphPad Prism 4.0 software (San Diego, CA, USA) was used to calculate the ligand binding parameters including maximal binding (B_{max}) and IC₅₀ as well as the cAMP

signaling parameters including maximal response (R_{max}) and EC_{50} . The significance of differences in binding and signaling parameters, receptor cell-surface expression levels, and pERK1/2 activities between the WT and mutant hMC3Rs were determined by Student's *t*-test using GraphPad Prism 4.0.

2.3 Results

NDP-MSH, an analog of endogenous α -MSH (Sawyer et al. 1980), has been shown to be a superpotent agonist for MC3R and was used in the present study. To determine the function of each residue of the DPLIY motif and helix 8 of MC3R, we generated 20 mutants using alanine-scanning mutagenesis (Table 2.1, Fig. 2.1). Since the mutant I335A has been studied previously (Tao 2007), its ligand binding and signaling property studies are not included herein.

2.3.1 Cell surface expression of the WT and mutant hMC3Rs

Retention of the mutant receptors in the endoplasmic reticulum (ER) resulting from failing to pass the quality control system has been recognized as the major defect of inactivating mutations in numerous GPCRs (reviewed in (Tao and Conn 2014)). To investigate the cell surface expression of the mutants, flow cytometry was performed. As shown in Fig. 2.2, all mutants were expressed normally on cell surface and no significant differences were observed between the WT hMC3R and the mutants. The data for I335A here were in agreement with previous results using confocal microscopy (Tao 2007). To confirm the results, we further tested the HA-tagged I335S and D158E. Our data was consistent with previous reports that I335S is intracellular retained (with only ~5% WT

hMC3R expression at the cell surface) while D158E is partially retained with decreased cell surface expression compared to the WT hMC3R (Fig. 2.2) (Tao 2007; Wang et al. 2008).

2.3.2 Ligand binding of the WT and mutant hMC3Rs

To compete with different concentrations of unlabeled NDP-MSH, ¹²⁵I-NDP-MSH was used as the radioligand in the binding assay. The result showed that P333A, L343A, and R344A had no detectable binding. Nine mutants (L334A, Y336A, S340A, E342A, F347A, R348A, E349A, I350A, and L351A) showed decreased IC₅₀ while only one mutant, L341, had increased IC₅₀. There were no significant differences between the IC₅₀s of all the other mutants and that of the WT hMC3R (Table 2.2, Fig. 2.3).

As shown in Fig. 2.4, nine mutants (D332A, L344A, Y336A, S340A, E342A, F347A, R348A, I350A, and L351A) had significantly decreased maximal binding compared with WT hMC3R while one mutant (F338A) showed significantly, although very slightly, increased maximal binding. All the other mutants had similar maximal binding as the WT hMC3R.

2.3.3 Signaling properties of the WT and mutant hMC3Rs

Whether the hMC3R variants could respond to NDP-MSH stimulation with enhanced cAMP generation was investigated. As expected, NDP-MSH could stimulate dose-dependent increase of intracellular cAMP in HEK293T cells transfected with WT hMC3R. As shown in Table 2.2, two mutants (Y336A and E342A), in addition to three binding-defective mutants (P333A, L343A, and R344A), had no measurable signaling. F338A and R339A showed increased EC₅₀ while F347A displayed reduced EC₅₀. No

significant differences were observed between the EC₅₀s of all the other mutants and that of the WT hMC3R.

Our data of maximal response in Fig. 2.5 also showed that six mutants (R339A, S340A, F347A, R348A, I350A, and L351A) had significantly decreased maximal response compared with WT hMC3R. All the other mutants had apparent normal signaling.

2.3.4 Constitutive activity of hMC3Rs

Unlike the human MC4R, which has been previously shown to be constitutively active (Nijenhuis et al. 2001; Tao 2014), the hMC3R has been recognized with little or no constitutive activity (Tao 2007). In present study, the constitutive activities of the 19 mutants of hMC3R (I335A not included) were analyzed. We demonstrated that the F347A had significantly increased basal activity compared with the WT hMC3R. As shown in Fig. 2.6, the nearly 4.6 times elevation of basal activity demonstrated that the F347A was a constitutively active mutant, consistent with our previous report (Tao et al. 2010). All the other mutants had similar basal activities compared with that of the WT hMC3R. The dose-response curve in Fig. 2.6B highlights that F347A, although constitutively active, had reduced maximal response compared with the WT hMC3R.

2.3.5 ERK1/2 signaling of the WT and mutant hMC3Rs

To further investigate the ERK1/2 signaling pathway, especially the newly-identified biased signaling of hMC3R, the WT hMC3R together with nine mutants, including one constitutively active mutant (F347A) and eight binding-defective or signaling-defective mutants (P333A, I335A, Y336A, E342A, L343A, R344A, I350A, and

L351A) which led to < 20% the WT receptor cAMP production, were studied using western blots. Empty vector pcDNA3.1 and WT hMC3R without the 3×HA tag were also studied, excluding non-specific stimulation.

Our result showed that the basal pERK1/2 levels in cells transfected with either WT MC3Rs (with or without 3×HA-tag) were similar to that of cells transfected with the empty vector (Fig. 2.7B). Upon 1 μ M NDP-MSH stimulation, WT MC3Rs showed significant ERK1/2 phosphorylation but no ERK1/2 activation was detected in cells transfected with the empty vector. There was no significant difference between the pERK1/2 levels of HA-tagged and non-tagged WT MC3Rs (Fig. 2.7C).

The constitutive activities of WT and mutant hMC3Rs were studied. Our results showed that all the mutants, including the F347A that was shown to be constitutively active in cAMP pathway, had similar basal pERK1/2 levels as the WT hMC3R (Fig. 2.7D).

We then investigated the pERK1/2 levels of WT and mutant hMC3Rs after 1 μ M NDP-MSH stimulation. As expected, the ERK1/2 phosphorylation was significantly enhanced in HEK 293T cells transfected with WT hMC3R. P333A and L343A did not respond to NDP-MSH stimulation with ERK1/2 activation while all other mutants had significantly increased pERK1/2 levels compared to the corresponding basal activity upon 1 μ M NDP-MSH stimulation (Fig. 2.7E).

2.4 Discussion

Although several physiological functions of the MC3R have now been described, it remains to be the most enigmatic member of the melanocortin receptor family. The

recent identification of an additional helix 8, linking the TM7 and the C-terminus of GPCRs, has also aroused great interest. Analysis of the role of helix 8 in both family A and B GPCRs confirmed its importance in receptor expression, ligand binding, signal transduction and internalization (Ernst et al. 2000; Faussner et al. 2005; Kuwasako et al. 2011; Marin et al. 2000; Tetsuka et al. 2004). In the present study, we performed detailed study of the 20 residues in helix 8 and the DPLIY motif of hMC3R using the classical method of site-directed alanine-scanning mutagenesis (Cunningham and Wells 1989).

The flow cytometric analysis demonstrated that all mutants were expressed normally on cell surface. Receptor retention or mislocalization, which has previously been described as the major defect for human disease caused by GPCR mutations (Tao and Conn 2014), was not observed in the present study. Similarly, in our studies on the second and third intracellular loops, we also showed that the alanine mutants are expressed normally at the cell surface (Huang and Tao 2014a; Wang and Tao 2013).

Ligand binding studies revealed that 11 residues (P333, L334, Y336, S340, E342, L343, R344, F347, R348, I350 and L351) were important for NDP-MSH binding. Although it is uncommon that residues important for ligand binding are located in the cytoplasmic side of receptor, observations have been reported previously in MC3R (Huang and Tao 2014a) as well as other GPCRs, such as the gonadotropin-releasing hormone receptor (Lu et al. 2005), angiotensin II type 2 receptor (Moore et al. 2002), and V2 vasopressin receptor (Pan et al. 1994). These residues are not expected to directly participate in ligand binding. Rather they might indirectly participate in the ligand–receptor interaction through intramolecular interactions and conformational changes (Kobilka and Deupi 2007; Rovati et al. 2007).

The highly conserved DPLIY motif is one of the two crucial motifs (the other one is D/ERY motif in TM3) for receptor stabilization and activation (Park et al. 2008). The P^{7.50} (the superscript number represents Ballesteros-Weinstein numbering (Ballesteros and Weinstein 1995)) has been regarded as critical in inducing structurally important helical break due to its distinctive cyclic structure of side chain. Although L^{7.51} was not studied in detail before, its adjacent residue I^{7.52} has been recognized to be important in multiple aspects of MC3R function (Tao 2007). L^{7.51} might contribute to the supposed interaction between I^{7.52} and hydrophobic residues in helix 8 to maintain the receptor conformation necessary for ligand binding. Y^{7.53} has been shown to be critical for receptor activation and signal transduction in many GPCRs, such as MC4R, gonadotropin-releasing receptor, and 5HT_{2c} receptor (Arora et al. 1996; Prioleau et al. 2002; Roth et al. 2009). The conformational change of tyrosine enables it to insert into the space previously occupied by TM6 and hinder its inward tilt to stabilize the active state (Scheerer et al. 2008). Mutations of the tyrosine residue in some other receptors result in either no ligand binding or defective signaling (Feng and Song 2001). Our results here highlighted the importance of Y^{7.53} in hMC3R.

The D/E(x)₇LL motif, composed of highly conserved di-leucine sequence with an upstream acidic residue, has been shown to be present in the C-terminus of several GPCRs such as α_1 -, α_2 -adrenergic receptors and the dopamine receptor (Schulein et al. 1998). The corresponding residue of glutamate (E335^{7.63}) in V₂ vasopressin receptor was reported to be crucial for establishing a transport-competent folding state to support the escape of the receptor from the ER (Schulein et al. 1998). The di-leucine motif, which should be strictly defined as dihydrophobic pair due to mainly consisting of leucine,

isoleucine or valine, was shown to play an important role in cell surface targeting, receptor internalization and protein trafficking (Gabilondo et al. 1997; Ho and MacKenzie 1999). The alanine mutation of di-leucine pair was reported to have no effect on ligand affinity in β_2 -adrenergic receptor (Gabilondo et al. 1997), whereas the same mutation of di-isoleucine was shown to impair NDP-MSH binding to MC4R (VanLeeuwen et al. 2003). Our data demonstrated that the maximal binding of E342^{7.59}A, I350^{7.67}A, and L351^{7.68}A had decreased by 90%, 87% and 83% respectively, compared with WT hMC3R, although all the mutant receptors could be normally expressed on the cell surface.

The interaction between two highly conserved aromatic residues, tyrosine in the D/NPxxY motif and phenylalanine in Helix 8 has been highlighted in several studies. However, the conserved phenylalanine in the melanocortin receptor family is substituted by a leucine residue (L343^{7.60} in hMC3R) and the function of replaced phenylalanine might be taken by another aromatic residue, F347^{7.64}. As previously discussed, the I^{7.52} in DPLIY motif of MC3R might interact with hydrophobic residues in helix 8 to maintain the receptor conformation. The L343^{7.60} is a preferable choice for taking part in the hydrophobic interaction since the mutant L343^{7.60}A did not exhibit any binding or signaling in response to NDP-MSH stimulation in our study.

One residue, R339^{7.56}, was demonstrated to be crucial for normal receptor signaling. Alanine mutation of this arginine resulted in normal ligand binding but severely decreased signaling potency. Missense mutations of the corresponding residue of arginine in hMC4R, including R305^{7.56}S, R305^{7.56}Q, and R305^{7.56}W, have been identified from obese patient (Tao 2009). R305^{7.56}S and R305^{7.56}Q have been shown to have partial cAMP response to α -MSH stimulation (Calton et al. 2009; Stutzmann et al. 2008) and

R305^{7.56}W exhibits normal binding but severely impaired signaling in response to α -MSH stimulation (Roubert et al. 2010), which is in agreement with our results of R339^{7.56}A hMC3R.

The MC3R has been reported to activate the ERK1/2 signaling pathway (see Introduction). Unbalanced cAMP and ERK1/2 signaling was reported in some human MC4R mutants (He and Tao 2014; Huang and Tao 2012; Mo et al. 2012) (reviewed in (Tao 2014)). In our recent study of the DRY motif and intracellular loop 2 of hMC3R, the presence of this biased signaling was also suggested (Huang and Tao 2014a). In the current study to test it further, the ERK1/2 phosphorylation levels of nine hMC3R mutants, including eight binding- or signaling-defective mutants (P333A, I335A, Y336A, E342A, L343A, R344A, I350A, and L351A) which led to < 20% cAMP production compared with WT hMC3R, and one constitutively active mutant (F347A) with enhanced basal cAMP activity, were investigated using western blots.

Non-specific effects were excluded in the present study by using the empty vector pcDNA3.1 and non-tagged WT hMC3R. The result indicated that there was no endogenous receptor in HEK293T cells responding to NDP-MSH stimulation and HA tag did not have any effect on MC3R activation of ERK1/2 (Fig. 2.7A, B, and C). All nine mutants had similar basal pERK1/2 levels as the WT MC3R. Notably, the F347A, which had increased basal cAMP level, did not show any increase in basal pERK1/2 level compared with the WT hMC3R. Upon 1 μ M NDP-MSH stimulation, two binding-defective mutants, P333A and L343A, did not activate ERK1/2. However, all the other tested mutants, especially I335A, Y336A, E342A, and R344A that had almost no cAMP response, had shown significant ERK1/2 activation. Two mutants with no detectable

binding, I335A (Tao 2007) and R344A (present study), responded to NDP-MSH stimulation with increased pERK1/2 levels. Although it is uncommon, similar observations have been previously reported in other GPCRs, such as MC4R (Huang and Tao 2012) and gonadotropin-releasing hormone receptor (Bedecarrats et al. 2003). This might be due to the faster dissociation of the ligand from the MC3R mutants compared with WT receptor making the binding difficult to detect, but ERK1/2 could still be activated by this transient binding (Huang and Tao 2014a). NDP-MSH was reported to induce ERK1/2 phosphorylation in HEK293 cells transfected with WT MC3R in a very fast and potent pattern, peaking at only 5 min with an EC_{50} of 3.3 ± 1.5 nM (Chai et al. 2007). These data suggest these mutants were biased receptors, supporting two recent studies on biased signaling at the MC3R (Montero-Melendez et al. 2015; Yang et al. 2015a).

In summary, for the systemic study of the DPLIY motif and helix 8 of hMC3R, we have identified residues that are crucial for ligand binding and cAMP generation. One constitutively active mutant was identified and the existence of biased signaling was confirmed in MC3R mutants. The data obtained in our present research indicated that the highly conserved DPLIY motif and helix 8 play important roles in MC3R activation and signal transduction. Our result established a theoretical basis for the structure-functional relationship of MC3R and will be useful for further investigation of its physiological role in energy homeostasis.

Table 2.1 Forward primer sequences used for site-directed mutagenesis studies of hMC3R. The mutated codons are underlined.

Constructs	Primer sequences
D332A	CAACTCCGTCATCG <u>CCC</u> CACTCATCTACG
P333A	CTCCGTCATCGAC <u>GCA</u> CTCATCTACGC
L334A	CTCCGTCATCGACCCAG <u>CC</u> ATCTACGCTTTCCGG
I335A	CATCGACCCACTCG <u>CCT</u> TACGCTTTCC
Y336A	CATCGACCCACTCATCG <u>CC</u> GCTTTCCGGAGCCTG
A337G	CCACTCATCTAC <u>GGT</u> TTCCGGAGCCTG
F338A	CCCACTCATCTACGCT <u>GCC</u> CGGAGCCTGGAATTG
R339A	CTCATCTACGCTTT <u>CGG</u> AGCCTGGAATTGCG
S340A	CATCTACGCTTTCCGG <u>GCC</u> CTGGAATTGCGCAAC
L341A	CTACGCTTTCCGGAGCG <u>GGA</u> ATTGCGCAACACC
E342A	CGCTTTCCGGAGCCT <u>GGA</u> ATTGCGCAACACCTTTAG
L343A	CTTTCCGGAGCCTGGA <u>AGCG</u> GCAACACCTTTAGGG
R344A	CGGAGCCTGGAATT <u>GCC</u> AACACCTTTAGGGAG
N345A	GGAGCCTGGAATTGCGC <u>GCC</u> ACCTTTAGGGAGATTC
T346A	CTGGAATTGCGCAAC <u>GCC</u> TTTAGGGAGATTC
F347A	GAATTGCGCAACAC <u>CGCT</u> AGGGAGATTCTCTG
R348A	GCGCAACACCTTT <u>GCG</u> GAGATTCTCTGTG
E349A	CAACACCTTTAGG <u>GCG</u> ATTCTCTGTGGC
I350A	CACCTTTAGGGAG <u>GCT</u> CTCTGTGGCTGC
L351A	CACCTTTAGGGAGATT <u>GCC</u> TGTGGCTGCAACGGC

Table 2.2. The ligand binding and signaling properties of WT and mutant hMC3Rs.

hMC3R	NDP-MSH binding	NDP-MSH-stimulated cAMP	
	IC ₅₀ (nM)	EC ₅₀ (nM)	R _{max} (100%)
WT	2.63 ± 0.27	0.47 ± 0.05	100
D332A	1.59 ± 0.27	0.43 ± 0.04	98.50 ± 18.45
P333A	N/A ^a	N/A ^a	N/A ^a
L334A	1.09 ± 0.07 ^b	0.39 ± 0.10	79.50 ± 12.12
Y336A	0.79 ± 0.10 ^b	N/A ^a	N/A ^a
A337G	2.32 ± 0.42	0.57 ± 0.17	98.25 ± 11.27
F338A	3.17 ± 0.79	0.90 ± 0.24 ^c	94.75 ± 15.58
R339A	2.38 ± 0.28	2.34 ± 0.37 ^d	48.33 ± 20.00 ^c
S340A	0.84 ± 0.12 ^b	0.77 ± 0.22	52.29 ± 13.18 ^c
L341A	4.81 ± 1.13 ^c	0.50 ± 0.07	102.50 ± 19.31
E342A	0.58 ± 0.02 ^d	N/A ^a	N/A ^a
L343A	N/A ^a	N/A ^a	N/A ^a
R344A	N/A ^a	N/A ^a	N/A ^a
N345A	1.89 ± 0.17	0.36 ± 0.08	107.00 ± 12.13
T346A	2.08 ± 0.18	0.48 ± 0.16	91.80 ± 8.89
F347A	1.09 ± 0.19 ^b	0.25 ± 0.06 ^c	67.25 ± 8.38 ^c
R348A	0.91 ± 0.04 ^b	0.42 ± 0.11	53.40 ± 5.62 ^b
E349A	1.21 ± 0.18 ^c	0.47 ± 0.16	103.00 ± 23.34
I350A	0.61 ± 0.07 ^b	0.33 ± 0.07	15.75 ± 1.55 ^d
L351A	0.64 ± 0.03 ^b	0.32 ± 0.10	17.50 ± 2.02 ^d

Values are expressed as the mean ± SEM of at least four independent experiments. The

R_{max} of WT hMC3R was 2386.00 ± 239.95 pmol/10⁶ cells upon NDP-MSH stimulation.

^a Not detectable.

^b Significantly different from WT hMC3R, p<0.01.

^c Significantly different from WT hMC3R, $p < 0.05$.

^d Significantly different from WT hMC3R, $p < 0.001$.

Figure 2.1 Schematic model of the hMC3R with the 20 mutations characterized in present study highlighted.

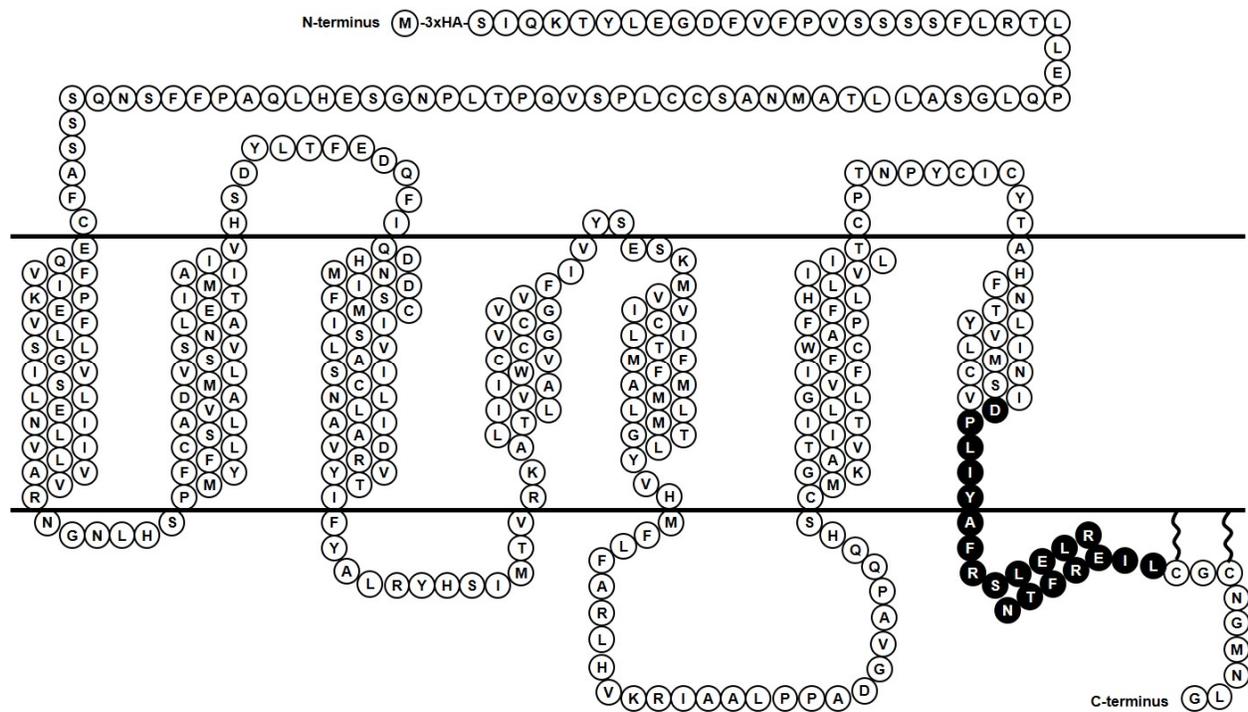


Figure 2.2 Cell surface expression of the WT and mutant hMC3Rs by flow cytometry.

The results are expressed as % of cell surface expression level of WT hMC3R after correction of the nonspecific staining in cells transiently transfected with the empty vector as described in Materials and methods. Values are mean \pm SEM of at least three independent experiments. * indicates significantly different from WT hMC3R ($P < 0.05$).

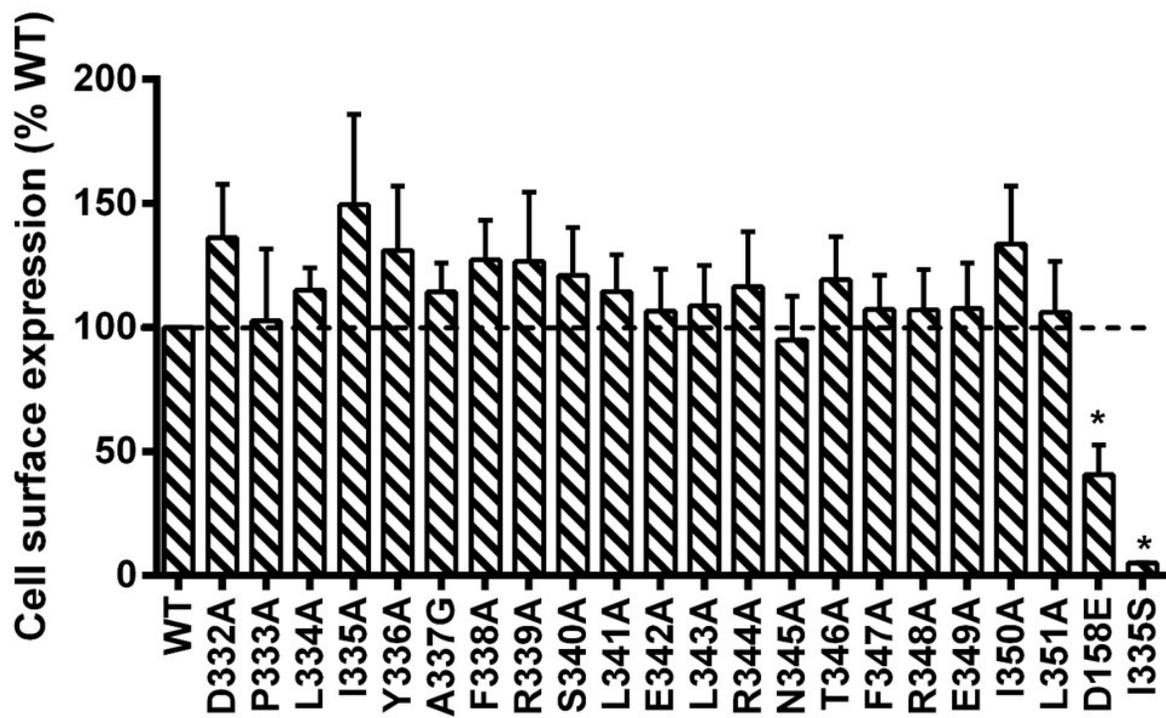


Figure 2.3 Ligand binding properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand.

HEK293T cells were transiently transfected with WT and mutant constructs, and the binding properties were measured 48 h later by displacing the binding of ¹²⁵I-NDP-MSH using different concentrations of unlabeled NDP-MSH as described in Materials and methods. Data are expressed as % of WT binding \pm range from duplicate measurements within one experiment. All experiments were performed as least three times independently.

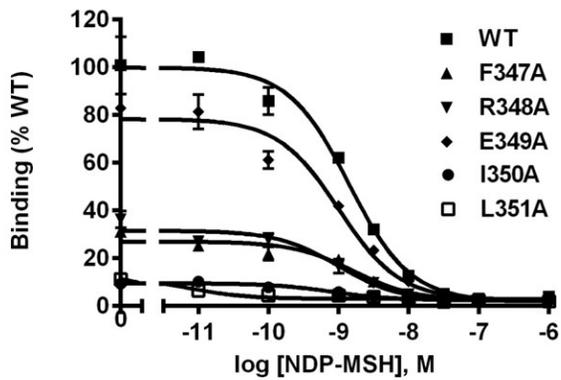
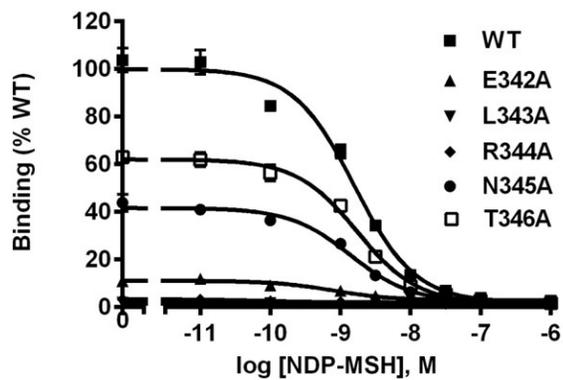
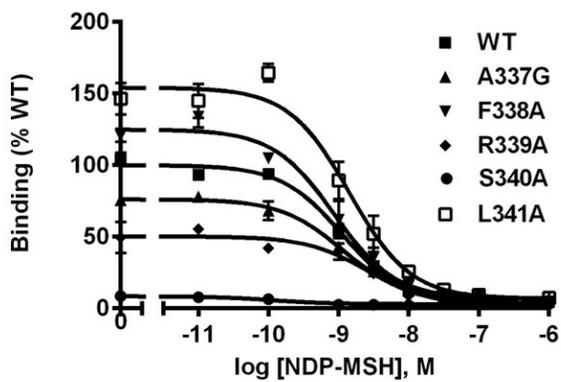
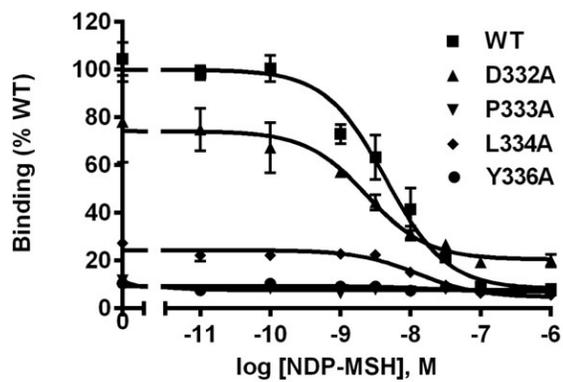


Figure 2.4 Total specific binding of WT and mutant hMC3Rs with NDP-MSH as the ligand.

Data are mean \pm SEM of at least three independent experiments. * indicates significantly different from WT hMC3R ($P < 0.05$).

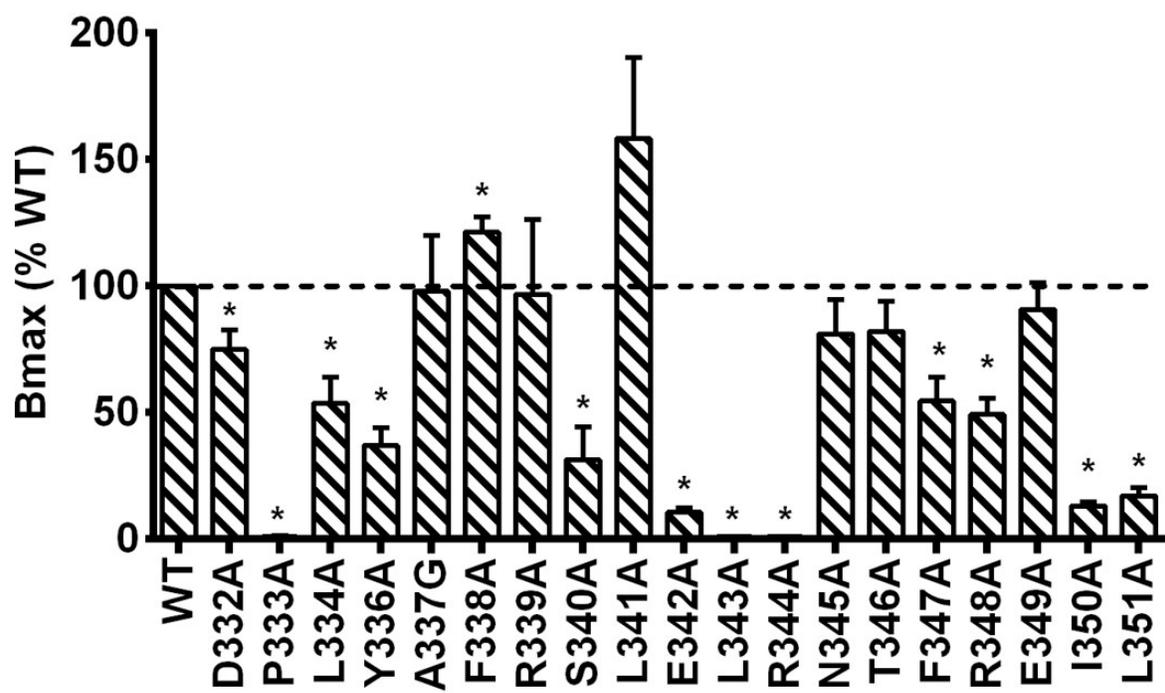


Figure 2.5 Signaling properties of the WT and mutant hMC3Rs with NDP-MSH as the ligand.

HEK293T cells were transiently transfected with WT and mutant constructs, and intracellular cAMP levels were measured by RIA after stimulation with different concentrations of NDP-MSH as described in Materials and methods. Data are mean \pm SEM from triplicate measurements within one experiment. All experiments were performed as least three times independently.

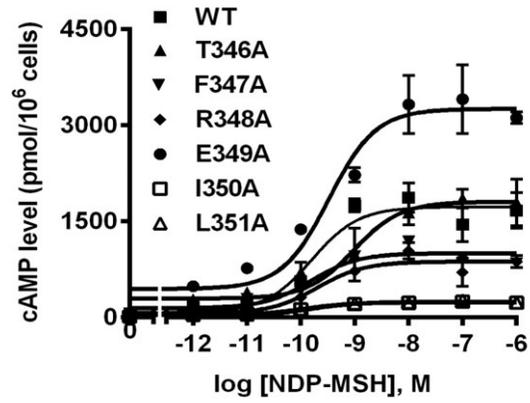
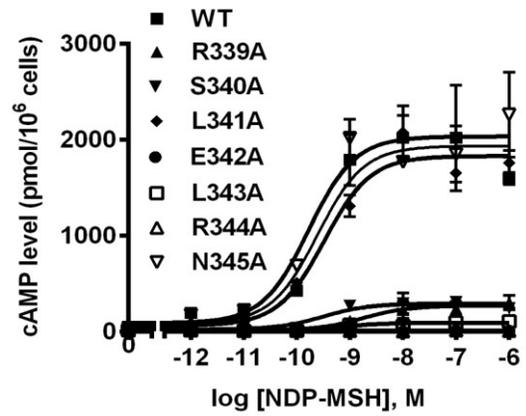
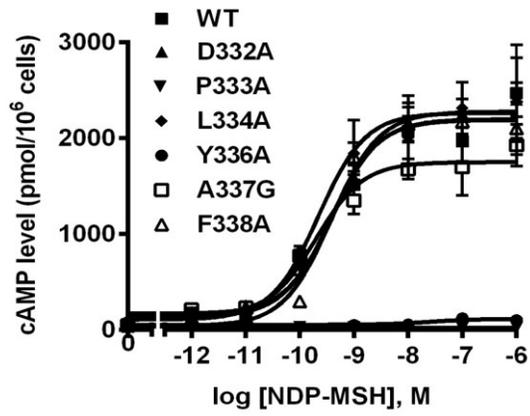


Figure 2.6 Basal activities of WT and mutant hMC3Rs.

(A) HEK293T cells were transiently transfected with WT and mutant constructs, and intracellular cAMP levels were measured without ligand stimulation. Data are mean \pm SEM of at least four independent experiments. The basal cAMP level of WT hMC3R was 25.79 ± 2.69 pmol/ 10^6 cells. * indicates significantly different from WT hMC3R ($P < 0.05$).

(B) A representative dose-response curve of WT and F347A hMC3Rs. Similar results were obtained in at least three independent experiments.

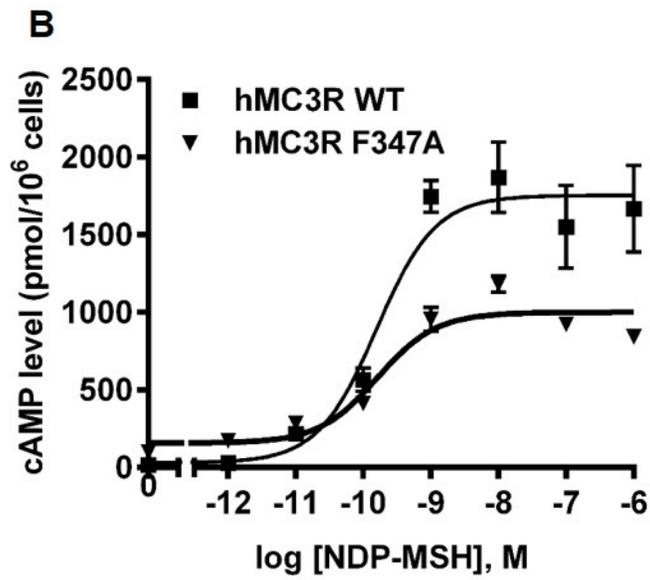
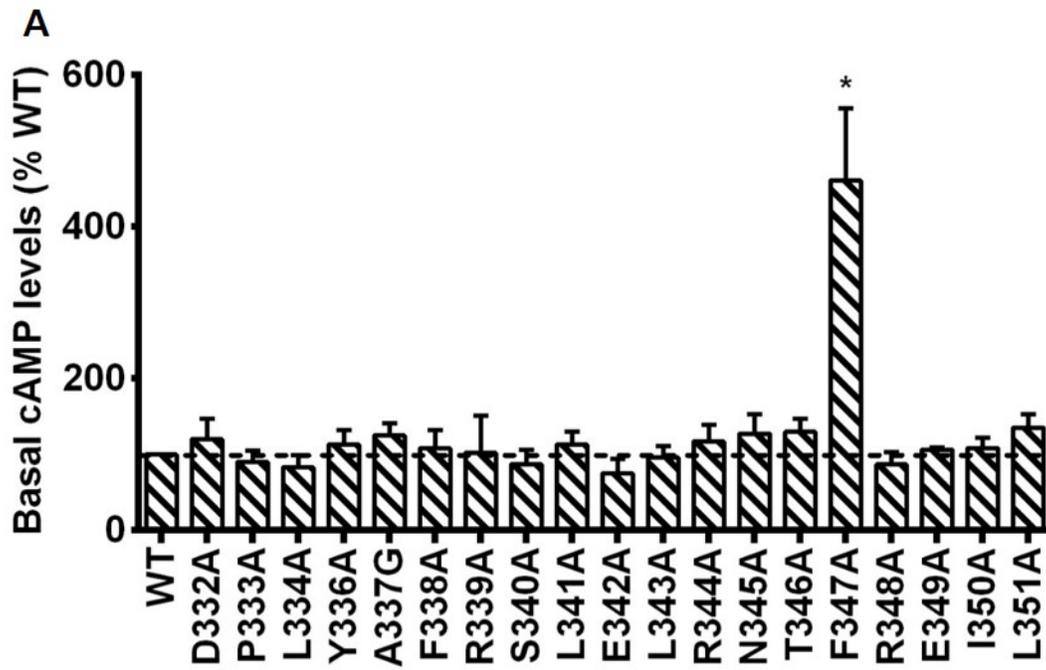
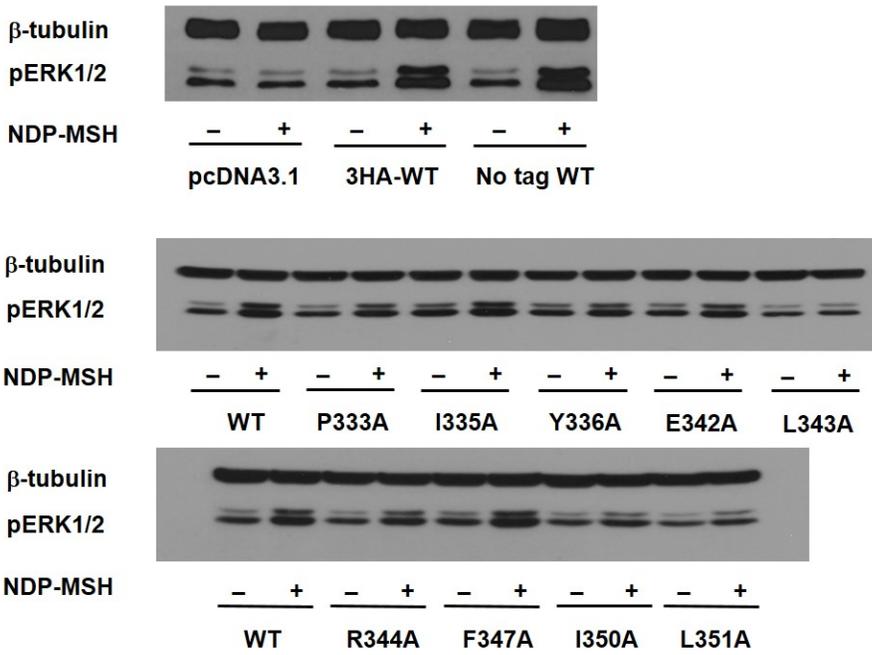


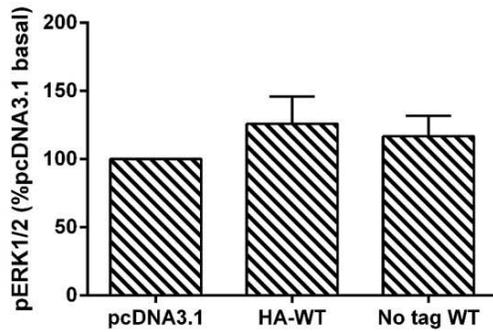
Figure 2.7 MAPK signaling of WT and mutant hMC3Rs.

(A) HEK293T cells were transiently transfected with pcDNA3.1, WT (with or without HA tag) or mutant constructs, and 24h later cells were starved overnight and then harvested after they were treated with or without 1 μ M NDP-MSH stimulation for 5 min. Western blot analysis was performed using antibody against pERK1/2 and β -tubulin as control. (B) Basal pERK1/2 levels of cells transfected with pcDNA3.1 or WT hMC3R (with or without HA tag); data are mean \pm SEM of three independent experiments. (C) Stimulated pERK1/2 levels in cells transfected with pcDNA3.1 or WT hMC3R (with or without HA tag) after 1 μ M NDP-MSH stimulation, expressed as a percentage of each basal pERK1/2 activity from three independent experiments. * indicates significantly different from corresponding basal activity ($P < 0.05$) and ns means no statistical difference between HA-tagged and non-tagged WT hMC3R ($P > 0.05$). (D) Data of the basal pERK1/2 of WT and mutant hMC3Rs are mean \pm SEM of at least four independent experiments. (E) Data of the pERK1/2 levels of WT and mutant hMC3Rs after 1 μ M NDP-MSH stimulation are expressed as percentage of each basal pERK1/2 activity from at least four independent experiments. * indicates significantly different from corresponding basal activity ($P < 0.05$).

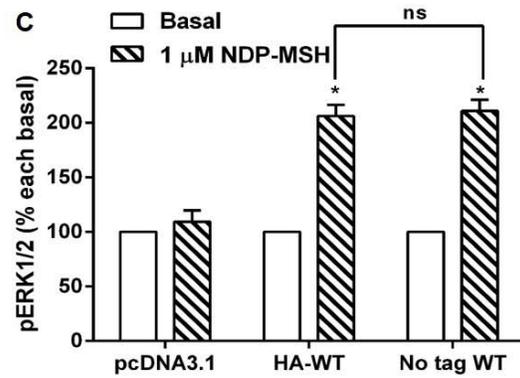
A



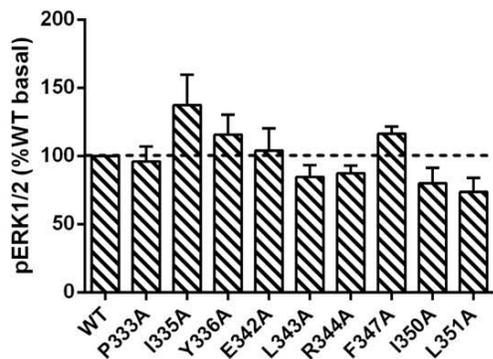
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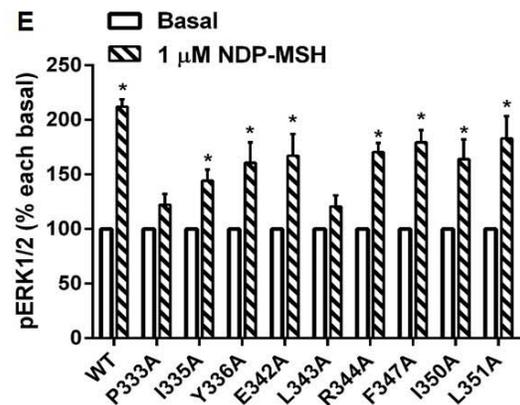
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D



E



Chapter 3

3.1 Introduction

Two of the five melanocortin receptors (MCRs), the melanocortin-3 (MC3R) and -4 receptor (MC4R), are referred as neural MCRs due to their high expression in the central nervous system. They are increasingly recognized as important regulators of energy homeostasis (reviewed in (Begrache et al. 2013; Tao 2009)). Genetic studies in rodent models revealed non-redundant roles of these two receptors in the regulation of energy homeostasis (Chen et al. 2000a; Rowland et al. 2010). The MC4R plays an essential role in both food intake and energy expenditure (Huszar et al. 1997; Tao 2010a), whereas the MC3R primarily participates in regulation of feed efficiency (Butler et al. 2000; Chen et al. 2000a) and maintenance of circadian rhythm (Begrache et al. 2012; Girardet and Butler 2014).

As typical members of rhodopsin-like family A G protein-coupled receptors (GPCRs), the two neural MCRs primarily couple to the stimulatory G protein (G_s) to activate adenylyl cyclase, leading to intracellular cAMP production and downstream protein kinase A (PKA) activation. In addition to the conventional G_s -cAMP-PKA signaling pathway, previous studies have also revealed that activation of both MC3R and MC4R lead to extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation (Chai et al. 2007; Chai et al. 2006; Vongs et al. 2004). The cAMP production and ERK1/2

activation are not necessarily related. We and others have reported the existence of unbalance cAMP and ERK1/2 signaling pathways in both MC3R and MC4R (He and Tao 2014; Mo and Tao 2013; Montero-Melendez et al. 2015; Patten et al. 2007; Yang et al. 2015a; Yang et al. 2015b).

The two neural MCRs are unique to share an endogenous agonist α -melanocyte stimulating hormone (α -MSH) and an endogenous antagonist agouti-related peptide (AgRP). AgRP, first cloned in 1997, was initially found to dose-dependently antagonize α -MSH binding to neural MCRs and inhibit α -MSH-induced anorectic signals (Fong et al. 1997; Ollmann et al. 1997). Subsequently, AgRP was identified to behave as an inverse agonist for both MC3R and MC4R by decreasing the basal cAMP activities (Nijenhuis et al. 2001; Tao et al. 2010). Studies using rodent models revealed that both overexpression of AgRP and intracerebroventricular AgRP administration increase food intake and decrease energy expenditure, leading to accumulated adiposity (Goto et al. 2003; Korner et al. 2003; Small et al. 2001). In humans, AgRP levels are decreased in obese individuals (Katsuki et al. 2001) and SNPs in AgRP gene have been shown to be associated with resistance to obesity (Marks et al. 2004).

In addition to its antagonizing effect, several lines of *in vivo* evidence indicated that AgRP could induce long-term orexigenic signals in a melanocortin-independent manner by an unknown mechanism (Hagan et al. 2000; Tolle and Low 2008). Recent *in vitro* studies also demonstrated that AgRP acts as a biased agonist that mediates MC4R activity through G_i protein activation and potassium channels regulation, independently of its inhibition of α -MSH binding, suggesting agonistic property (Buch et al. 2009; Ghamari-Langroudi et al. 2015). Therefore, the role of AgRP in melanocortin system needs to be

re-evaluated and detailed pharmacological studies focusing on signal transductions initiated by AgRP through neural MCRs would be undoubtedly helpful.

Our recent study revealed that AgRP indeed independently activates ERK1/2 signaling pathway through MC4R (Mo and Tao 2013). However, little is known about the underlying mechanism or whether this biased agonism of AgRP is universal in neural MCRs. To further investigate the agonistic property of AgRP in neural MCRs, in the present study, we investigated the potential of AgRP in regulating ERK1/2 activation through MC3R and we further clarified the mechanism by which AgRP initiated ERK1/2 activation through both neural MCRs. We also explored whether AgRP initiated other signaling pathways, in addition to cAMP and ERK1/2 pathways, through neural MCRs, including AKT and AMPK activation.

3.2 Materials and methods

3.2.1 Reagents and supplies

AgRP(83-132) was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and [Nle⁴, D-Phe⁷]- α -melanocyte stimulation hormone (NDP-MSH) from Peptides International (Louisville, KY, USA). N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), N-[(2R)-2,3-Dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-benzamide (PD325901), 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) and wortmannin were purchased from LC Laboratories (Woburn, MA, USA). Pertussis toxin

(PTX) was purchased from Sigma-Aldrich (St. Louis, MO, USA). [¹²⁵I]-cAMP was iodinated in our lab using chloramine T method.

3.2.2 Plasmids construction

The N-terminal c-myc-tagged WT hMC4R cloned into pcDNA3.1 was generated as previously described (Tao and Segaloff 2003). The N-terminal 3×HA-tagged WT hMC3R cloned into pcDNA3.1 was obtained from Missouri S&T cDNA Resource Center (<http://www.cDNA.org/>; Rolla, MO, USA). The F347A hMC3R mutant was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as described before (Yang et al. 2015b). The F347A mutation in hMC3R plasmid was confirmed by DNA sequencing performed by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA).

3.2.3 Cell culture and DNA transfection

Human Embryonic Kidney (HEK) 293T cells, purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% newborn calf serum, 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and 50 µg/ml gentamicin in a 5% CO₂-humidified atmosphere. GT1-7 cells, generously provided by Dr. Pamela Mellon (Mellon et al. 1990), were cultured in the same media for HEK293T cells except that it was supplemented with 10% fetal bovine serum. For cAMP assay, the HEK293T cells were plated into 6-well clusters (Corning, NY, USA) pre-coated with 0.1% gelatin and transfected approximately 24 hours later with plasmids at 50-70% confluence using calcium phosphate precipitation method (Chen and Okayama 1987). For preparation of

cellular lysates for immunoblotting, cells were seeded into gelatin-coated 100 mm dishes and starved for 18 h (or 24 h for GT1-7 cells) at 37 °C in Waymouth's MB752/1 media (Sigma-Aldrich) containing 1 mg/ml bovine serum albumin (Waymouth/BSA) 24 h after transfection (or 48 h after GT1-7 cells seeding).

3.2.4 cAMP assay

Forty-eight hours after transfection, HEK 293T cells were washed twice with warm Waymouth/BSA and then incubated with fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma-Aldrich) at 37°C for another 15 min. Then either buffer alone or different ligands (1 μM NDP-MSH or 0.1 μM AgRP) were added. After 60 min incubation at 37°C, the reaction was terminated on ice and the intracellular cAMP was collected by adding 0.5 M perchloric acid containing 180 μg/ml theophylline (Sigma-Aldrich) and 0.72 M KOH/0.6 M KHCO₃ into each well. The cAMP concentrations were determined by radioimmunoassay as previously described (Tao et al. 2010).

3.2.5 Cell treatment and protein preparation

Forty-two hours after transfection (or 72 h after GT1-7 cells seeding), pre-starved cells were treated for 5 min (or various times for time-course study) at 37°C with either buffer alone or different ligands (1 μM NDP-MSH or 0.1 μM AgRP, or various concentrations for dose-course study). The time points set for the present study were determined according to our previous report of MC4R-mediated ERK1/2 activation (Mo and Tao 2013). For signaling mechanism studies, cells were incubated with vehicle or different inhibitors (50 ng/ml PTX, 1 μM PD325901, 10 μM U0126, 10 μM H-89, 1 μM wortmannin or 10 μM LY294002) for 30 min (18 h for PTX) before ligand treatment. The

concentrations and duration of inhibitor incubation were determined based on the product datasheets and on available literature (Buch et al. 2009; Gao et al. 1999; Gioeli et al. 2011; Jones and Clague 1995; Rodrigues et al. 2009). Cells were subsequently washed with ice-cold PBS and lysed with lysis buffer containing phosphatase and protease inhibitors. The protein concentrations of the cellular lysates were determined by Bradford assay.

3.2.6 Western blotting analysis

The method for western blotting was described in detail in previous studies (He and Tao 2014; Mo and Tao 2013). Briefly, cellular extracts were separated on 10% SDS-PAGE gel and transferred onto PVDF membrane for immunoblotting. Specific rabbit primary antibodies were used for the detection of the phosphorylated forms of ERK1/2 (Thr²⁰²/Tyr²⁰⁴), AKT (Thr³⁰⁸), AKT (Ser⁴⁷³) and AMPK (Thr¹⁷²) (1:2000~1:5000, Cell Signaling Technology, Danvers, MA, USA). Equal protein loading was verified by mouse β -tubulin antibody (1:5000, Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA, USA). Blots were probed with horseradish peroxidase-conjugated anti-rabbit (1:2000, Jackson ImmunoResearch Inc., West Grove, PA, USA) and anti-mouse (1:5000, Jackson ImmunoResearch Inc.) antibodies at room temperature for 2 h. The bound antibodies were detected using ECL reagent (Thermo Scientific, Rockford, IL) and quantified by ImageJ 1.49 software (NIH, Bethesda, MD, USA).

3.2.7 Statistical analysis

The significance of the differences in cAMP assay and western blotting was determined by two-tailed Student's *t*-test using Graphpad Prism 4.0 software.

3.3 Results

3.3.1 ERK1/2 signaling efficacy of AgRP in hMC3R-transfected HEK293T cells

HEK293T cell has been widely used as a reliable mammalian expression system in MCR studies. Our published data demonstrated that there are no endogenous receptors in HEK293T cells; transfection with the empty vector pcDNA3.1 do not result in response to either NDP-MSH or AgRP treatment (Mo and Tao 2013; Yang et al. 2015b). Previous studies have also revealed that AgRP(83-132), an amidated carboxyl-terminal fragment of AgRP, retains the biological activity of full-length AgRP (Quillan et al. 1998; Yang et al. 1999). Therefore, HEK293T cells transfected with WT hMC3R were treated with AgRP(83-132) in current study to evaluate the potential of AgRP to initiate ERK1/2 activation through MC3R.

The dose-response study was first performed by incubating cells with increasing concentrations of AgRP ($10^{-12} \sim 10^{-7}$ M) for 5 min, using NDP-MSH ($10^{-11} \sim 10^{-6}$ M) for comparison. The incubation time was determined based on our previous study of AgRP-initiated ERK1/2 activation through hMC4R (Mo and Tao 2013). Our results demonstrated that ERK1/2 phosphorylation upon NDP-MSH stimulation was dose-dependent with an EC_{50} of 0.43 nM (Fig. 3.1A-B). Interestingly, significant elevation of phosphorylated ERK1/2 (pERK1/2) levels was also observed in hMC3R-transfected HEK293T cells treated with AgRP at the concentration of 0.01 μ M and 0.1 μ M with 1.5- and 1.9-fold increase, respectively (Fig. 3.1A-B).

The time-course study was subsequently performed by incubating cells with either 1 μ M NDP-MSH or 0.1 μ M AgRP, shown to induce maximal ERK1/2 phosphorylation in

the dose-response study, for different times (2 - 180 min). As shown in Fig 3.1C-D, our results showed that although both ligands induced maximal pERK1/2 levels in hMC3R-transfected HEK293T cells at 5 min, different time-course was observed. NDP-MSH stimulation induced a sustained activation of ERK1/2 for up to 45 min and the pERK1/2 level decreased to basal at 120 min, whereas AgRP administration led to a transient activation of ERK1/2 that peaked at 5 min and quickly returned to basal 30 min after stimulation (Fig. 3.1C-D). Since the pattern of ERK1/2 activation observed herein was similar to that of hMC4R-transfected HEK293T cells we previously reported (Mo and Tao 2013), a stimulation for 5 min with either 1 μ M NDP-MSH or 0.1 μ M AgRP was used in subsequent studies of both neural MCRs.

3.3.2 cAMP and ERK1/2 signaling properties of constitutively active hMC3R mutant

AgRP has been shown to be an inverse agonist that decreases the basal cAMP activity for the hMC4R (Nijenhuis et al. 2001). However, unlike hMC4R, the hMC3R has little or no constitutive cAMP activity, which makes the study of cAMP signaling efficacy of AgRP in hMC3R difficult. Therefore, the only constitutively active hMC3R mutant identified so far, F347A, was used in the present study to investigate the potential biased agonism of AgRP in hMC3R. As shown in Fig. 3.2A, the F347A hMC3R had a basal cAMP production that was 5-fold higher compared with WT hMC3R, consistent with our previous studies (Tao et al. 2010; Yang et al. 2015b). NDP-MSH stimulation to F347A hMC3R led to a robust increase of cAMP generation that was 17.2 times higher than the basal level, whereas AgRP treatment caused 75% decrease of basal cAMP activity (Fig. 3.2A).

Next, we tested the potential of AgRP to affect the ERK1/2 signaling of constitutively active mutant, F347A hMC3R. As shown in Fig. 3.2B-C, We found that

F347A had similar basal pERK1/2 level as the WT hMC3R. Upon 1 μ M NDP-MSH stimulation for 5 min, the ERK1/2 phosphorylation was significantly enhanced in HEK293T cells transfected with either WT or F347A hMC3R, which was in accordance with the cAMP signaling. However, after 5 min treatment with 0.1 μ M AgRP, significantly increased pERK1/2 levels were also detected for both WT and F347A hMC3R compared with corresponding basal ERK1/2 activity.

3.3.3 Signaling pathways involved in AgRP-initiated ERK1/2 activation through neural MCRs

To identify the mechanism by which AgRP induced ERK1/2 activation through both neural MCRs, different specific inhibitors, including G_i signaling inhibitor PTX (50 ng/ml), MEK1/2 inhibitors PD325901 (1 μ M) and U0126 (10 μ M), PKA inhibitor H-89 (10 μ M), and PI3K inhibitors wortmannin (1 μ M) and LY294002 (10 μ M), were used in the present study. HEK293T cells transfected with either hMC3R or hMC4R were incubated with different inhibitors for various times (see Section 3.2.5 for detail) before the 5 min treatment of 0.1 μ M AgRP and western blotting analysis was subsequently performed. It should be noted that all the concentrations and incubation times of the inhibitors used herein were determined based on or even higher than that reported in previous studies, which have been shown to be potent and effective enough to block corresponding signaling pathways (Gao et al. 1999; Gioeli et al. 2011; Jones and Clague 1995; Rodrigues et al. 2009).

As shown in Fig. 3.3A-B, in HEK293T cells transfected with hMC3R, pretreatment with 10 μ M LY294002 significantly decreased the basal pERK1/2 level of hMC3R while pretreatment of either 1 μ M PD325901 or 10 μ M U0126 nearly completely abolished the

basal ERK1/2 phosphorylation. No significant differences were observed between the basal pERK1/2 levels of the cells pretreated with the other inhibitors and that of the control group. Upon 0.1 μ M AgRP stimulation, the cells pretreated with 10 μ M H-89 did not respond with ERK1/2 phosphorylation while all the other groups showed significantly increased pERK1/2 levels compared to each basal, comparable with that of the control group (Fig. 3.3C).

As shown in Fig. 3.4A-B, in HEK293T cells transfected with hMC4R, basal pERK1/2 level was similar to that obtained in hMC3R-transfected HEK293T cells. The basal ERK1/2 phosphorylation was significantly decreased and nearly totally blocked by PI3K inhibitor LY294002 and two MEK1/2 inhibitors, respectively. However, pretreatment of the cells with 50 ng/ml PTX or 10 μ M H-89 did not significantly inhibit 0.1 μ M AgRP-stimulated ERK1/2 phosphorylation compared with the control group. In contrast, pretreatment of the cells with PI3K inhibitors, either 1 μ M wortmannin or 10 μ M LY294002, led to the abolishment of AgRP-initiated ERK1/2 phosphorylation (Fig. 3.4C).

3.3.4 Ligand-stimulated activation of AKT through neural MCRs

Since the involvement of PI3K in AgRP-initiated ERK1/2 activation through neural MCRs, especially MC4R, has been suggested in our results above, we explored whether AgRP could induce AKT activation, the main downstream kinase of PI3K. NDP-MSH was used for comparison.

The time-course study was performed as described in Materials and methods and the levels of phosphorylated AKT (pAKT) were analyzed. We found that upon 1 μ M NDP-MSH or 0.1 μ M AgRP treatment, AKT phosphorylation, at either Thr308 or Ser473, was

not significantly affected in HEK293T cells transiently transfected with hMC3R during a 180 min treatment period (Fig. 3.5A, Fig. 3.6). The same result was obtained in HEK293T cells expressing hMC4R (Fig. 3.5B, Fig. 3.6).

We also studied the potential of NDP-MSH and AgRP to affect AKT activation in a mouse hypothalamic cell line, GT1-7, that endogenously expresses murine MC4R (mMC4R). As shown in Fig. 3.5C1 and Fig. 3.6A-B, stimulation with 1 μ M NDP-MSH induced a sustained AKT activation at both Thr308 and Ser473 with a maximal increase at 30 min and the pAKT decreased to the basal level after 120 min incubation. Surprisingly, the similar sustained AKT activation was also observed in GT1-7 cells upon 0.1 μ M AgRP treatment. As shown in Fig. 3.5C2 and Fig. 3.6C-D, significant increase of pAKT level was detected 20 min after AgRP stimulation. A 2.2-fold maximal increase of pAKT (T308) and a 4.2-fold maximal increase of pAKT (S473) were observed 30 - 60 min after AgRP treatment. The pAKT (T308) decreased to basal level at 120 min while the pAKT (S473) returned to normal after 180 min incubation.

3.3.5 Ligand-stimulated activation of AMPK through neural MCRs

The hypothalamic AMP-activated protein kinase (AMPK) has been suggested to play a vital role in neural MCRs-mediated regulation of energy homeostasis. We assessed the potential of AgRP in affecting AMPK activity by regulating the phosphorylated AMPK (pAMPK) levels through neural MCRs. NDP-MSH was used for comparison.

We showed that in HEK293T cells transfected with either hMC3R or hMC4R, 1 μ M NDP-MSH stimulation significantly decreased the pAMPK levels by 50% after 45 min

incubation (Fig. 3.5A1-B1, Fig. 3.7A-B). The NDP-MSH-induced dephosphorylation of AMPK was persistent and the pAMPK levels remained reduced up to 180 min. In contrast, no significant changes of pAMPK levels were observed upon 0.1 μ M AgRP treatment during the 180 min study period (Fig. 3.5A2-B2, Fig. 3.7A-B).

In MC4R-expressing GT1-7 cells, stimulation with 1 μ M NDP-MSH induced significant AMPK dephosphorylation 60 min after incubation (Fig. 3.5C1, Fig. 3.7C). The pAMPK remained at a level that was approximately half the basal up to 180 min. Similar as that in HEK293T cells, the pAMPK levels in GT1-7 cells were not significantly affected upon 0.1 μ M AgRP treatment during the 180 min study period (Fig. 3.5C2, Fig. 3.7C).

3.4 Discussion

The neural MCRs, including MC3R and MC4R, have been recognized to play crucial roles in the regulation of energy homeostasis. Recent studies have revealed that AgRP, which was traditionally considered to act as an antagonist of neural MCRs, could regulate the MC4R activity through G_i protein (Buch et al. 2009) and potassium channel (Ghamari-Langroudi et al. 2015), independently of its competitive inhibition of α -MSH binding, suggesting agonistic property. Our previous study also showed that AgRP stimulates ERK1/2 activation in WT and constitutively active mutant MC4Rs (Mo and Tao 2013). To deepen our understanding of the role AgRP plays in neural melanocortin system, in the present study, we further assessed the potential of AgRP in regulating ERK1/2 activation through MC3R. We also performed detailed study of the mechanism by which AgRP initiated ERK1/2 activation through both neural MCRs. We were also

interested in whether signaling pathways other than cAMP and ERK1/2 would be activated by AgRP through neural MCRs.

Our result showed that NDP-MSH induced a dose-dependent and sustained ERK1/2 activation in hMC3R-transfected HEK293T cells, AgRP also behaved as an agonist transiently activating ERK1/2 signaling through hMC3R. Moreover, using the artificially-generated constitutively active hMC3R mutant F347A, we further demonstrated that AgRP functioned as a biased agonist in cAMP and ERK1/2 signaling pathways by decreasing cAMP accumulation while activating ERK1/2 phosphorylation. To the best of our knowledge, this is the first study demonstrating biased agonism of AgRP at the MC3R. The ERK1/2 signaling mediated by GPCRs has been demonstrated to play a pivotal role in multiple essential cellular processes such as development, differentiation and proliferation (Aouadi et al. 2006; Johnson and Lapadat 2002; Nishimoto and Nishida 2006). In neural MCRs, in addition to its function in mediating cell proliferation (Chai et al. 2007; Chai et al. 2006), the ERK1/2 signaling cascade was also reported to be involved in the regulation of energy homeostasis in terms of food intake, feeding behaviors and long-lasting effects of melanocortins (Begrache et al. 2012; Daniels et al. 2003; Sutton et al. 2005). Therefore, the biased agonism of AgRP in ERK1/2 signaling through neural MCRs deserves further investigation.

Although MC4R-mediated ERK1/2 signaling has been extensively studied, the mechanism remained enigmatic since discrepant results have been reported (Breit et al. 2011). NDP-MSH-initiated ERK1/2 activation has been attributed to G_i protein regulation in HEK293 cells expressing MC4R (Chai et al. 2006), or to Ca^{2+} and PKC pathway in hypothalamic GT1-1 cells that endogenously express MC4R (Chai et al. 2006), or to

PI3K-involved signaling in CHO cells stably transfected with MC4R (Vongs et al. 2004), or to be only PKA-dependent in GT1-7 cells (Damm et al. 2012). Previous studies have suggested that AgRP and NDP-MSH might have different binding sites in MC4R, implying divergent signaling pathways (Chen et al. 2006; Yang et al. 2000). In the present study, we sought to identify the mechanism by which AgRP stimulated ERK1/2 signaling in HEK293T cells transfected with hMC4R using specific inhibitors for MEK1/2, PKA, PI3K and G_i protein. Our result demonstrated that only PI3K inhibitors wortmannin and LY294002 specifically abolished AgRP-induced ERK1/2 activation and LY294002 pretreatment also decreased the basal ERK1/2 activity. Therefore, in HEK293T cells expressing hMC4R, the AgRP stimulated ERK1/2 activation in a PI3K-dependent manner.

Unlike MC4R, the MC3R-mediated ERK1/2 signaling has not been extensively studied. Although an earlier report suggested that MC3R activation does not induce ERK1/2 phosphorylation, later studies suggested otherwise (Chai et al. 2007; Yang et al. 2015a; Yang et al. 2015b; Montero-Melendez et al. 2015; Huang and Tao 2014). One report suggested that NDP-MSH-induced ERK1/2 activation in HEK293 cells stably expressing hMC3R is PI3K- and G_i-dependent (Chai et al. 2007). In the current study, our result showed that although PI3K inhibitor LY294002 significantly decreased basal ERK1/2 activity in hMC3R-transfected HEK293T cells, PI3K inhibition did not interfere with ERK1/2 phosphorylation upon AgRP treatment. In contrast, AgRP-induced ERK1/2 activation through MC3R was completely blocked by pretreatment of PKA inhibitor H-89. PKA-dependent ERK1/2 activation has been reported in other GPCRs, such as melanocortin-2 receptor (Roy et al. 2011) and β_2 -adrenergic receptor (Shenoy et al. 2006). However, considering that AgRP acts as an inverse agonist in cAMP signaling pathway

in MC3R, the cAMP-regulated PKA activation would not be applicable in the present study. We suggest that cAMP-independent PKA-activation, which has been reported in many other GPCRs, such as α 2-adrenergic receptor (DeBock et al. 2003), glucagon-like peptide 1 receptor (Kim Chung le et al. 2009) and endothelin receptor type A (Dulin et al. 2001), might be involved.

AKT, also known as protein kinase B, is the main downstream kinase of PI3K and the PI3K/AKT pathway has been shown to play a pivotal role in cell metabolism (Reviewed in (Manning and Cantley 2007)). Although AKT is reported to be associated with an increase of cellular proliferation in neuronal CAD cells regulated by MC3R (Nyan et al. 2008), our result revealed that pAKT levels were not significantly affected by either NDP-MSH or AgRP treatment in HEK293T cells expressing hMC3R, which is consistent with our finding that AgRP-initiated ERK1/2 activation through hMC3R is PI3K-independent. However, the negative result of AKT activation was also obtained in hMC4R-transfected HEK293T cells. Similar findings were previously reported that NDP-MSH alone does not stimulate AKT phosphorylation in MC4R-transfected HEK293 cells (Chai et al. 2009) or MC4R-expressing murine astrocytes (Ramirez et al. 2015). The difference of cellular context might be responsible for this phenomenon since we indeed observed AKT activation induced by NDP-MSH in GT1-7 cells, in agreement with previous reports in the same cell line (Perino et al. 2014) and murine osteoblasts that endogenously express MC4R (Guo et al. 2016). Moreover, we found that AgRP could also initiate sustained AKT phosphorylation independently in GT1-7 cells, which has not been reported before and further indicates the agonistic property of AgRP, providing new signaling pathway for AgRP as a biased agonist.

AMPK has been recognized as a sensor of cellular energy and a crucial regulator of energy homeostasis (Lage et al. 2008). Studies *in vivo* found that neural MCR agonist MT-II decreases AMPK activity in the paraventricular area of hypothalamus while AgRP increases AMPK activity (Minokoshi et al. 2004). Our data indicated that, in addition to its inhibitory effect of AMPK activity through hMC4R as previously reported (Molden et al. 2015), NDP-MSH could also induce sustained decrease of pAMPK levels in hMC3R-transfected HEK293T cells. In contrast, no significant changes of AMPK activity in either HEK293T cells or GT1-7 cells were observed upon AgRP treatment in the present study. Previous *in vivo* study suggested that AgRP does not alter AMPK activity in the hypothalamus of fasted mice owing to the activity already being high (Minokoshi et al. 2004). This explanation is also applicable in our study considering the high basal pAMPK levels in both cell types. The inhibitory effect of α -MSH in GT1-7 cells was reported to be ERK1/2-dependent (Damm et al. 2012). We showed that AgRP treatment did not affect AMPK activity although it independently initiated ERK1/2 activation. There are also studies using other cell lines suggesting that AMPK acts upstream of ERK1/2 (Chen et al. 2002; Soltoff and Hedden 2008). Therefore, the relationship between ERK1/2 and AMPK remains to be clarified.

AgRP has been suggested to independently exert agonist activity through activating G_i protein (Buch et al. 2009) and through opening potassium channels (Ghamari-Langroudi et al. 2015). It is also reported that AgRP could induce endocytosis of MC3R and MC4R through β -arrestin to reduce the amount of MCRs molecules accessible to melanocortins (Breit et al. 2006). Our finding of agonistic property of AgRP in ERK1/2 and AKT signaling pathways through neural MCRs enriched our understanding

of the role AgRP plays in the melanocortin system and in the regulation of energy homeostasis beyond its antagonism of α -MSH. These discoveries of AgRP agonism might also provide a basis for understanding the long-term action of AgRP on food intake (Tao 2014). However, considering the strong cell-line-dependency of kinases activation, further *in vitro* studies using more cell lines together with *in vivo* studies would be necessary to further dissect the mechanism by which AgPR initiates multiple signaling pathways through neural MCRs.

Pharmaceuticals targeting MCRs, especially MC4R, have been extensively studied for obesity treatment. However, no MC4R agonists or their analogs have progressed into clinic for obesity treatment due to the adverse effects on cardiovascular functions caused by MC4R activation (da Silva et al. 2008; Fani et al. 2014; Greenfield et al. 2009). One small-molecule compound that acts as selective AgRP inhibitor has recently been reported to decrease food intake and adiposity in rodent models without arousing side effects, and thus might represent a novel strategy for anti-obesity drugs development (Dutia et al. 2013). Our finding of AgRP-initiated multiple intracellular signaling pathways through neural MCRs would provide a foundation at the molecular level for the development of potential AgRP inhibitors.

Although the presence of both MC3R and MC4R mRNA was suggested in GT1-7 cells (Khong et al. 2001), subsequent studies have confirmed that only MC4R is endogenously expressed in GT1-7 cells while no functional MC3R is detectable (Buch et al. 2009; Shinyama et al. 2003). Therefore, the effects of both NDP-MSH and AgRP in GT1-7 cells in the present study should be mediated through MC4R. The MC3R is less studied compared with MC4R and the role of MC3R in the regulation of energy

homeostasis is still controversial, partly because of the lack of stable cell lines endogenously expressing MC3R and ligands selectively targeting MC3R. Our result, that NDP-MSH could induce persistent AMPK dephosphorylation through MC3R, might suggest a new link between MC3R and energy metabolism but remains to be further explored by *in vivo* studies.

In summary, the results of the present study demonstrated that AgRP acted as a biased agonist in both neural MCRs, decreasing cAMP activity but stimulating ERK1/2 activation. AgRP initiated ERK1/2 phosphorylation through MC3R and MC4R by different mechanisms. We also observed ligands-initiated AKT and/or AMPK activation through neural MCRs. Biased intracellular signaling cascades contribute to a better understanding of AgRP activity and pharmacology of neural MCRs.

Figure 3.1 ERK1/2 signaling initiated by NDP-MSH or AgRP in hMC3R-transfected HEK293T cells.

ERK1/2 phosphorylation was measured as described in Materials and methods. A represent blot (A) and densitometry analysis (B) of ligand-stimulated ERK1/2 phosphorylation from the dosage-response study. Representative image (C) and densitometry result (D) of ligand-stimulated ERK1/2 phosphorylation from the time-course study. Results are expressed as the percentage of the value obtained in non-stimulated HEK293T cells transfected with WT hMC3R. Shown are mean \pm SEM of at least three independent experiments. * indicates significantly different from basal pERK1/2 level (* P < 0.05).

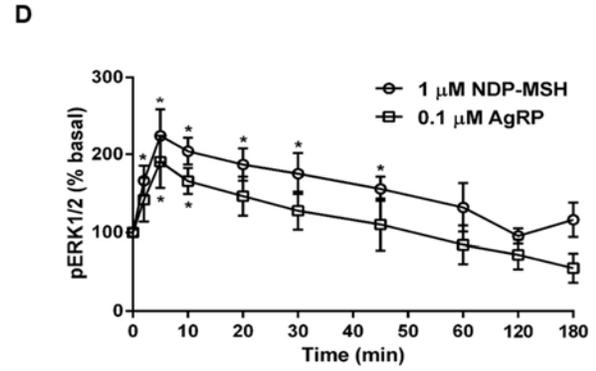
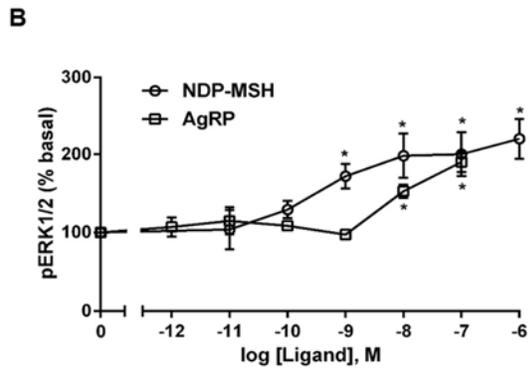
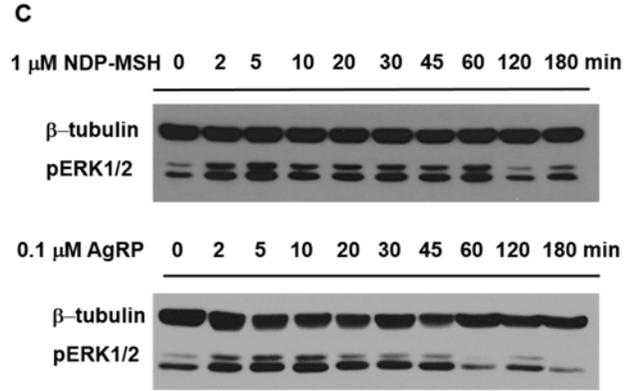
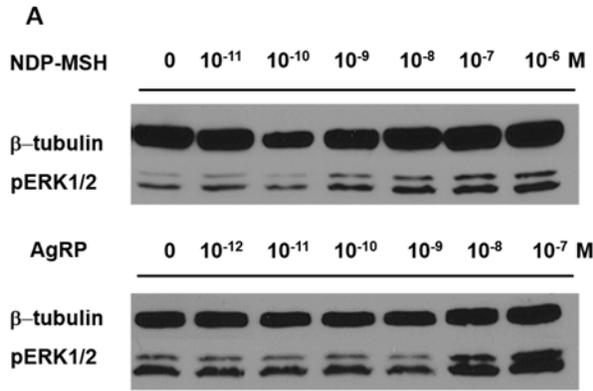


Figure 3.2 Effects of NDP-MSH and AgRP on cAMP and ERK1/2 signaling of WT and constitutively active mutant hMC3Rs.

Intracellular cAMP accumulation (A) and ERK1/2 phosphorylation (B-C) were measured as described in Materials and methods. Shown are representative image (B) and densitometry analysis (C) of ligand-stimulated ERK1/2 phosphorylation of WT and F347A MC3Rs and results are expressed as the percentage of WT basal pERK1/2 level. Shown are mean \pm SEM of at least three independent experiments. * indicates significant difference from F347A basal cAMP or pERK1/2 level (* $P < 0.05$; *** $P < 0.001$) and ns means no statistical difference ($P > 0.05$).

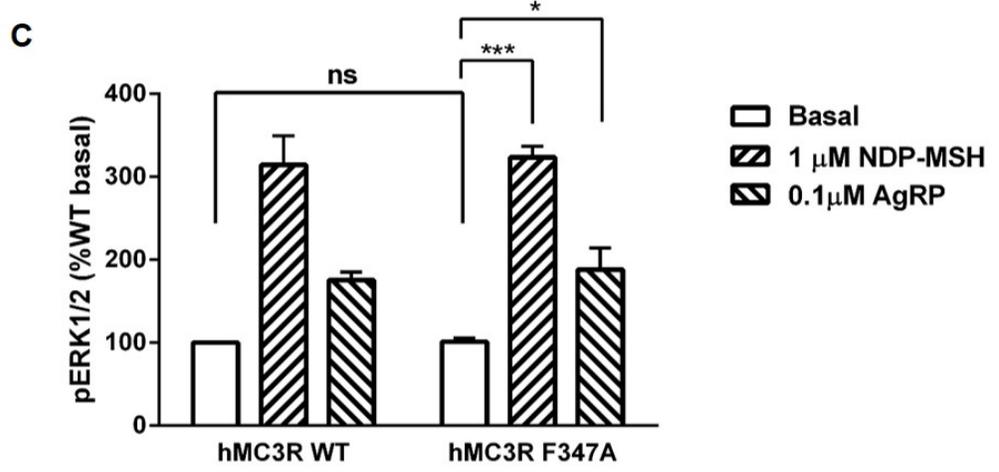
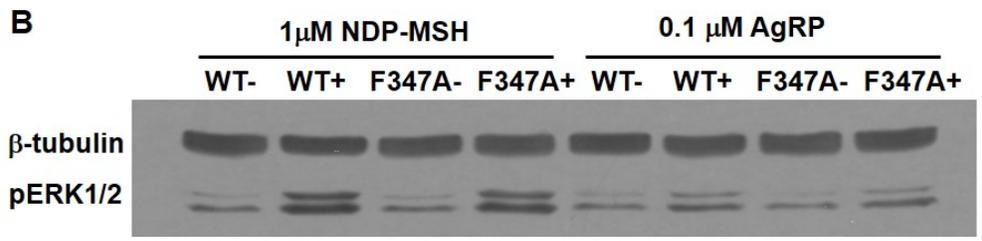
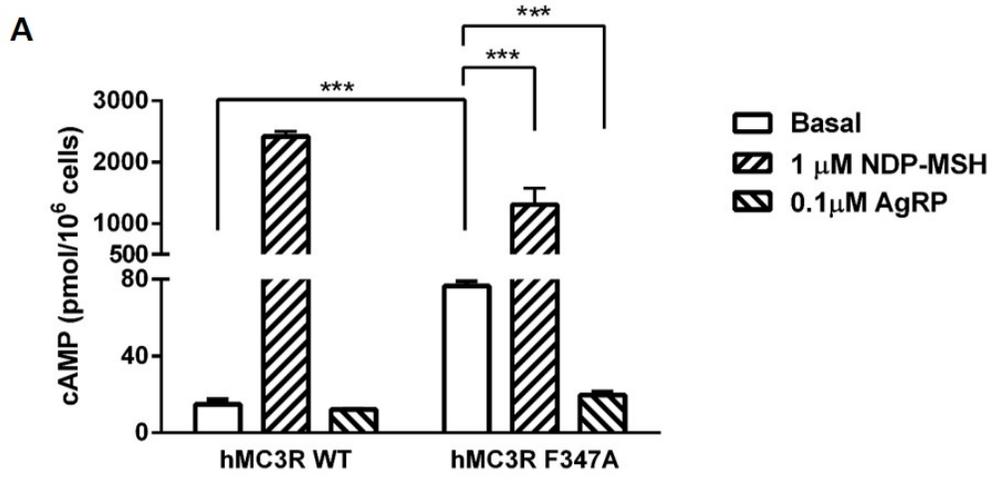


Figure 3.3 Effects of different specific inhibitors on AgRP-initiated ERK1/2 activation through MC3R.

Inhibitors pretreatment and ERK1/2 phosphorylation measurement were performed as described in Materials and methods. (A) Representative image of AgRP-stimulated ERK1/2 phosphorylation in hMC3R-transfected HEK293T cells pretreated without or with different inhibitors. (B) Basal pERK1/2 levels of hMC3R-transfected HEK293T cells and results are expressed as percentage of basal pERK1/2 level of hMC3R without inhibitor treatment (control). * indicates significant difference from the basal pERK1/2 level of control (** $P < 0.01$; *** $P < 0.001$). (C) Densitometry analysis of AgRP-stimulated ERK1/2 phosphorylation of hMC3R-transfected HEK293T cells pretreated without or with different inhibitors. Results are expressed as the percentage of each basal pERK1/2 levels and shown as mean \pm SEM from at least three independent experiments. * indicates significant difference from corresponding basal pERK1/2 level or from the stimulated pERK1/2 level of control (* $P < 0.05$; ** $P < 0.01$) and ns means no statistical difference ($P > 0.05$).

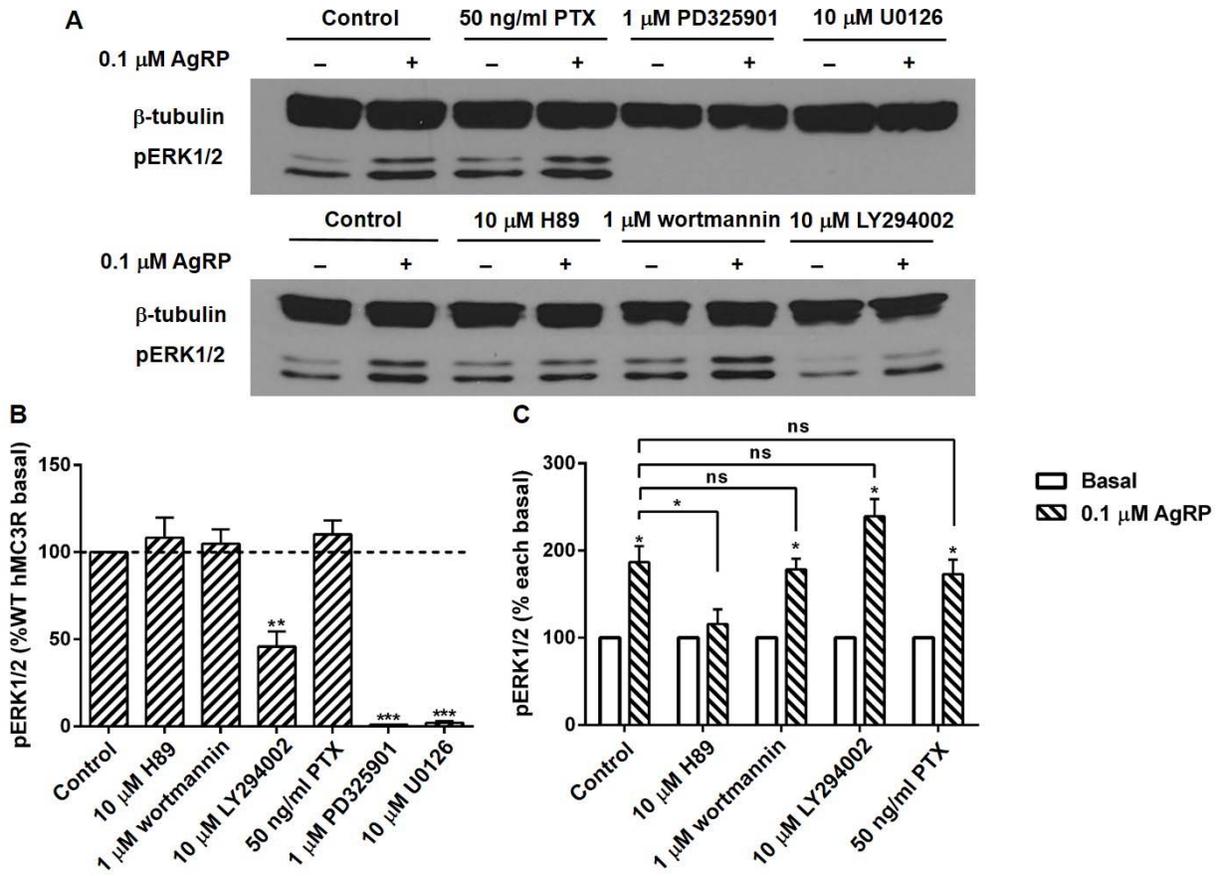


Figure 3.4 Effects of different specific inhibitors on AgRP-initiated ERK1/2 activation through MC4R.

Inhibitors pretreatment and ERK1/2 phosphorylation measurement were performed as described in Materials and methods. See the legend to Figure 3.3 for detail.

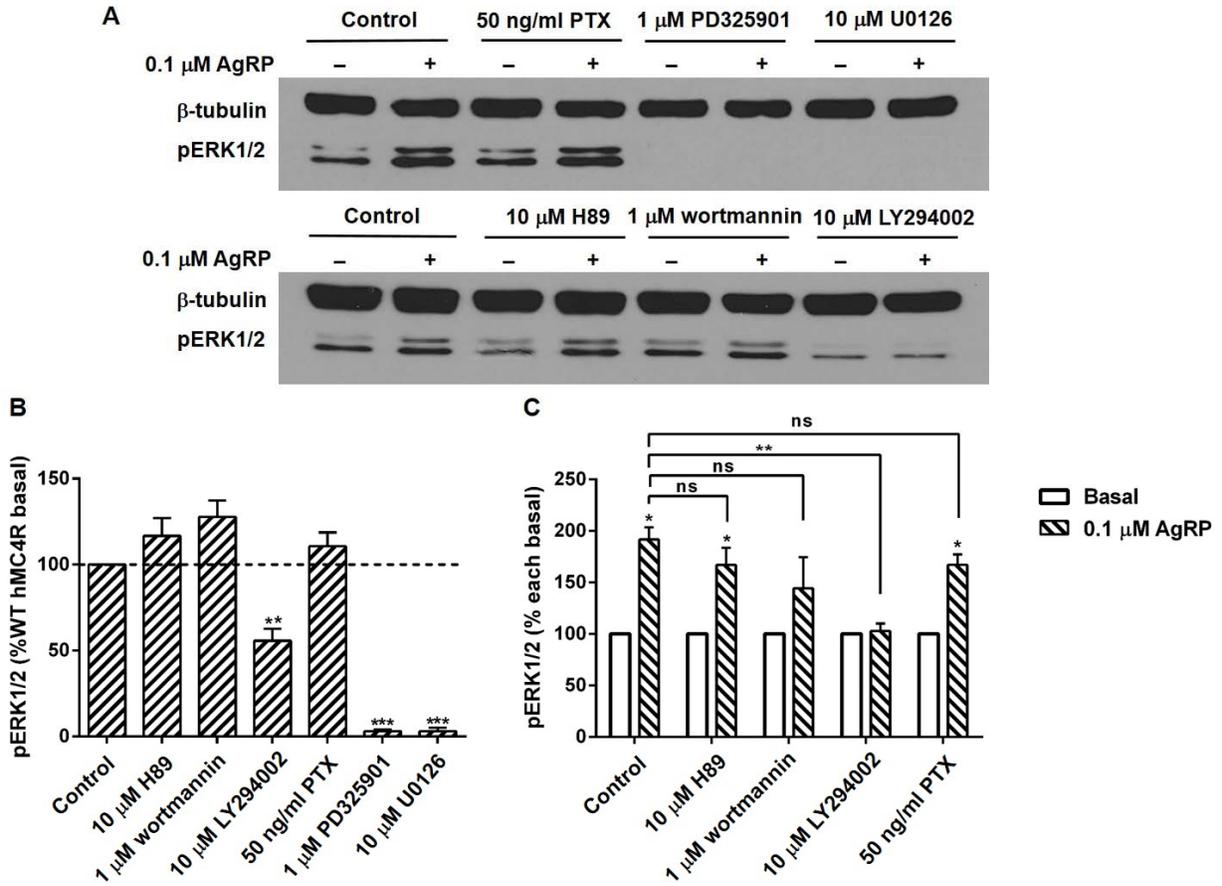


Figure 3.5 Representative images of NDP-MSH-stimulated (1) and AgRP-stimulated (2) AKT and AMPK phosphorylation in HEK293T cells transiently transfected with hMC3R (A) or hMC4R (B), and in GT1-7 cells (C).

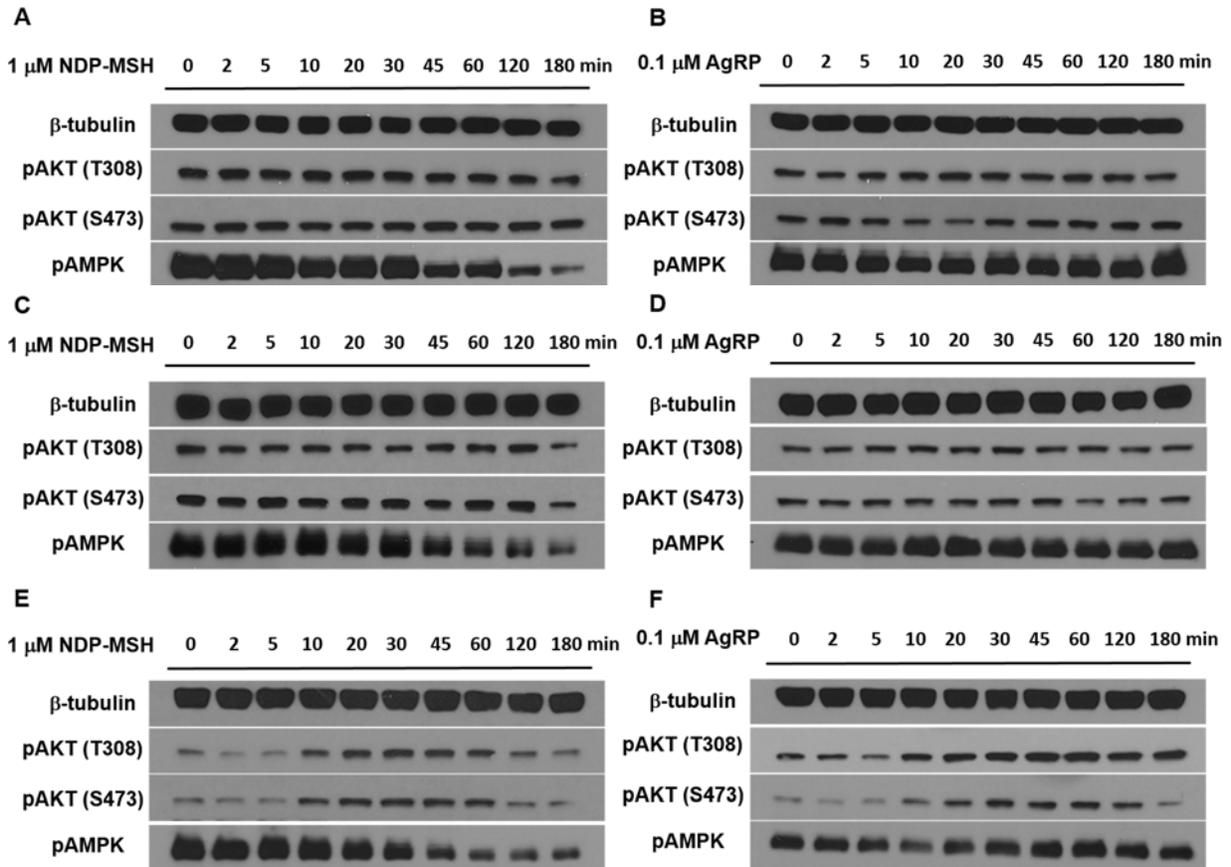


Figure 3.6 Ligand-stimulated AKT phosphorylation through neural MCRs.

AKT phosphorylation was measured as described in Materials and methods. Time-course of NDP-MSH-induced AKT(T308) (A) and AKT(S473) (B) phosphorylation and AgRP-induced AKT(T308) (C) and AKT(S473) (D) phosphorylation in HEK293T cells transfected with hMC3R or hMC4R, and in GT1-7 cells. Results are expressed as the percentage of each basal pAKT level in non-stimulated HEK293T cells or GT1-7 cells. Shown are mean \pm SEM of at least three independent experiments. * indicates significantly different from basal pAKT level in GT1-7 cells (* $P < 0.05$).

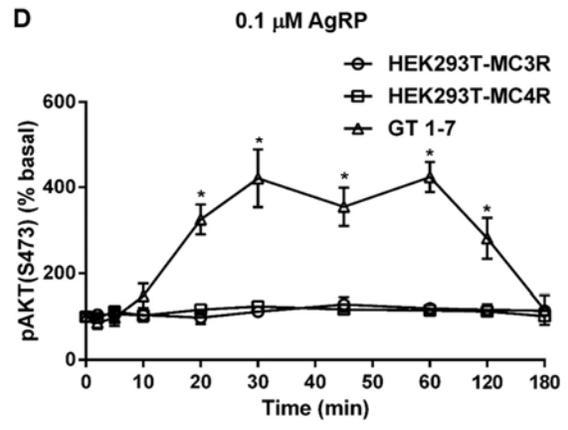
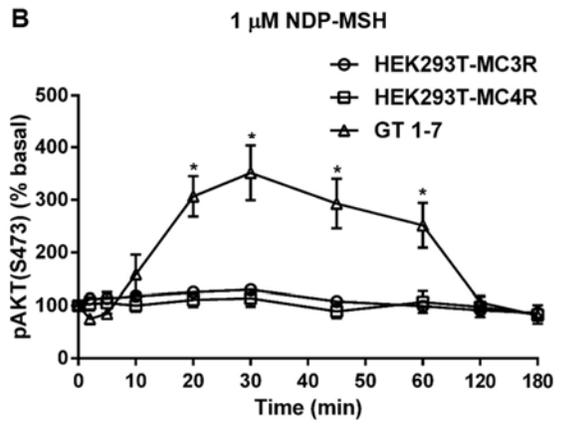
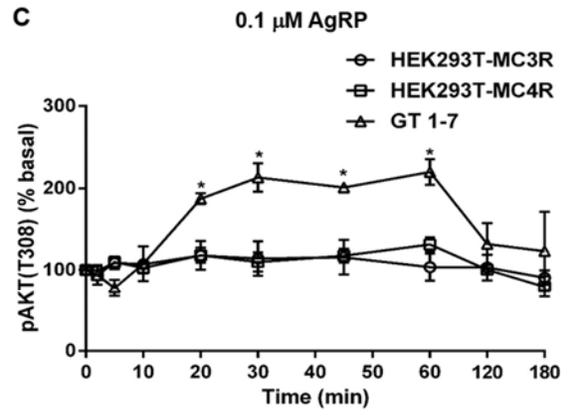
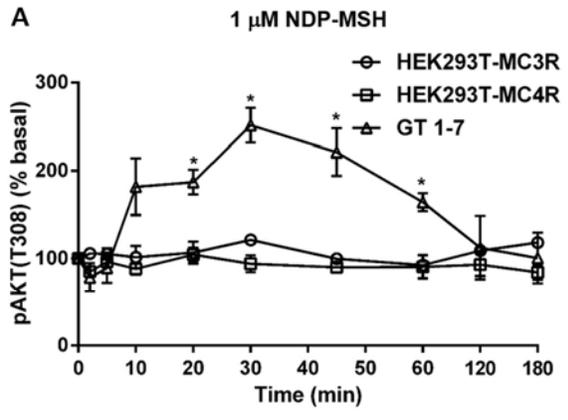
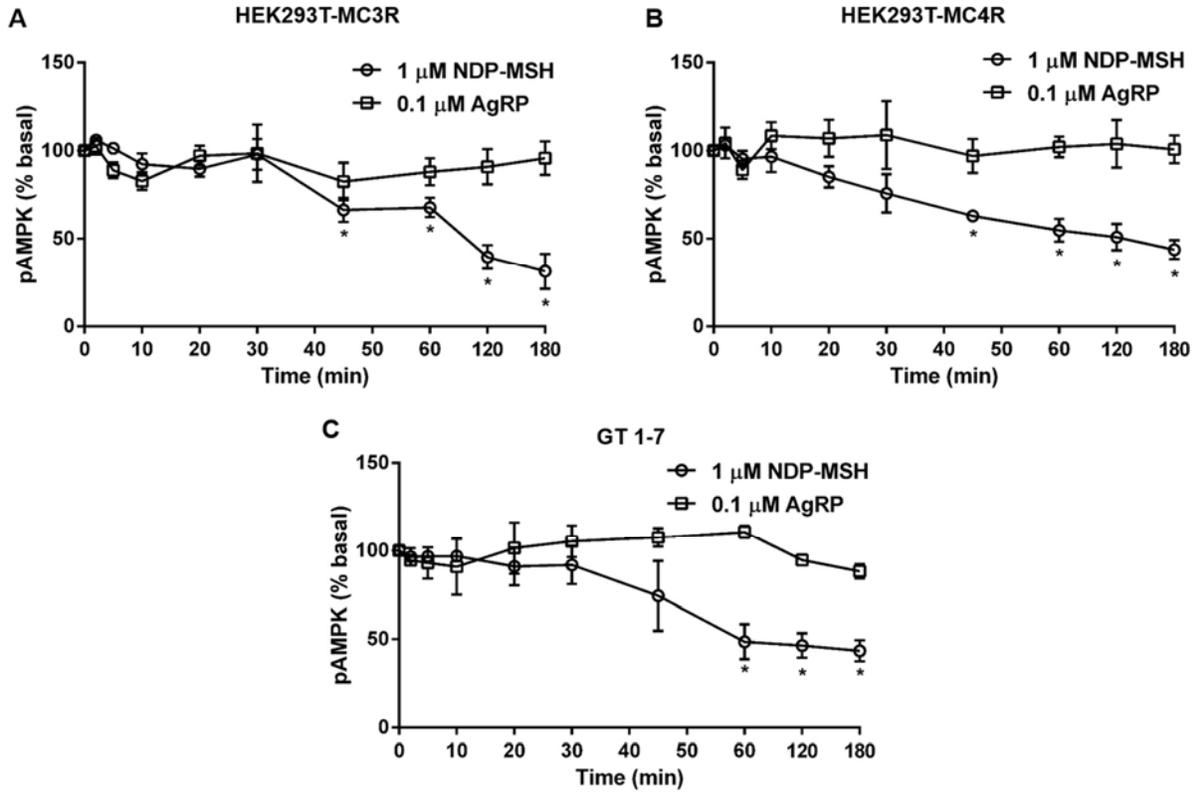


Figure 3.7 Ligand-stimulated AMPK phosphorylation in HEK293T cells transfected with MC3R (A) or MC4R (B), and in GT1-7 cells (C).

AMPK phosphorylation was measured as described in Materials and methods. Results are expressed as the percentage of the basal pAMPK level obtained in non-stimulated HEK293T cells or GT1-7 cells. Shown are mean \pm SEM of at least three independent experiments. * indicates significantly different from corresponding basal pAMPK levels (* $P < 0.05$).



Conclusions and Future Prospective

Obesity is a multifactorial epidemic with enormous health, economic and social burdens. Therapeutic options for obesity available now are far from ideal and novel avenues are urgently sought. The MC3R is increasingly recognized as an important regulator of energy homeostasis. Although a number of significant studies have been published, the pathogenic role of MC3R in human obesity pathogenesis remains controversial.

Our systematic study of the twenty residues in the DPLIY motif and helix 8 of MC3R demonstrated that eleven residues were important for ligand binding and one was critical for downstream cAMP generation. We also identified one mutant F347A to be constitutively active in cAMP signaling. Moreover, six binding- or signaling-defective mutants displayed unbalanced cAMP and pERK1/2 signaling pathways, suggesting the existence of biased signaling in MC3R mutants. Our data highlighted the functional importance of this domain in the activation and signal transduction and contributed to a better understanding of the structure-function relationship of MC3R.

In addition to biased mutants, biased ligands of many GPCRs have also been established and extensively studied, representing a frontier in GPCR pharmacology and drug discovery. Our detailed study of AgRP in Chapter 3 demonstrated that AgRP acted as a biased agonist in MC3R, decreasing cAMP activity but stimulating ERK1/2 activation.

We further found that AgRP initiated ERK1/2 phosphorylation through MC3R and MC4R by different mechanisms and we also observed ligand-stimulated AKT and/or AMPK activation through neural MCRs. These novel findings added a new layer of complexity to the pharmacology of neural MCRs, especially MC3R. Our data would provide new insights into the role of AgRP in neural melanocortin system.

Future *in vitro* studies using more cell lines together with *in vivo* studies will enrich our understanding of neural MCR-mediated multiple intracellular signaling pathways and the related physiological effects. Recently identified ligands selectively targeting MC3R will be helpful to study the molecular mechanism of the MC3R in regulating energy homeostasis.

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