Hypothalamic Melanocortin Regulation of Energy Balance and Metabolism

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ABSTRACT

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Genetic and environmental factors both contribute to obesity, however studies in twins and adoptees demonstrate that genetic predisposition and susceptibility are driving factors in the development of this disease. Although numerous human mutations are associated with an increase in obesity prevalence, melanocortin-4 receptor (MC4-R) mutations are the most common monogenic form of severe obesity and genetic deletion of this receptor in rodents also leads to an obese phenotype. The G-protein coupled MC4-R is a target for the peptide products of Proopiomelanocortin (POMC) and Agouti-related peptide (AgRP) neurons residing in the arcuate nucleus of the hypothalamus. POMC-derived α-melanocyte-stimulating hormone (MSH) is an agonist for the MC4-R and promotes negative energy balance, while the melanocortin-receptor antagonist AgRP promotes positive energy balance. Given the strong influence of the hypothalamic melanocortin system on energy balance, this thesis sought to investigate unexplored aspects of this system in relation to obesity.

POMC is post-translationally processed to biologically active peptides with opposing actions. α-MSH is well established to decrease food intake and increase energy expenditure, however POMC-derived β-endorphin (β-EP) has been shown in limited studies to increase food intake. Our experiments in intracerebroventricular (icv) cannulated rats demonstrate that the effects of β-EP alone on feeding are complex. β-EP acutely stimulated food intake during both the light and dark cycle, however orexigenic effects were not sustained in a chronic model; in fact, higher doses of chronic β-EP decreased food intake. Subthreshold doses of β-EP also reversed α-MSH-

induced suppression in feeding and weight gain in an acute fasting and refeeding model as well as a chronic infusion model. β-EP 1-27, a product of C-terminal β-EP cleavage reported to have reduced opioid activity, did not stimulate food intake alone, nor could it reverse α-MSH-induced suppression in feeding. These studies show that POMC-derived peptides α-MSH and β-EP can interact to regulate food intake and body weight and highlight the importance of understanding how the balance between these peptides is maintained, as well as the potential role of differential POMC processing in regulating energy balance.

AgRP is also a critical component of the melanocortin system; however, studies in which the AgRP peptide was deleted show only a mild phenotype suggesting that developmental compensation exists in this model. Consequently the role of the AgRP peptide was investigated using a novel AgRP inhibitor developed by TransTech Pharma, Inc. Results show that this inhibitor was extremely effective in reversing exogenous icv AgRP-induced metabolic and neuroendocrine parameters in rats, and these parameters were unaffected in saline infused rats receiving this drug. This AgRP inhibitor also reduced food intake, weight gain and adiposity in diet-induced obese (DIO) and *ob/ob* mice and increased thyroxine (T4) levels in DIO mice, consistent with AgRP's reported effects; however this drug did not affect food intake or weight gain in lean chow fed mice. The AgRP inhibitor also suppressed rebound feeding and potently reduced food intake in mice immediately upon initiation of a high fat diet (HFD). As some of these effects were also observed in AgRP knockout (KO) mice, this indicates that there are clear off-target effects that are not due to AgRP antagonism. Although there are many potential reasons why a drug may yield anorexia and weight loss, the fact that these effects were only observed in obese models or in the presence of increased dietary fat, suggests the possibility that another molecule that promotes positive energy balance and fat intake is also being targeted.

As the melanocortin system can also regulate pituitary function, this thesis investigated circulating and pituitary prolactin levels in models with genetic manipulation of this system. Previous studies have shown that acute stimulation of the melanocortin system by α-MSH or antagonism by AgRP decreases and increases prolactin levels, respectively. However, the effects of chronic melanocortin manipulation were unknown. Male mice with selective MSH overexpression and AgRP deletion were found to have decreased blood prolactin levels under both stressed and unstressed conditions, and mice with AgRP deletion also had lower prolactin content in the pituitary. As prolactin is tonically inhibited by dopamine and acute melanocortin regulation of prolactin release has been shown to be dopamine-mediated, we sought to show this mechanism in these genetic models. Measurements of mediobasal hypothalamic (MBH) *tyrosine hydroxylase* mRNA levels as well as dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) content did not differ between groups, nor did prolactin measurements after functional dopamine receptor antagonism. However, these results do not preclude changes in dopamine activity as dopamine turnover was not directly investigated. This is the first report of reduced baseline and stress-induced prolactin release and pituitary prolactin content in mice with genetic alterations of the melanocortin system and suggests that changes in hypothalamic melanocortin activity may be reflected in measurements of serum prolactin levels.

The melanocortin system has been shown to mediate some of the adaptive responses after introduction to the HFD including attenuation of hyperphagia and weight gain; thus, preliminary studies sought to investigate the effects of the HFD on hypothalamic melanocortin gene and peptide expression, as well as expression of enzymes responsible for POMC post-translational processing. Mice administered a 60% HFD were found to have increased levels of POMC precursor peptide after both acute (3 and 7 days) and chronic (8 week) HFD administration.

After 3 days of HFD feeding, increased POMC peptide levels were accompanied by increased *Pomc* mRNA and α-MSH and β-EP, however after chronic HFD feeding POMC peptide levels were elevated without an increase in these parameters. Additionally, chronic HFD feeding suppressed AgRP mRNA and peptide levels and tended to increase the α-MSH/β-EP ratio and suppress mRNA levels of prolylcarboxypeptidase (*Prcp)*, an enzyme responsible for inactivating α-MSH. These data suggest that the HFD can modulate melanocortin gene and peptide expression with distinct time-related changes and are consistent with the hypothesis that POMC-derived peptides act in concert to regulate energy balance and metabolism. However, these results are preliminary and further investigation of the effects of HFD feeding on peptide and processing enzyme expression in more anatomically discrete hypothalamic nuclei as well as dynamic studies of peptide release are required.

These data provide a contribution to the field of melanocortin regulation of energy balance by further elucidating the effects of POMC processed peptides both alone and in interaction, by providing insight into the effects of a novel AgRP inhibitor on energy balance and metabolism, by investigating the effects of genetic models of chronic melanocortin manipulation on the pituitary hormone prolactin, as well as by examining the effects of HFD feeding on melanocortin gene and peptide expression.

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ABBREVIATIONS

3V	Third Ventricle
4V	Fourth Ventricle
ACTH	Adrenocorticotropic Hormone
AgRP	Agouti Related Peptide
AH	Anterior Hypothalamus
ATGL	Adipose Triglyceride Lipase
A^y	Agouti Yellow
BAT	Brown Adipose Tissue
BDNF	Brain-Derived Neurotrophic Factor
β-EP	β-Endorphin
β-LPH	β-Lipotropin
cAMP	Cyclic Adenosine Monophosphate
BMI	Body Mass Index
CB	Cannabinoid
CDC	Center for Disease Control

CLIP	Corticotropin-Like Intermediate Peptide
CMV	Cytomegalovirus
CNS	Central Nervous System
CPE	Carboxypeptidase E
CRE	Cyclic Adenosine Monophosphate Response Element
CREB	Cyclic Adenosine Monophosphate Response Element-Binding
CRH	Corticotropin-Releasing Hormone
D2R	Dopamine Type-2 Receptor
DIO	Diet-Induced Obese
DMH	Dorsomedial Hypothalamus
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic Acid
DR	Diet Resistant
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EE	Energy Expenditure

ELISA	Enzyme-Linked Immunosorbent Assay
FFA	Free Fatty Acids
FOXO1	Forkhead Box O1
GABA	γ-Aminobutyric Acid
GHRH	Growth Hormone Releasing Hormone
GLP	Glucagon-Like Peptide
GLUT4	Glucose Transporter Type 4
GTT	Glucose Tolerance Test
h	Hours
HFD	High Fat Diet
HPLC	High Performance Liquid Chromatography
HPLC-EC	High Performance Liquid Chromatography/Electrochemical Detection
HSL	Hormone Sensitive Lipase
Icv	Intracerebroventricular
IR	Insulin Receptor
IRS	Insulin Receptor Substrate

KO	Knockout
LH/A	Lateral Hypothalamic/Area
MC	Melanocortin Receptor
MCH	Melanin-Concentrating Hormone
MOR	μ-Opioid Receptor
MSH	Melanocyte-Stimulating Hormone
MTII	Melanotan II
NPY	Neuropeptide Y
NTS	Nucleus of the Solitary Tract
PAM	Peptidylglycine Amidating Monooxygenase
PBN	Parabrachial Nucleus
PC/Pcsk	Proconvertase
PCA	Perchloric Acid
POMC	Proopiomelanocortin
PRCP	Prolylcarboxypeptidase
PVN	Paraventricular Nucleus

qRTPCR	Quantitative Real Time Polymerase Chain Reaction
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RNAi	RNA Interference
RQ	Respiratory Quotient
Sim1	Single-Minded Homolog 1
SNS	Sympathetic Nervous System
STAT3	Signal Transducer and Activator of Transcription 3
TG/TAG	Triacylglycerol/Triacylglyceride
Tg	Transgenic
TIDA	Tuberoinfundibular Dopaminergic
T3	Triiodothyronine
T4	Thyroxine
TRH	Thyrotropin Releasing Hormone
TSH	Thyrotropin Stimulating Hormone
UCP-1	Uncoupling Protein 1

VCO2	Carbon Dioxide Production
VMH	Ventromedial Hypothalamus
VO2	Oxygen Consumption
WAT	White Adipose Tissue

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CHAPTER 1: LITERATURE REVIEW

OBESITY: RATIONALE AND SIGNIFICANCE

A recent report from the Center for Disease Control (CDC) estimates that from 2009-2010, about 36% of adults and nearly 17% of youth qualified as obese, with obesity in adults defined as a Body Mass Index (BMI) of 30 kg/m^2 or greater, in children greater than 2 years of age as greater than the 95^{th} percentile for BMI-for-age, and in children less than 2 years as greater than the 95^{th} percentile on growth charts (weight-for-recumbent length) (1, 2). This disease is associated with a number of co-morbidities, including heart disease, hypertension, type 2 diabetes, sleep disorders, cancer, osteoarthritis and others (6), and some of these are leading causes of death in United States (7). It is well-recognized that the etiology of this epidemic is complex and stems from the interaction of countless genetic and environmental factors which are delineated in **Figure 1.1**. The heterogeneity of response to the obesogenic environment suggests that some individuals are genetically pre-disposed for increased susceptibility.

Isolating the relative contributions of genetics versus environment to obesity is challenging as a reciprocal relationship clearly exists. However, studies in monozygotic twins and adoptees provide unique models which allow researchers to separate the genetic and environmental contributions to obesity. These studies consistently show that genetics strongly influences BMI (8-10), with BMI heritability estimated between 50-70% (8, 9). Studies in monozygotic twins reared apart show that these twins have similar BMI and body composition measurements, similar to what is observed in twins reared together (8-10). Additionally, overfeeding in male twins showed that body weight, fat mass and body fat distribution variability is 3-6 times greater

between twin pairs than among twin pairs (11). It has also been shown that the BMI of adoptees strongly reflects the BMI of their biological, rather than adoptive, parents (12-15). For example, the correlation of adoptees with biological mothers, fathers and full siblings was 0.17, 0.16 and 0.59, respectively, while the correlations of adoptees with adoptive mothers, fathers and siblings was 0.10, 0.03 and 0.14 respectively (14).

Numerous studies in both humans and animal models have sought to uncover the genetic components that contribute to obesity. The contribution of the hypothalamus to obesity has been observed over many decades, and seminal studies noted that hypothalamic tumors and lesions can lead to obesity (16, 17). However more recent data have revealed a specific contribution of the hypothalamic melanocortin system to the regulation of energy balance and metabolism. In fact, mutations in the melanocortin 4 receptor (MC4-R) are considered the most common monogenic form of severe obesity (18). This thesis focuses on further uncovering how the melanocortin system contributes to energy balance and metabolism.

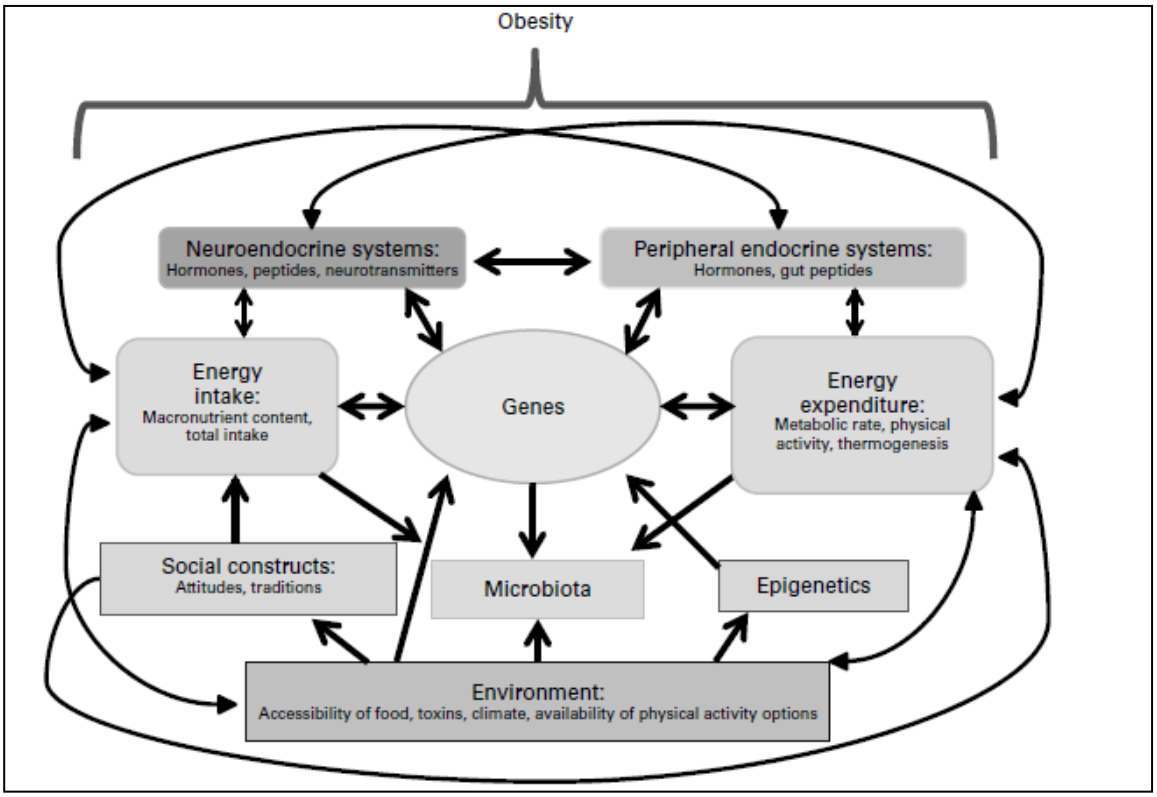

Figure 1.1. Genetic and Environmental Factors Contributing to Obesity. (4)

INTRODUCTION TO THE HYPOTHALAMIC MELANOCORTIN SYSTEM

The hypothalamic melanocortin system, which consists of both the anorexigenic Proopiomelanocortin (POMC) neurons and orexigenic Agouti-related protein/Neuropeptide Y (AgRP/NPY) neurons, plays a central role in the regulation of energy balance. Both sets of neurons are primarily located in the arcuate nucleus which allows them to receive signals from the periphery (19). These neurons also project to a number of different areas in the brain where their peptide products interact at MC3-R and MC4-Rs on downstream neurons (3). Activation of the MC3-R and MC4-Rs by POMC-derived α-melanocyte-stimulating hormone (α-MSH) and antagonism of these receptors by AgRP functions to promote negative and positive energy balance, respectively. In addition MC3-R and MC4-Rs modulate the thyroid and adrenal axes as well as regulate prolactin secretion (3). Genetic manipulation of this system including deletion of either POMC, the MC3-R, or MC4-R or overexpression of AgRP produces varying degrees of obesity, while MSH overexpression yields a lean phenotype (3). These neurons are subject to modulation by various hormones and nutrients including insulin and leptin, which have been shown to stimulate Pomc and inhibit Agrp expression (3, 19) (**Figure 1.2**).

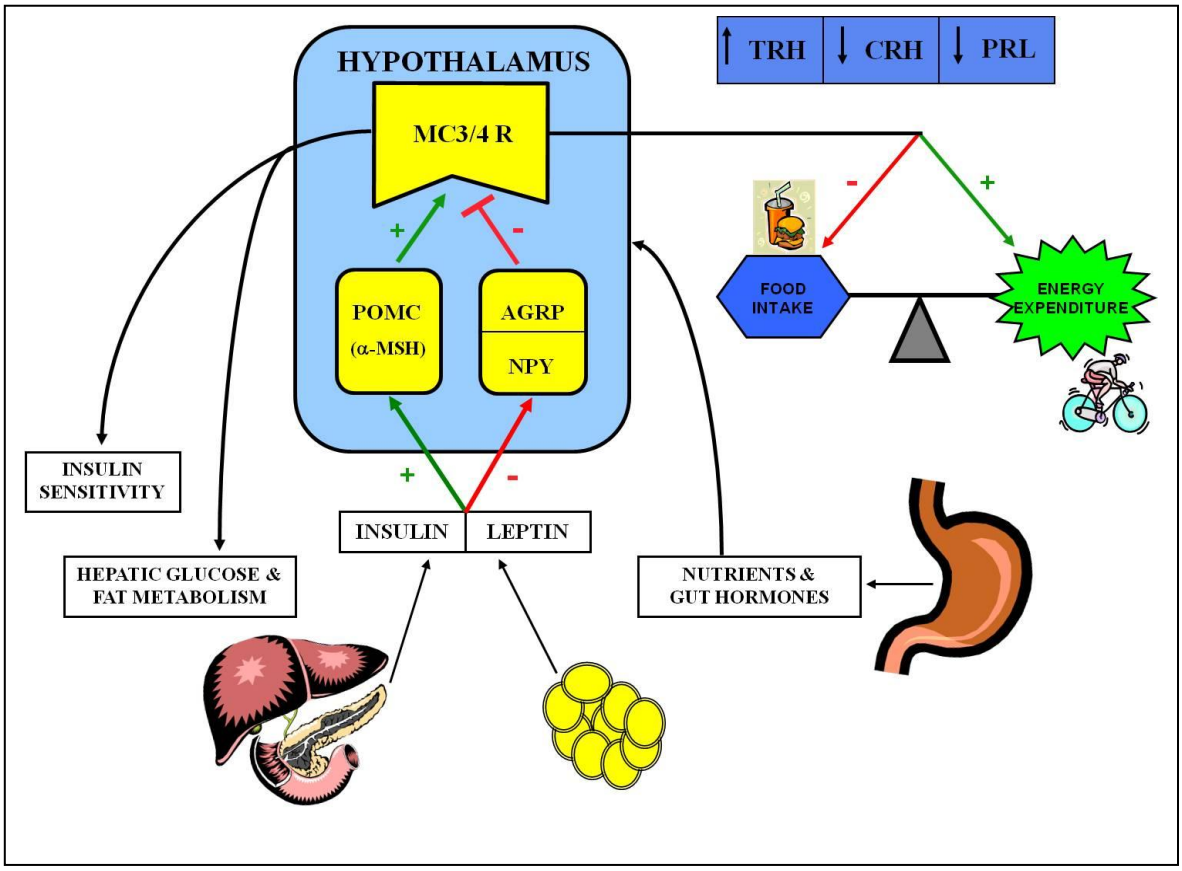

Figure 1.2. Hypothalamic Melanocortin Regulation of Energy Balance and Metabolism (3)

POMC AND POMC-DERIVED PEPTIDES IN ENERGY BALANCE AND METABOLISM

POMC is expressed both centrally and peripherally. In the central nervous system (CNS), POMC is primarily synthesized in the arcuate and nucleus of the solitary tract (NTS), and in the periphery is synthesized in the pituitary, skin and hair follicle (19). The POMC precursor peptide is posttranslationally processed to yield a number of different peptides, many of which are relevant to energy balance and metabolism (**Figure 1.3**).

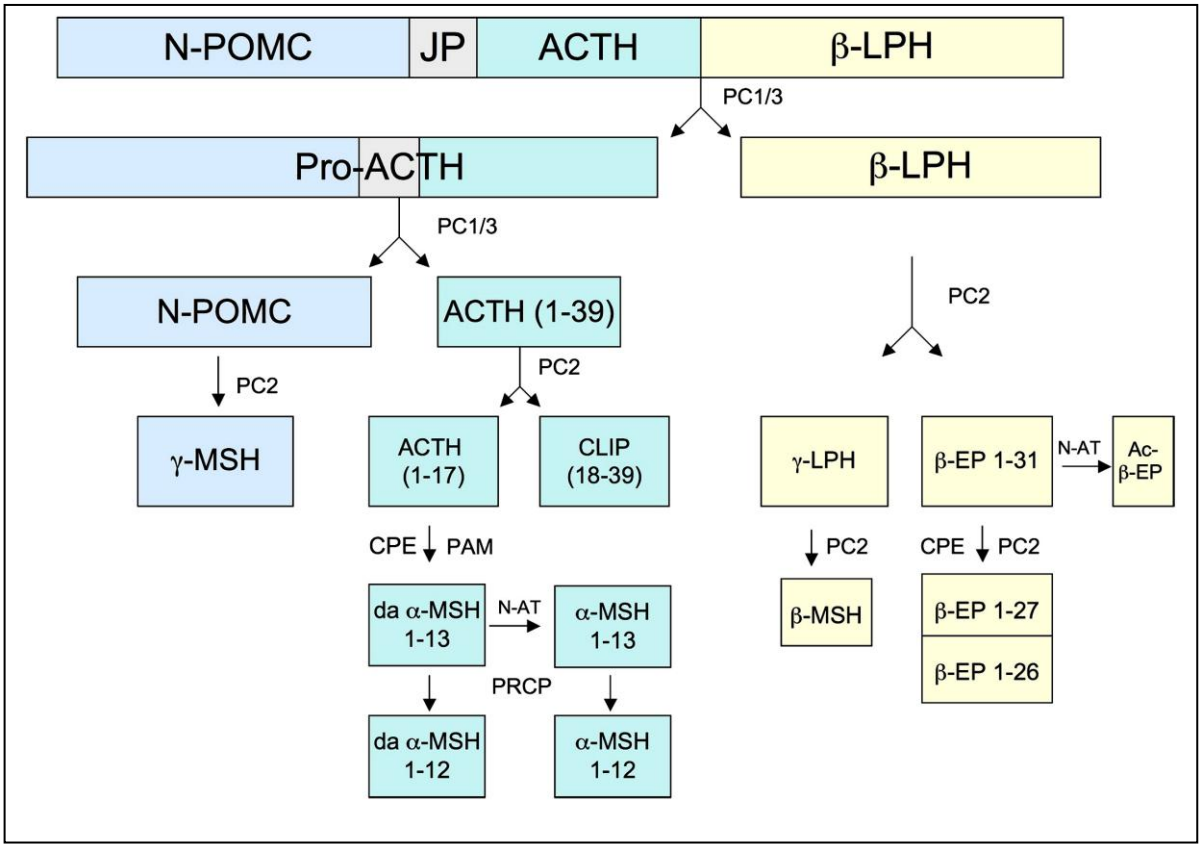

Figure 1.3. POMC Processing in the Hypothalamus (5)

GENETIC MODELS OF POMC OVEREXPRESSION AND DELETION

Pomc overexpression in wild-type (WT) mice has minimal effects on food intake and body weight gain, although it does attenuate fasting-induced hyperphagia (20). However, Pomc overexpression in *ob/ob* mice partially reverses the obese phenotype; these mice were characterized by reduced body weight, adiposity, hyperphagia, liver weight, and serum free fatty acids (FFA) and increased oxygen consumption (VO2) and body temperature (20). Impairments in glucose metabolism were also normalized independent of changes in energy balance (20). Similarly, POMC overexpression in the hypothalamus of aged obese Sprague-Dawley rats and high fat diet (HFD)-fed rats as well as Pomc overexpression in the arcuate of Zucker rats reduces food intake and adiposity (21-23).

Numerous studies have characterized the phenotype associated with genetic deletion of POMC. The POMC -/- null mouse is hyperphagic and obese, with increased adiposity and lean mass, hyperleptinemia and reduced VO2 (corrected for total body mass) (24, 25). Phenotypically these mice also have a lighter coat color, as the MC1-R in the skin controls pigmentation (24). Exposure to the HFD augmented the obese phenotype in Pomc null mice, and although Pomc +/- heterozygotes were not obese on normal chow they became obese on a HFD (25). POMC null and heterozygous mice have also been shown to have increased preference for fat intake and an impaired adaptive (both thermogenic and activity-related) response after HFD exposure (26). Thyroid function in POMC null mice is controversial—lower total thyroxine (T4) levels have been reported (25), however, another study showed increased plasma total T4 and triiodothyronine (T3) levels as well as reduced plasma thyroid-stimulating hormone (TSH), pituitary TSH

content, and hypothalamic thyroid-releasing hormone (TRH) content (27). Although glucose homeostasis was originally reported as relatively normal in these knockout (KO) mice, more recent reports have shown that glucose homeostasis is abnormal (28-30). These mice also have an enhanced response to an insulin challenge and become hypoglycemic; this is likely due to the low circulating corticosterone concentration and this hypoglycemic effect can be mitigated with α-MSH or corticosterone supplementation (28).

The adrenal gland is poorly developed in the Pomc null mouse and these animals have undetectable corticosterone levels despite their obese phenotype (24, 31). Corticosterone supplementation in POMC null mice also exacerbates the obese phenotype and increases plasma insulin levels 50-fold (corticosterone also increases insulin levels 14-fold in WT mice) (29, 30). Similarly, selective restoration of POMC in the pituitary and periphery of POMC null mice exacerbates the obesity phenotype independent of hyperphagia and yields severe insulin resistance (30). Pomc null mice also have low hypothalamic *Agrp* levels which are increased upon corticosterone supplementation or restoration of POMC in the periphery and pituitary (29, 32). It is possible that exacerbation of the obese phenotype in Pomc null mice after corticosterone supplementation or POMC rescue in the pituitary or periphery is due to increased AgRP levels (29, 32).

POMC-DERIVED PEPTIDES: α-MSH

Central/peripheral administration: α-MSH is well established to favor negative energy balance in rodents. Central administration of α-MSH or melanotan II (MTII), an MSH agonist, decreases food intake, body weight, and adiposity, increases VO2 and activity, and suppresses respiratory

quotient (RQ) (33-37). MTII is particularly effective in promoting negative energy balance in models in which AgRP levels are elevated such as during a fast and in leptin and leptin-receptor deficient rodents such as *ob/ob* mice and obese Zucker rats (38-40). MTII is also effective in agouti yellow (A^y) mice, an obese mouse strain with ectopic Agouti overexpression, and in POMC null mice (32, 40). MTII offers an additional advantage as it can be administered peripherally, and this route of administration yields effects similar to central administration. The effects of MTII have been shown in numerous rodent models including lean BL6 and *ob/ob* mice as well as lean and diet-induced obese (DIO) rats under both acute and chronic settings (41, 42). Furthermore, some of the effects of MTII on weight gain, adiposity, energy expenditure (EE) and reductions in insulin and leptin levels have been shown to be independent of food intake (43, 44). MTII can also modulate peripheral lipid metabolism. Centrally administered MTII has been shown to stimulate enzymes involved in lipid catabolism in white adipose tissue (WAT), including adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) (44). Moreover, these effects were independent of food intake (44). MTII also increases sympathetic nervous system (SNS) activity to WAT in rats (44). Similarly in Siberian hamsters MTII stimulates WAT lipolysis through SNS activation and increases body temperature through regulation of intrascapular brown adipose tissue (BAT) activity (45, 46).

α-MSH and MTII have also been shown to affect glucose metabolism. Obici et al showed that icv α-MSH increases the rate of glucose uptake and enhances insulin's ability to inhibit hepatic glucose production (35). This is further supported by an increase in glucose transporter type 4 (Glut4) mRNA observed in skeletal muscle of MTII-treated mice (47). MTII was also shown to improve glucose tolerance and decrease basal insulin levels in lean BL6 mice (48), as well as

increase insulin sensitivity in DIO obese rats (49). MTII has also been shown to decrease hepatic lipid content in DIO obese rats (49).

Genetic studies: The effects observed with exogenous α-MSH or MSH agonists are further supported by genetic studies in mice with overexpression of α- and γ_3-MSH (in which these MSH peptides are overexpressed in tissues with the ability to cleave the N-terminal POMC transgene). Transgenic overexpression of MSH improved the obese phenotype of leptin receptor deficient mice and improved glucose tolerance in obese A^y mice (50). Overexpression of MSH in DIO mice also improved the metabolic phenotype--transgenic mice had reduced body weight and adiposity as well as increased VO2, decreased RQ scores, and attenuated hepatic fat accumulation (51). Both lean and DIO mice overexpressing MSH had improved glucose tolerance in a glucose tolerance test (GTT) and reduced fasting insulin levels versus WT mice (51). This is noteworthy as this was observed in lean mice with similar adiposity and body weight (51).

<u>POMC-DERIVED PEPTIDES: γ2/γ3-MSH & β-MSH</u>

γ2/γ3-MSH: *In vitro* studies have shown that γ2-MSH has a greater binding affinity for the MC3-R versus the MC4-R (52). *In vivo* studies have not always consistently demonstrated γ2-MSH's effects on feeding (52-54), however the peripheral MC3-R agonist [D-Trp(8)]-γ-MSH stimulates feeding (55). Lee et al showed that this effect is centrally mediated as central infusion of [D-Trp(8)]-γ-MSH transiently stimulated food intake while central infusion of a low dose of an MC3-R antagonist decreased food intake and body weight gain (56). Consistent with MC3-

R's reported autoinhibitory function on POMC *in vitro*, [D-Trp(8)]-γ-MSH suppressed hypothalamic *Pomc* mRNA expression (56, 57).

β-MSH: Although β-MSH is not endogenously present in rodents as they do not possess the dibasic cleavage site required for β-MSH production (58), this peptide is shown to bind to the MC4-R with equal molar affinity as α-MSH (52). Icv β-MSH has also been shown to decrease food intake in rodents (52, 53), although injection after an extended fast reported no effect on feeding (54).

POMC-DERIVED PEPTIDES: β-ENDORPHIN

β-Endorphin (β-EP) 1-31 is a μ-opioid receptor (MOR) agonist that stimulates food intake after acute injection into the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH) and lateral ventricle (59-62), however the effects of chronic β-EP administration are unknown. Contrary to the orexigenic effects observed with acute β-EP, a mouse with selective deletion of β-EP possesses a hyperphagic phenotype with mild obesity, increased WAT mass and impaired glucose metabolism without any change in EE (63, 64). Furthermore, under non-deprived conditions there is evidence that β-EP controls reward behavior as β-EP KO mice exert less work for food reinforcers than WT mice (63). Additionally, mice deficient in both the MC4-R and β-EP are more obese than mice deficient in either gene alone (64).

β-EP 1-31 is C-terminally cleaved to β-EP 1-27 and β-EP 1-26, peptides reported to have reduced opioid activity (65). The effects of these peptides on feeding is limited to a broiler chick model, in which a high dose of β-EP 1-27 was shown to stimulate food intake alone and block β-EP 1-31 induced increased feeding (66). This is in agreement with studies that have shown that

the stimulatory effects of β-EP 1-31 on feeding can be blocked by opioid receptor antagonists (59-62).

POMC IN THE BRAINSTEM

There is evidence that melanocortin action in the brainstem also regulates energy balance. Injection of either α-MSH or MTII into the fourth ventricle (4V) decreases food intake and body weight and conversely NTS and 4V injection of SHU9119, an MC3/4-R antagonist, increases food intake and decreases core body temperature, respectively (67-69). Additionally, 4V and NTS injection of SHU9119 can block the anorectic effects of arcuate leptin injections (70). Furthermore, overexpression of Pomc in the NTS decreases food intake, body weight, adiposity, serum triglycerides (TG) and increases uncoupling protein 1 (UCP1) expression in BAT and attenuates obesity in a HFD rodent model (71, 72). However the relative contribution of POMC projections to the brainstem versus POMC neurons residing in the brainstem is still under investigation. Evidence from one publication shows that the majority (70%) of α-MSH in the NTS is derived from the hypothalamus (73). Whether NTS Pomc cells are leptin responsive is also controversial, and although one paper has shown that leptin stimulates phosphorylated signal transducer and activator of transcription 3 (STAT-3) in NTS Pomc cells (74), another group did not replicate this finding (75). Furthermore, although fasting suppresses *Pomc* mRNA expression in the NTS, this cannot be reversed by leptin (as it can in the arcuate) (75). In stark contrast to fasting-induced suppression in POMC-derived peptides adrenocorticotropin hormone (ACTH) and desacetyl-α-MSH and proconvertase (PC) 1/3 in the arcuate, which can be reversed

by leptin; in the NTS, fasting increases POMC-derived peptides ACTH and desacetyl-α-MSH and this is unaltered by leptin (76).

ENZYMES RESPONSIBLE FOR POST-TRANSLATIONAL PROCESSING OF POMC

Overview: PC1 initiates the first cleavage step of the POMC precursor peptide to Pro-ACTH and β-lipotropin (β-LPH). Pro-ACTH is further cleaved by PC1 to yield N-terminal POMC and ACTH 1-39. N-terminal POMC, ACTH 1-39 , and β-LPH are all cleaved by PC2 to yield γ-MSH, ACTH 1-17 and corticotropin-like intermediate peptide (CLIP), and γ-LPH and β-EP 1-31, respectively (5). ACTH 1-17 is cleaved by carboxypeptidase E (CPE), amidated by peptidylglycine amidating monooxygenase (PAM), and acetylated by an N-transferase to yield α-MSH, a product which can be degraded by prolylcarboxypeptidase (PRCP) (5). β-EP 1-31 is C-terminally cleaved by PC2 and CPE to β-EP 1-27 and 1-26 (5). γ-LPH is converted to β-MSH by PC2, however this does not occur in rodents as rodent γ-LPH do not possess the essential paired lysine cleavage site required to generate β-MSH (77) (**Figure 1.3**).

PC1 and PC2. PC1 and PC2 are serine proteases that function in secretory granules to cleave precursor peptides, such as POMC, at dibasic amino acid residues (77). The expression of these enzymes is regulated by a number of different factors. For example leptin stimulates PC1 and PC2 mRNA and protein as well as promoter activity *in vitro*. *In vivo*, fasting decreases PC1 and PC2 gene expression in the PVN and median eminence, as well as decreases PC1 protein in these nuclei; the effects on PC1 protein can be restored by leptin (76, 78). No difference in PC2 protein in the PVN or median was observed in these studies (78). Furthermore, dopamine, thyroid hormone and corticosteroids have all been shown to regulate PC1 and PC2 gene

expression in the pituitary, although their direct relevance to POMC processing in the hypothalamus is yet unknown (79). Additionally, the neuroendocrine peptide proSAAS has been shown to inhibit PC1 *in vitro* (80, 81), although *in vivo* adult ProSAAS KO mice have similar PC1/3 and PC2 expression compared to WT mice (82).

PC1 is responsible for cleaving a number of prohormones in addition to POMC, including AgRP and the precursors for growth hormone releasing hormone (GHRH), insulin, glucagon, and TRH (78, 83). PC1/3 KO mice are characterized by reduced growth due to defective pro-GHRH processing, hyperproinsulinemia without abnormal glucose metabolism, and impaired proglucagon processing to GLP-1 and GLP-2 in the intestinal tract (84). Furthermore, although these mice have impaired processing of POMC to ACTH, corticosterone levels are normal (84). In a separate study which disrupted the Pcsk1 locus (which encodes PC1), heterozygote females were more susceptible to DIO (homozygotes were embryonic lethal) (85). Furthermore, mice with a mutation in PC1/3 that renders the enzyme partially inactive are able to process pro-GHRH, but not pro-insulin or POMC, leading to an obese phenotype characterized by hyperphagia, increased adiposity and decreased lean mass, and glucose intolerance (86). These mice also have reduced hypothalamic α-MSH content which may contribute to the obese phenotype (86).

PC2 cleaves a number of prohormones that are cleaved by PC1, including POMC. The PC2 KO mouse has a slightly decreased growth rate and an inability to maintain normoglycemia during fasting as well as appropriately increase glucose during a GTT; these effects have been attributed to impaired prohormone processing of pro-glucagon in the pancreas (87) . PC2 deficiency greatly

impairs post-translational POMC processing; only one-third of β-LPH is converted to γ-LPH and β-EP 1-31, and C-terminal processing of β-EP is non-existent in this model leading to increased β-EP 1-31 (88, 89). Furthermore these mice have increased levels of ACTH and depleted levels of α-MSH (89). Although the increased β-EP 1-31 and decreased α-MSH do not contribute to the phenotype of this mouse in relation to obesity, these mice have increased stress-induced analgesia, potentially due to impaired β-EP 1-31 C-terminal processing (90)

CPE. CPE is an enzyme responsible for cleaving C-terminal basic amino acid residues in a number of pro-peptides including the precursors for insulin, glucagon, TRH, and melanin-concentrating hormone (MCH) as well as POMC (91-93). Cpe KO mice are characterized by hyperphagia, increased adiposity, reduced activity and EE, increased RQ, and impaired glucose metabolism (94) and have been shown to have decreased PC1 expression, increased PC2 expression, increased POMC precursor peptide, decreased α-MSH levels and decreased C-terminal processing of β-EP (95), of which the latter may contribute to their obese phenotype. CPE, located in the secretory granules (96), is also important for cell sorting for a number of peptides including POMC (97). CPE targets POMC to the regulated secretory pathway and in Cpe-deficient mice POMC is sorted to the constitutive pathway (97).

PRCP. This serine carboxypeptidase is widely expressed and cleaves peptides with a penultimate proline residue (98), including the C-terminal valine from α-MSH 1-13, which biologically inactivates the peptide (99). Prcp KO mice have increased α-MSH levels in the hypothalamus and are characterized by a hypophagic lean phenotype with reduced growth and adiposity, increased lean mass, as well as increased EE, UCP1 activity in BAT, and thyroid

function without any change in activity (99, 100). The lean phenotype is evident in both chow and HFD-fed mice (99, 100). Treatment with Prcp inhibitors decreases food intake in a number of different models and the effects of these inhibitors can be blocked by the MC3/4-R antagonist SHU9119 (99). In the hypothalamus, *Prcp* mRNA is most densely expressed in the lateral hypothalamic area (LHA), dorsomedial hypothalamus (DMH), perifornical region, and zona incerta, with more limited expression in the arcuate. It is not known if PRCP acts intracellularly or extracellularly. Extracellularly, PRCP derived from MCH and hypocretin/orexin terminals that project to the PVN may act on arcuate-derived α-MSH that also projects to the PVN (98, 99). Intracellularly in the DMH, PRCP could degrade α-MSH, as POMC is not co-expressed with PRCP in the arcuate (98, 99).

MSH acetylation. α-MSH is acetylated by a yet unknown N-acetyltransferase. Although deacetylated α-MSH is the primary form of α-MSH in rodent brains (101-104), in some studies only the acetylated form is shown to decrease food intake, likely because the acetylated form possesses greater stability while the deacetylated form is subject to degradation (101, 105). Leptin has been shown in one study to increase acetylated α-MSH, however, other reports have not been able to reproduce this effect (76, 101, 106) .

THE MELANOCORTIN ANTAGONIST AGRP

AgRP is expressed primarily in the hypothalamus and adrenal medulla, however lower levels are present in the testis, lung and kidney (107). This peptide is post-translationally modified to its C-terminal $AgRP_{83-132}$ form by PC1/3, which is coexpressed in AgRP neurons. However, evidence from PC1/3 null mice suggests that in its absence other proteases can compensate (108). It has

been shown that in rat homogenate, AgRP is mostly present in the C-terminally cleaved form (109) and *in vitro*, $AgRP_{83-132}$ is a more potent melanocortin inhibitor than full-length AgRP (108). Central AgRP injections have been shown to stimulate increases in food intake, body weight gain, adiposity, leptin and insulin levels (110-112). Icv AgRP decreases EE, VO2, body temperature, plasma TSH and UCP-1 protein in BAT and locomotor activity (111, 113, 114). Some effects on adiposity, leptin, insulin, TSH and UCP-1 activity in BAT are all shown to be independent of food intake (111, 112). The effects of a single third ventricle (3V) AgRP injection on food intake are prolonged—increases have been shown to persist for an entire week. Furthermore AgRP can block MSH-induced suppression in feeding if peptides are injected together, however if MTII is administered 24 h after 3V AgRP, MTII cannot block AgRP's orexigenic effects suggesting that the prolonged orexigenic effects of AgRP are melanocortin-independent (110, 115). Furthermore, AgRP stimulated neuronal activation in brain areas 24 h after injection (116). Icv AgRP was also shown to stimulate neuronal activity in orexin neurons in the LHA 23 h after injection, suggesting a mechanism by which AgRP may mediate prolonged increases in food intake (117).

The effects of the melanocortin receptor agonist SHU9119 are similar to AgRP. Icv SHU9119 has been shown to dose-dependently increase food intake, body weight, fat pad mass, and serum TG levels and TAG content in WAT (37, 44, 118, 119). Pair-feeding studies suggest that the effects of SHU9119 on body weight are primarily due to food intake, however increases in adiposity and triacylglyceride (TAG) content in WAT are somewhat independent of food intake (44). Although SHU9119 was not reported to decrease EE or activity, it did increase RQ during the dark cycle independent of food intake (44). SHU9119 infusion also lead to increased

expression of enzymes involved in lipogenesis in WAT (44, 118) as well as increased liver triglyceride (TG) content, and increased lipogenic gene expression in the liver (118, 119). SHU9119 was also able to block MTII-induced increases in SNS activity and SHU9119-induced increases in lipogenic genes in WAT was absent in triple adrenoceptor-deficient mice without SNS signaling, suggesting that the SNS plays an important role in mediating the melanocortin system's effects on lipid metabolism (44).

Agrp bears 25% sequence homology to *Agouti* (120), which is primarily expressed in the skin of rodents and serves a paracrine function on pigmentation via melanocortin receptor antagonism (107, 121). *In vitro*, AgRP possesses approximately 100-fold greater binding affinity than Agouti at the MC3-R and MC4-Rs (122) . However, overexpression of both AgRP and Agouti are associated with an obese phenotype (123, 124). Specific overexpression of AgRP in the PVN, VMH and LH increases food intake, body weight and adiposity and stimulates leptin and insulin levels (125). AgRP overexpression in the PVN and VMH also decreased core body temperature however this was not evident after overexpression in the LHA, suggesting that AgRP in specific nuclei can contribute to different aspects of energy balance (125).

Genetic deletion of AgRP yields a much milder phenotype than has been reported with other forms of genetic or pharmacological AgRP manipulation. The first study which reported on the phenotype of the AgRP KO mouse found that these animals were nearly identical to their WT counterparts, and furthermore deleting both AgRP and NPY (an orexigenic neuropeptide colocalized in AgRP neurons in the arcuate (126)) did not produce a lean phenotype (127) . A separate study generated an AgRP KO mouse line and found that while younger AgRP KO mice

were nearly identical to their WT counterparts, older mice had a leaner phenotype (128). Mice at 6 months of age had reduced body weight and adiposity, increased VO2, body temperature and activity, decreased RQ during the light cycle and increased total T3 and T4 levels. AgRP KO mice were also more sensitive to the anorectic effects of MTII (128). Similarly, 50% suppression in hypothalamic *Agrp* mRNA by RNAi increased metabolic rate and decreased body weight (129).

AgRP neuronal ablation, which removes not only the AgRP and NPY peptide but also other neurotransmitters released from the neuron, favors negative energy balance; although the severity of the effects reported hinge on the technique and timing of ablation. Bewick et al ablated 47% of AgRP neurons in adult mice by transgenic neurotoxin expression and found these mice to be hypophagic with decreased body weight, adiposity, leptin and insulin levels and increased BAT UCP-1 mRNA expression; these mice were also unresponsive to ghrelin (130). Other studies using either directed deletion of an essential mitochondrial transcription factor *Tfam,* or incorporation of the human diphtheria toxin into AgRP cells to induce neuronal death by 85%, observed a mildly leaner phenotype (131, 132). Luquet et al, using the same diphtheria toxin, found that AgRP neuronal ablation in adults yields a dramatic starvation phenotype, however neonates were able to compensate for AgRP neuronal deletion (133). Another study using designer receptor technology to inhibit AgRP neuronal activity observed decreased feeding during the dark cycle (134). Oppositely, activation of AgRP neurons using the same technique induced hyperphagia, increased body weight and adiposity and reduced EE and increased food-seeking behavior (134). Activation of AgRP neurons using photostimulation produced similar

effects; stimulation induced voracious feeding which had a dose-responsive effect dependent on the number of activated neurons (135).

Studies which manipulate the entire AgRP neuron not only remove AgRP, but NPY in AgRP cells, as well as neurotransmitters such as γ-aminobutyric acid (GABA), released from the AgRP neuron. There is evidence that the dramatic phenotype observed with AgRP neuronal ablation is due to inhibitory inputs from GABA. It was first observed that AgRP-neuronal ablation in A^y mice still produced a starvation phenotype, suggesting that the melanocortin system was not involved in this effect (136). Further studies showed that treatment with a GABA partial agonist ameliorated the starvation phenotype induced by AgRP neuronal ablation and that GABA activity in the parabrachial nucleus (PBN) was responsible for this phenotype (137) . This concept is supported by another study in which mice with specific deletion of the vesicular GABA transporter in AgRP neurons have a leaner phenotype with normophagia, increased VO2, activity and RQ (138). These mice are also resistant to HFD-induced weight gain, potentially via increased HFD-induced thermogenesis, and are not as responsive to ghrelin's orexigenic effects (138) .

MELANOCORTIN RECEPTOR 3 AND 4 KNOCKOUT MOUSE MODELS

MC4-R are widely expressed in the rodent brain including the cortex, thalamus, brainstem, spinal cord, and hypothalamus and are particularly highly expressed in the PVN (139). Several studies have utilized the MC4-R KO mouse to further understand the effects of the melanocortin system on energy balance. The MC4-R KO mouse is characterized by mature-onset obesity; this phenotype is less severe in heterozygote mice, indicating a gene-dosage effect (140). MC4-R

KO mice also have increased naso-anal length, late-onset hyperphagia, increased adiposity, leptin and insulin levels that are somewhat independent of increases in food intake, decreased activity and decreased metabolic rate (adjusted for body weight) (140-142). Furthermore, young MC4-R KO mice with equivalent body weights were reported to have lower total VO2 levels, but normal core body temperature (142). Deletion of the MC4-R in rats produces a similar obese phenotype, with hyperphagia and decreased activity (143). Restoration of MC4R expression in the PVH and a subpopulation of amygdala neurons in MC4-R KO mice prevented approximately 60% of obesity development (144), suggesting that neurons in these brain regions are important, but not exclusively responsible for the obese phenotype of these mice.

MC3-R are predominantly expressed in the hypothalamus, but these receptors are detected in the thalamus and brainstem as well (139). MC3-R KO mice have been reported as either normophagic or hypophagic, with equivalent body weight to WT mice but with increased adiposity, decreased lean mass, and elevated leptin levels (145, 146). MC3-R KO mice also have similar VO2 levels, but decreased activity (145, 146). Although MC3-R KO mice have normal RQ on a chow diet, an increase in RQ occurred when they were switched to a higher fat diet (145, 146). Furthermore, MC3-R KO mice have reduced numbers of mitochondrial-rich multilocular brown adipocytes and an increased fraction of larger unilocular cells in intrascapular BAT (146). Effects on glucose homeostasis are conflicting; some report abnormal glucose metabolism while others report normal levels (145-147). Restoring MC3-R expression in the VMH of MC3-R KO mice partly improved the metabolic phenotype, suggesting that MC3-Rs in this area are important but not exclusively responsible for the obese phenotype of these mice (148).

PERIPHERAL SIGNALS THAT INFLUENCE MELANOCORTIN ACTIVITY

As the arcuate is uniquely situated to receive input from the bloodstream, it is subject to regulation by a variety of different hormones and nutrients including, but by no means limited to leptin, insulin, ghrelin, estrogen, opioids, FFA, glucocorticoids, leucine, and resistant starch (3, 149-157). Further detail of peripheral signals regulating melanocortin activity is limited to hormones and nutrients pertinent to this thesis.

Leptin. There are several lines of evidence to support that leptin modulates melanocortin activity. Firstly, there are leptin receptors on POMC and AgRP neurons (158, 159). Deletion of the leptin receptor on POMC neurons yields a mildly obese phenotype with increased adiposity and leptin levels, although the phenotype is not nearly as severe as in the leptin-receptor deficient *db/db* mouse (159-161). Furthermore, deletion of this receptor in Pomc neurons suppresses *Pomc* mRNA levels and increases *AgRP* mRNA (160). Deletion of the leptin receptor in AgRP neurons or both AgRP and POMC neurons also yields an obese phenotype (159). These double KO mice have decreased VO2, core body temperature and increased RQ (159). Oppositely, rescued expression of the leptin receptor in Pomc neurons of *db/db* mice partially rescues the obese phenotype, improves EE, increases activity and normalizes glucose metabolism (162, 163) as well as increases *Pomc* mRNA, decreases *Agrp* mRNA and increases α-MSH peptide levels (163). Leptin has also been shown to directly regulate POMC and AgRP. Exogenous leptin stimulates POMC and downregulates AgRP (149, 150, 164), and leptin can prevent fasting-induced suppression in Pomc mRNA (149, 164). Electrophysiology studies show that leptin increases POMC neuronal activity by depolarizing POMC cells through a nonspecific cation

channel and by reducing GABAergic tone onto POMC neurons (57). Additionally, leptin and leptin-receptor deficient rodent models such as *ob/ob* mice and Zucker rats are hypersensitive to the anorectic and metabolic effects of melanocortin agonists (38, 41).

Insulin. Evidence suggests that insulin's central effects on energy balance are partly mediated by the melanocortin system. Central administration of insulin decreases food intake and this is blocked by a subthreshold dose of SHU9119 (151). Central insulin is also shown to increase activity (165) and restoring IR action in POMC neurons increases EE and activity (166). Central insulin administration also stimulates *Pomc* mRNA (151). Yet, deletion of insulin receptors in POMC or AgRP neurons does not affect metabolic parameters or glucose homeostasis; although, IR deletion in AgRP neurons results in a reduced ability to suppress hepatic glucose production after insulin infusion which can be normalized by restoring receptor expression (166-168). Thus, the effects of insulin on energy balance and how these effects are mediated by the melanocortin system is not precisely understood. Several targets of insulin signaling have been implicated in the regulation of energy balance including FOXO1, a transcription factor expressed robustly in the hypothalamus including in Pomc and Agrp neurons (169). Arcuate injections of constitutively nuclear/active forms of FOXO1 are shown to promote positive energy balance while repression/loss-of-function of FOXO1 yields a lean phenotype (169, 170). Furthermore, FOXO1 has been shown to stimulate Agrp and inhibit Pomc mRNA and promoter activity (169, 170). In support of this, Foxo1 deletion in Pomc neurons leads to a lean phenotype with decreased *Agrp* mRNA and increased Cpe coupled with an anorexigenic POMC processing profile including increased α-MSH and increased β-EP C-terminal cleavage products (171).

These studies demonstrate the importance of FOXO1 to the regulation of melanocortin-mediated effects on energy balance.

G*hrelin.* Central administration of ghrelin increases food intake, weight gain and adiposity and increases RQ (152, 153, 172). One of the mechanisms by which ghrelin promotes positive energy balance is via stimulation of AgRP. The ghrelin receptor is co-expressed with 94% of neurons expressing NPY in the arcuate (173) and icv ghrelin has been shown to increase *Agrp* mRNA (152, 153).

Opioids: Morphine has been shown to downregulate *Pomc* mRNA (154), while naltrexone stimulates *Pomc* mRNA (174-176); this is thought to be mediated via auto-inhibitory MOR on POMC neurons (177).

Glucocorticoids. Adrenalectomy has been shown to normalize food intake, attenuate the obese phenotype, improve glucose metabolism and decrease elevated corticosterone levels in *ob/ob* mice, and the effects of adrenalectomy on food intake and body weight gain are shown to be reversed by corticosterone (178-180). There is evidence that some of these effects are mediated by the melanocortin system. For example, adrenalectomized *ob/ob* mice have higher *Pomc* mRNA and lower *Agrp* mRNA levels than non-adrenalectomized *ob/ob* mice (179). Adrenalectomy has been shown to decrease *Pomc* and *Agrp* mRNA and corticosterone replacement reversed these effects (181). Adrenalectomy has also been shown to decrease the number of inhibitory synapses on POMC neurons and excitatory synapses on AgRP/NPY neurons—an effect that is also reversed by corticosterone replacement (178). Two different studies have examined the effects of adrenalectomy on fasting. In rats, adrenalectomy did not

block fasting-induced increases in *Agrp* mRNA; in fact these increases in *Agrp* mRNA persisted despite a fasting-induced fall in leptin and insulin levels (181). However in another study adrenalectomy blunted both fasting-induced increases and decreases in *Agrp* and *Pomc* mRNA, respectively, and corticosterone supplementation during fasting increased levels of both these genes (155). It has also been shown that the anorectic effects of MTII and leptin are amplified in adrenalectomized rats and this is normalized by glucocorticoid replacement; furthermore AgRP is not orexigenic in adrenalectomized rats unless they are replaced with glucocorticoids (182). Additionally, CRH-overexpressing mice are characterized by a hyperphagic phenotype with slightly increased fat mass (but similar body weight) and increased *Agrp* mRNA levels (183).

DOWNSTREAM TARGETS OF THE MELANOCORTIN SYSTEM

TRH. TRH is one of the most well-described targets of the melanocortin system. Hypophysiotrophic TRH neurons located in the PVN project to the external zone of the median eminence and pituitary stalk where TRH is released and transported to the anterior pituitary, binds to TRH receptors, and regulates secretion of TSH from thyrotropes (184, 185). Regulation of thyroid hormones is controlled by a negative feedback loop in which T4 and T3 negatively regulate TSH secretion (186). Leptin has been shown to directly regulate thyroid function via leptin receptors on TRH neurons as well as indirectly via the hypothalamic melanocortin system (185). α-MSH and AgRP fibers project to TRH neurons in the PVN (111, 187-189) and central α-MSH has been shown to increase pro-TRH in the PVN, as well as increase blood levels of TSH and free and total T4 (187, 188). One mechanism by which this may occur is via phosphorylated cyclic adenosine monophosphate response element-binding (CREB), which is

increased in TRH and corticotropin-releasing hormone (CRH) neurons in the PVN after ICV α-MSH (190). It was also shown *in vitro* that phosphorylated CREB can bind to the cyclic adenosine monophosphate response element (CRE) of the TRH promoter and deletion of CRE in the TRH promoter reduces stimulation of the TRH gene by α-MSH (191). Oppositely, central AgRP suppresses proTRH in the PVN and plasma T4 and T3 levels, however effects on TSH are inconsistent (111, 188, 189). *In vitro*, α-MSH increases TRH release from hypothalamic explants, and this was blocked by AgRP (188) . Similarly, leptin stimulated TRH release in rats and this was blocked by AgRP (188). Despite the important role of the melanocortin system in thyroid function, this system is intact in MC4-R and MC3-R KO mice (146, 192); however icv AgRP in MC4R KO mice does not suppress proTRH mRNA in the PVN as it does in WT mice, further supporting that the MC4-R mediates these effects on the HPT axis (192). The thyroid axis is also regulated by fasting. Starvation downregulates TRH in the PVN and median eminence as well as serum TSH, T4 and T3 levels and this suppression is rescued by exogenous leptin (78), but not insulin or glucose (193). Interestingly, fasting-induced suppression of *Trh* mRNA in the PVN, and fasting-induced suppression in T4 and T3 levels are not evident in DIO mice (194).

CRH. There is also evidence that CRH is downstream of the MC4-R as these receptors are present on CRH neurons and POMC neurons innervate CRH neurons (195, 196). However, the effects of melanocortin stimulation on CRH activity are somewhat conflicting. Icv MTII has been shown to stimulate *Crh* mRNA in the PVN and increase plasma corticosterone levels, and the anorectic effects of MTII are partly reversed by a CRH receptor antagonist (195). In chicks, β-MSH induced suppression of feeding can be blocked by corticotrophin-releasing factor type 2

receptor antagonism (197). However, other studies have shown that icv α-MSH inhibits CRH in the median eminence and *in vitro* α-MSH is shown to decrease CRH release (198, 199). Furthermore, overexpression of MSH has been shown to suppress high-fat diet induced increases in corticosterone (51). This discrepancy of melanocortin action on CRH has been hypothesized to be due to differential regulation of distinct populations of CRH neurons as CRH neurons in the PVN which project to the median eminence and control ACTH release may be regulated differently than CRH neurons in the PVN which project to autonomic centers in the brain (200).

MCH. There is also evidence that MCH, an orexigenic peptide expressed in the LHA, is downstream of the MC4-R (201). This is despite the fact that MCH and MC4-R co-localization has not been shown (202). A^y mice have increased levels of MCH and icv injection of AgRP or SHU9119 increases MCH mRNA levels (203). Furthermore, MCH expression is increased in POMC null mice (25).

BDNF. There is also evidence that brain-derived neurotrophic factor (BDNF), a neurotrophin widely expressed in peripheral and central tissues including the hypothalamus, is an anorectic peptide downstream of melanocortin signaling (204). Fasting suppresses BDNF expression specifically in the VMH and this is partly reversed by MTII treatment (205). Furthermore, A^y and MC4-R KO mice have reduced VMH BDNF expression (205). *In vitro*, a selective MC4-R agonist MK1 stimulates hypothalamic BDNF release, and this effect is blocked by the melanocortin antagonist SHU9119; additionally *in vivo*, the anorectic effects of MK1 is blocked by an anti-BDNF antibody, but not a CB-1 antagonist (206).

Sim1. SIM 1, a transcription factor associated with obesity in humans (207), yields an obese phenotype with increased linear growth if deleted in mice (208); this is reminiscent of the phenotype observed in MC4-R KO and A^y mice. Furthermore, SIM1 haploinsufficiency renders mice less responsive to MTII-induced anorexia and c-fos activation in the PVN (209). Additionally, both leptin and MTII increase hypothalamic SIM1 mRNA expression, and overexpession of Sim1 is protective against DIO and partly ameliorates obesity in A^y mice (209, 210).

HYPOTHALAMIC MELANOCORTIN REGULATION OF PROLACTIN

As mentioned above, the hypothalamic melanocortin system has been shown to regulate neuroendocrine parameters such as the hypothalamic-pituitary-thyroid axis and the hypothalamic-pituitary-adrenal axis. However, the hypothalamic melanocortin system has also been shown to regulate prolactin release. Icv α-MSH has been shown to decrease prolactin release in OVX rats by 50% and suppress increases in prolactin release due to stress, estrogen treatment, and on the day of proestrus (211-213). This is further supported by studies in which treatment with α-MSH anti-serum increased prolactin secretion (214). In a primate model, icv AgRP infusion stimulates prolactin release and this is blocked by α-MSH (215). β-EP and opioids are also well-described to stimulate prolactin release. β-EP stimulates prolactin release in a primate model and this can be blocked by α-MSH (216). Furthermore, icv β-EP anti-serum decreases baseline prolactin and blunts stress-induced increases in prolactin levels (217).

The effects of melanocortin peptides on prolactin regulation is shown to be mediated via TIDA neurons. TIDA neurons in the median eminence release dopamine, which binds to D2 receptors

in the lactotrophs of the pituitary to tonically inhibit prolactin release (218). Dopamine receptor antagonists stimulate prolactin release and these effects cannot be blocked by icv α-MSH, suggesting that α-MSH mediates its effects on prolactin release via dopamine (211, 213). Furthermore, icv α-MSH increased DOPA and DOPAC content in the median eminence (but not other regions of the brain with dopamine neuron terminals) (219). β-EP and other opioids also mediate prolactin release via a dopaminergic mechanism, as they have been shown to significantly decrease dopamine content in the hypophyseal portal blood and dopamine turnover in the median eminence (220-222). Furthermore in rats, POMC-immunoreactive terminals are shown to contact tyrosine hydroxylase-positive cell bodies and dendrites in the hypothalamus (223), demonstrating a physical interaction between POMC and dopamine.

HUMAN MUTATIONS IN THE MELANOCORTIN SYSTEM

MC4-R mutations. MC4-R mRNA has been found in human brain and adipose tissue (224). Greater than 150 mutations in the MC4-R have been identified in humans, predominantly as missense mutations, however frameshift, nonsense, and in-frame mutations have been identified as well (225). The prevalence of receptor mutations in severely obese populations is variable and contingent upon the population studied, although large studies using North American and Western European populations report that between 2-6% of the obese population carry the mutation (226-228). However, not all studies report an association between MC4-R mutations and incidence of obesity; in fact a study in a German population with significant (ie-leading to reduced function) nonsynonymous heterozygous mutation alleles found no increase in BMI compared to wild-type homozygotes (229). Individuals with mutations are characterized by

severe obesity with hyperphagia, increased linear growth as well as increased fat and lean mass, and hyperinsulinemia (228). However, thyroid function is normal and reduced EE and sympathetic function as well as increased RQ have also been reported (44, 228, 230, 231). Not surprisingly, individuals with non-functional receptors had a more severe phenotype than individuals with partially functioning receptors (228). Most individuals with MC4-R mutations are heterozygotes, however homozygotes exist and possess a more severe phenotype (225, 228). Despite the severe phenotype of these individuals, lifestyle intervention in obese children with functional MC4-R gene mutations has been shown to be effective, and furthermore gastric bypass is reported to be equally effective in functional MC4-R heterozygote individuals (232, 233).

MC3-R mutations. Mutations in the MC3-R have been shown to be associated with obesity, however the results are not as convincing as those for MC4-R mutations. Individuals with heterozygote mutations in the MC3-R receptor are characterized by obesity, increased levels of adiposity and leptin levels (234). However, unlike what has been observed in mice, mutations in the MC3-R are associated with increased food intake (235). A large study of obese and lean European individuals found that functional mutations in the MC3-R were more prevalent in the obese population, compared to the lean population (236); however a separate study in an obese population in North America reported no difference in MC3-R mutation prevalence between lean and obese subjects (227). The literature is further complicated by the fact that while obese individuals may display MC3-R mutations, these mutations may be not be of functional consequence (227, 234, 236, 237). Future studies are required to further understand the impact of functional MC3-R mutations in humans.

POMC mutations. Heterozygote and homozygote POMC deficient humans are reported to have red hair, fair skin, and an obese, hyperphagic phenotype with adrenal insufficiency (238-240), although pigmentation deficits were not obvious in individuals from non-Western European ancestry (240-242). ACTH is the sole agonist for the MC2-R in the adrenal gland and hence these individuals are glucocorticoid deficient; it is surprising that these individuals are obese despite their glucocorticoid deficiency (238). Mutations in the N-terminal section of POMC, an area that is suggested to possess a sorting signal motif responsible for directing POMC to the regulatory secretory pathway, was found in two severe early-onset obese individuals (83). These individuals had varying degrees of impaired POMC processing, resulting in reduced levels of α-MSH and β-EP (83).

α-MSH and β-MSH mutations. There is one report of a mutation in the POMC peptide which yielded an aberrant α-MSH peptide with reduced MC4-R affinity and reduced ability to stimulate the MC4-R (58). Three groups have reported on the contribution of β-MSH to human obesity. The first study found that the aberrant β-MSH peptide generated from a POMC missense mutation was not able to properly stimulate MC4-R signaling (58). This study also found that obese children with mutated β-MSH are hyperphagic with increased linear growth and lean body mass, similar to MC4-R null mice (58). The mutation in this peptide was also found to be more common in obese subjects compared to controls (58). Another group reported a different mutation in the POMC peptide that resulted in a mutated β-MSH form that also had reduced binding affinity and ability to stimulate the MC4-R (243). This mutated β-MSH was administered icv to rats and was not able to suppress food intake (as unmutated β-MSH can) (243). Furthermore, a missense POMC mutation in the dibasic cleavage site critical for β-MSH

and β-EP cleavage has been reported which produces a β-MSH/β-EP fusion protein with markedly reduced MC4-R signaling activity *in vitro* (244). This fusion protein is found more frequently in subjects with early-onset obesity compared to normal-weight controls (244). The contribution of β-MSH to obesity is further supported by data from the same study which showed that β-MSH, α-MSH and ACTH are all present in post-mortem human brains from normal-weight individuals, however density of β-MSH was reported as the greatest (243).

Mutations in POMC processing enzymes PC1, PC2, Cpe, Prcp. Two studies have shown that mutations in *Pcsk1* are associated with obesity; in fact, one study found that heterozygous carriers of any Pcsk1 mutation have an 8.7 fold higher risk of obesity (245, 246). Compound heterozygote PC1 deficiency in humans is associated with extreme obesity and abnormal glucose homeostasis, hypogonadotrophic hypogonadism, hypocortisolism, increased plasma proinsulin and POMC peptides and low insulin levels (247), however small-intestinal malabsorptive function is also reported in some cases (248, 249). Although human studies have not directly examined the link between PC2 and obesity, two individuals with Prader-Willi syndrome, characterized by extreme obesity, were found to have no detectable 7B2 or PC2 immunoreactivity in the PVN and supraoptic nucleus, and slightly decreased PC1 immunoreactivity, suggesting that PC2 may be associated with obesity in humans (250). One study has examined the correlation between Cpe mutations and obesity but found no significant association (251). Although the correlation between PRCP and obesity in humans has not been directly investigated, PRCP variants were associated with metabolic syndrome in men (252).

SUMMARY

Data from both animal models and humans strongly support the critical role of the melanocortin system in the regulation of energy balance and metabolism. Studies utilizing both genetic and pharmacological manipulation show that α-MSH promotes negative energy balance via increased MC3/4-R signaling while AgRP promotes positive energy balance via antagonism at these melanocortin receptors. This is further supported by studies in which Pomc overexpression reduces obesity phenotypes and deletion of either POMC or MC4-R in mouse models yields a massively obese phenotype. These mouse phenotypes bear similarity to humans as individuals with the inability to produce or process the POMC peptide or with defective MC4-R signaling have a severe obese phenotype. γ2 and γ3-MSH peptides also appear to play a contributory role in regulating energy balance at the MC3-R, although the effects are much less than those observed with α-MSH agonists. β-MSH, which is not endogenously present in rodents, yields effects on energy balance similar to α-MSH and has important effects in humans. The effects of β-EP, an agonist for the MOR, are somewhat inconsistent and sparse, as icv injections acutely stimulate food intake while chronic β-EP deletion yields an obese phenotype; the effects of β-EP 1-27 on feeding is unknown in mammals. Numerous inputs regulate melanocortin tone including various hormones and nutrients and some of the most well-described include leptin and insulin, which have been shown to stimulate POMC and inhibit AgRP. There are also a number of potential downstream targets of melanocortins that have been described including TRH and CRH neurons.

Given the importance of the melanocortin system to obesity in both animal and human models, this thesis sought to focus on unexplored aspects of melanocortin regulation of energy balance. In **Chapter 2,** we hypothesized that POMC-derived peptides modulate food intake and energy balance both individually and in an interactive manner. This chapter focuses on further understanding both the acute and chronic effects of icv β-EP under different feeding conditions as well as investigates the acute effects of β-EP 1-27 on food intake. This chapter also examines the ability of β-EP to antagonize α-MSH's effects on energy balance under both acute and chronic conditions. In **Chapter 3,** we hypothesized that the novel AgRP inhibitor, TTP2515, would decrease food intake, body weight and adiposity as well as increase EE and thyroid hormone levels. This AgRP inhibitor was utilized in a number of different models including rats receiving icv AgRP as well as under conditions in which AgRP is elevated such as during fasting and in leptin-deficient *ob/ob* mice. In **Chapter 4,** we hypothesized that blood and pituitary levels would be lower in genetic models of increased melanocortin signaling. In this chapter we studied mice overexpressing MSH and mice with genetic AgRP deletion to further explore this hypothesis. Finally, in **Chapter 5** we hypothesized that HFD feeding would modulate melanocortin activity and this chapter reports on some preliminary findings on the effects of the HFD on melanocortin gene and peptide expression, as well as expression of enzymes involved in post-translational POMC processing.

CHAPTER 2: EFFECTS OF β-ENDORPHIN ON ENERGY BALANCE AND INTERACTION WITH α-MSH

Abstract

Proopiomelanocortin (POMC) is posttranslationally processed to several peptides including α-MSH, a primary regulator of energy balance that inhibits food intake and stimulates energy expenditure (EE). However another POMC-derived peptide, β-endorphin (β-EP), has been shown to stimulate food intake. In this study we examined the effects of intracerebroventricular (icv) ß-EP on food intake and its ability to antagonize the negative effects of α-MSH on energy balance in male rats. A single icv injection of ß-EP stimulated food intake over a 2-6 h period during both the light and dark cycles. This effect was, however, not sustained with chronic icv ß-EP infusion. In the next study a subthreshold dose of ß-EP was injected together with NDP-MSH after a 16 h fast and the negative effects of NDP-MSH on refeeding and body weight gain were partially reversed. Finally, peptide interactions were studied in a chronic icv infusion model. Weight gain and food intake were significantly suppressed in the NDP-MSH group during the entire study. A subthreshold dose of β-EP antagonized these suppressive effects on food intake and weight gain for the first 3 days. However on days 4-7, β-EP no longer blocked these effects. Of note, the stimulatory effect of ß-EP on feeding and its ability to antagonize MSH was specific for ß-EP$_{1-31}$ and was not observed with ß-EP $_{1-27}$. This study highlights the importance of understanding how the balance between α-MSH and β-EP is maintained and the potential role of differential POMC processing in regulating energy balance.

Introduction

The hypothalamic melanocortin system, which includes both anorexigenic proopiomelanocortin (POMC) neurons and orexigenic agouti-related protein (AgRP) neurons, plays an important role in the regulation of energy balance and metabolism (3, 253). These neurons oppositely regulate food intake and EE via G-protein coupled melanocortin receptors in the brain. The POMC precursor protein is posttranslationally processed to a number of smaller peptides including α-MSH that promotes negative energy balance by both decreasing food intake and increasing EE (33, 43, 51, 254). POMC is also processed to the endogenous opioid peptide β-endorphin (β-EP) that binds to the inhibitory G-protein coupled mu opioid receptor (59, 255). The endogenous opioid system, consisting of the enkephalins and dynorphins, in addition to β-EP, and the mu, delta and kappa opioid receptors, exerts complex effects on feeding, metabolism, nutrient selection and reward via distinct pathways (256). However the specific role of β-EP in energy balance and how it interacts with α-MSH is not well established. β-EP has been shown to acutely stimulate food intake after intracerebroventricular (icv) or hypothalamic injection during the light cycle (59, 60, 62) and deletion of the mu opioid receptor protects from diet-induced obesity (257). In contrast, specific genetic deletion of β-EP yields a mildly obese phenotype (64), although under certain conditions the hedonic aspects of feeding were decreased (258). Furthermore, both POMC and AgRP neurons express mu opioid receptors and there is evidence that these neurons can be regulated by opioids (177, 259, 260). Hence the role of β-EP in the regulation of energy balance is still puzzling. Although previous studies have examined the acute effects of icv β-EP during the light cycle in satiated rats, little is known about the longer term effects of icv β-EP injection during the light and dark cycles and the effects of chronic icv

β-EP infusion. β-EP is a 31 amino acid peptide that can be further processed by C-terminal cleavage to yield β-EP $_{1-27}$ and β-EP $_{1-26}$ which have markedly reduced opioid activity (261). β-EP $_{1-27}$ binds to brain opioid receptors but diminishes β-EP induced analgesia (261). However, the effect of these peptides on feeding is relatively unknown.

Despite the fact that α-MSH and β-EP are cleaved from the same precursor peptide, their biological effects are quite different and in some cases even antagonistic. While β-EP is an analgesic (262), α-MSH produces hyperalgesia and can blunt the analgesic effects of morphine (263, 264). In addition, α-MSH has been shown to block some of the neuroendocrine and behavioral effects of β-EP (265-268). There is also some evidence that α-MSH and β-EP can interact with respect to feeding and metabolism. For example, pre-treatment with the melanocortin-receptor agonist MTII has been shown to block the acute orexigenic effects of a relatively large dose of icv β-EP (60). While these data suggest that opioid and melanocortin systems can interact, it is yet unknown if β-EP can antagonize the effects of α-MSH on feeding and energy balance under more physiological conditions in both an acute and chronic model. This study sought to further investigate the metabolic effects of β-EP as compared with β-EP $_{1-27}$ either alone or in combination with α-MSH. First, we examined the acute effects of icv β-EP injection during both the light and dark cycles as well as the chronic effects of icv β-EP infusion. We next investigated the ability of a subthreshold dose of β-EP to attenuate the effects of α-MSH on feeding and metabolism in both an acute and chronic setting. The differences between β-EP and β-EP $_{1-27}$ were also studied. Finally, we examined how infusion of these peptides modulates hypothalamic gene expression including *Pomc* and *Agrp*, as well as enzymes involved in processing the full-length POMC precursor peptide to its active metabolites, and

prolylcarboxypeptidase *(Prcp)* which inactivates α-MSH (5, 99).

Materials and Methods

Animals: All experiments were approved by the Columbia University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 200–250 grams (g) were purchased from Charles River (Wilmington, MA, USA). Animals had *ad libitum* access to water and LabDiet® Rodent Chow 5001 (PMI Nutrition International, St. Louis, MO). Rats were acclimatized to a natural light/dark cycle.

Surgery: Rats were anesthetized with pentobarbital for icv cannula placement. For the acute icv injections (*Exp 1 and 3*), a 22-gauge stainless steel cannula was inserted stereotaxically into the right lateral ventricle (56). In the chronic infusion experiments (*Exp 2 and 4*), a 28-gauge stainless steel cannula connected to a 7-day osmotic pump (ALZET model 2001, Cupertino, CA, USA) delivering 1 µl/h of saline was inserted stereotaxically into the right lateral ventricle (56). Animals were individually housed and allowed to recover for 5-7 days after cannula placement. After recovery, in *Exp 2* and *4*, minipumps were switched under isoflurane anesthesia to either 3-day pumps (ALZET model 1003D) or 7-day pumps delivering 1µl/h. Peptides were dissolved in sterile normal saline immediately before use. Before each experiment, rats were divided into treatment groups of equivalent weight and food intake. Animals exhibiting signs of illness and consuming less than 10 g of food per day were excluded from analyses.

Peptides: Rat β-EP $_{1\text{-}31}$ (022-33), β-EP $_{1\text{-}27}$ (022-08) and [Nle^4, D-Phe^7]-α-MSH (NDP-MSH, 043-06) were obtained from Phoenix Pharmaceuticals, Inc (Burlingame, CA).

Experimental protocols

Experiment 1: Acute effects of a single icv injection of ß-EP $_{1-31}$ during the light and dark cycle

Experiment 1a: *Effects of 0.5 and 5 µg β-EP during the light cycle.* Three groups of rats were studied during the light cycle (n=9/group). β-EP $_{1-31}$ (5 µg or 0.5 µg) or saline was injected icv between 0830-1100 h. Food was removed 1 h prior to the icv injection and returned immediately after injection. Measurements were obtained 4, 6 and 24 h after injection.

Experiment 1b: *Effects of 0.5, 1 and 5 µg of β-EP during the dark cycle.* In the first experiment, three groups of rats were studied (n=7-9/group). β-EP $_{1-31}$ (5 µg or 0.5 µg) or saline was injected icv between 1800-2000 h. Food was removed 2 h prior to icv injections and returned immediately after injection. Measurements were obtained 2, 4 and 14 h after injection. In the second experiment two groups of rats were studied (n=8/group). β-EP $_{1-31}$ (1 µg) or saline was injected icv between 1800-2000 h. Measurements were obtained 2, 4 and 12 h after injection.

Experiment 2: Chronic effects of icv infusion of β-EP $_{1-31}$ for 4 days

Rats received 3-day ALZET pumps delivering either 2 or 10 µg/day β-EP $_{1-31}$ or saline icv (n=7-9/group). Food intake and body weight were recorded daily between 0900-1000 h. On Day 4, rats were sacrificed and trunk blood was collected. A 3 mm mediobasal hypothalamic (MBH) section was dissected using a rat brain block and frozen in liquid nitrogen for quantitation of neuropeptide (*Pomc*, *Agrp*) and processing enzyme (prohormone convertase 1, *Pcsk1*; prohormone convertase 2, *Pcsk2*) and degrading enzyme (*Prcp*) mRNA.

Experiment 3: Acute effects of a single icv injection of β-EP $_{1-31}$ or β-EP $_{1-27}$ alone or in combination with NDP-MSH

For *Exp 3 a&b,* rats were fasted at 1700 h and received an icv injection approximately 16 h later. Rats were refed one hour after injection and food intake was monitored at 2, 4, 8 and 24 h and body weight was measured at 4, 8 and 24 h.

Experiment 3a: In the first experiment, rats received an icv injection of either 1 µg of β-EP$_{1-31}$ or saline (n=7-8). In the next experiment rats received an icv injection of either 1 µg NDP-MSH, 1 µg NDP-MSH+1 µg β-EP $_{1-31}$ or saline (n=6-7/group).

Experiment 3b: Rats received an icv injection of either 1 µg NDP-MSH, 1 µg NDP-MSH+1 µg β-EP $_{1-31}$, 1 µg NDP-MSH+1 µg β-EP $_{1-27}$, or saline (n=6-8/group).

Experiment 3c: Rats from *Exp 3b* were re-randomized and used for this experiment 3 days later. Rats received an icv injection of either 5 µg β-EP $_{1-27}$ or saline (n=8/group) between 0900-1130 h. Measurements were obtained at 2, 4, 8 and 24 h after injection.

Experiment 4: Chronic effects of continuous icv infusion of NDP-MSH alone and in combination with β-EP $_{1-31}$

Rats received 7-day pumps delivering either 2.4 µg/day MSH-NDP, 2.4 µg/day MSH-NDP+2.4 µg/day β-EP $_{1-31}$ or saline (n=6-8/group). Food intake and body weight were recorded daily between 0900-1000 h. After 7 days rats were sacrificed; trunk blood was collected and inguinal and retroperitoneal fat pads were weighed. A 3 mm MBH section was dissected for quantitation of neuropeptide (*Pomc*, *Agrp*) and processing enzyme (*Pcsk1*, *Pcsk2* and carboxypeptidase E, *Cpe*) and degrading enzyme (*Prcp*) mRNA.

Hormone assays: Trunk blood was collected into tubes containing EDTA immediately after decapitation and plasma was stored at −20 °C. Plasma insulin and leptin concentrations were measured using RIA kits (Millipore Corporation, Billerica, MA).

Measurement of hypothalamic mRNA levels: RNA isolation was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen USA, Valencia, CA) in conjunction with the RNase-Free DNase set (Qiagen USA) and total RNA was quantified using spectrophotometry. cDNA was synthesized using the Superscript III First-Strand cDNA Synthesis Kit (Life Technologies Corporation/Invitrogen, Grand Island, NY) and was analyzed using quantitative RT- PCR performed in the Lightcycler 480 Real-Time PCR system (Roche Applied Science). Samples were normalized to β-actin. Primer sequences are as follows: *β-actin* F5'ctctgaaccctaaggccaaccgtgaaaa3' R5' tctccggagtccatcacaatgccagtg3'; *Pomc* F5'cagtgccaggacctcaccacgg3' R5'cggtcccagcggaagtgaccc3'; *Agrp* F5'catgccctagctacaggaag3' R5' gcagtgccagcaggaca3'; *Prcp* F5'gcttccgcccctatctggcagc3' R5'gggccaagcaggcaaaggct3'; *Pcsk1* F5' cgttcagttccaagagactc3' R5' ggcagagatgcagtcattct 3'; *Pcsk2* F5' ccaagcgaaaccagcttcacg3' R5' catgctcgaggtagcggacg3'; *Cpe* F5'gcccagggaatagatctgaac3' R5'gaatgacagccttggtctc3'

Statistical analysis: Statistical analysis was performed with Student's *t* test when two groups were compared. Analysis of variance (ANOVA) followed by Fisher's protected least squares difference test was used when comparing more than two groups. $P < 0.05$ was considered statistically significant. Results are reported as mean values ± SEM.

Results

Experiment 1: Acute effects of a single icv injection of ß-EP $_{1-31}$ during the light and dark cycle

Experiment 1a: To investigate the effects of β-EP on food intake during the light cycle in satiated rats, we administered a single icv injection of β-EP $_{1-31}$ (0.5 or 5 µg dose) or saline in the morning. The 5 µg dose of β-EP stimulated food intake to 240% and 221% compared to saline at the 4 and 6 h timepoints ($p<0.05$; **Fig 2.1A**). The 0.5 µg dose increased cumulative food intake to 175% and 171% at 4 and 6 h but this did not reach significance. Overnight (from 6-24 h) food intake tended to decrease to 88% of saline levels in the 5 µg group ($p=0.06$; **Fig 2.1A**), but this was not evident in the 0.5 µg group which consumed 96% of saline levels. Cumulative food intake was not different between groups 24 h after injection.

Experiment 1b: To investigate the effects of β-EP on food intake during the dark cycle, we initially administered a single icv injection of β-EP 1-31 (0.5 or 5 µg) or saline in the evening. The 0.5 µg dose tended to increase food intake at 2 h vs. saline (5.4±0.7 vs. 4.0±0.56 g, $p=0.14$) while food intake in the 5 µg group (4.0±0.6g) was unchanged from saline. No differences were noted between groups at 4 h (*Saline* 7.1±0.7 vs. *0.5 µg* 7.6±0.8 vs. *5 µg* 6.3±0.6 g). At 14 h cumulative food intake in the 0.5 µg group (20.8±1.4g) was not different from saline (23.1±0.7, $p=0.13$). However 14 h cumulative food intake was significantly less than saline in the 5 µg group (18.9±0.6g, $p<0.01$). We next studied the effect of 1 µg of β-EP $_{1-31}$ or saline in the evening. This dose of β-EP increased food intake to 158% and 132% of saline levels at both 2 and 4 h ($p<0.01$; **Fig 2.1B**), respectively. However, between 4 and 12 h, the β-EP group

consumed only 72% of saline's intake (p=0.06) such that cumulative food intake at 12 h was similar between the saline and β-EP group.

Experiment 2: Chronic effects of icv infusion of β-EP $_{1-31}$ for 4 days

To examine the chronic effects of icv β-EP we continuously infused either a low (2 μg/day) or high (10 μg/day) dose of β-EP $_{1-31}$ via osmotic minipump. Although we did not detect any significant increases in food intake or body weight gain during the entire treatment period, on day 1, body weight gain tended to increase with 10 μg/day β-EP (**Fig 2.2B**). In contrast, on days 3 and 4, cumulative food intake was significantly suppressed in the 10 μg β-EP group to 90% and 89% of saline ($p<0.05$, **Fig 2.2A**). At sacrifice on day 4, body weight was similar between groups (Saline 355.4±4.3g, β-EP low 356.2 ±8.4g, β-EP high 352.0 ±4.4g).

Hormone levels and hypothalamic mRNA analyses: At sacrifice, leptin was suppressed to 80% of saline in the 10 μg/day β-EP group ($p<0.05$; **Fig 2.2C**); insulin was 73% of saline (p=0.10). Leptin and insulin were not different in the 2 μg/day β-EP group *vs.* saline. The 10 μg dose of β-EP suppressed *Agrp* mRNA levels ($p<0.05$; **Fig 2.2D**) while *Pomc* mRNA levels were unchanged between groups. Furthermore, *Prcp* mRNA levels tended to be lower in both the 2 μg (0.93±0.02, p=0.07 *vs.* saline) and 10 μg β-EP (0.94±0.03, p=0.08 *vs.* saline) groups. *Pcsk1* and *Pcsk2* mRNA were also measured, but no differences were detected between any of the groups (data not shown).

Experiment 3: Acute effects of a single icv injection of β-EP $_{1-31}$ or β-EP $_{1-27}$ alone or in combination with NDP-MSH

Experiment 3a: In the first experiment, a 1 µg icv injection of β-EP$_{1-31}$ alone in a fasting and refeeding model did not stimulate food intake or body weight gain (*Saline vs. β-EP*, 2 h: 8.8±1.0 vs. 6.8±0.7g, p=0.10; 4 h: 9.6±1.0 vs. 7.4±0.7, p=0.09); in fact food intake tended to be lower. In the next experiment, three groups of rats were fasted overnight and received an icv injection of NDP-MSH (1 µg), NDP-MSH (1 µg)+β-EP $_{1-31}$ (1 µg), or saline the next morning and were refed 1 h later. After 2 h, refeeding was suppressed in the MSH group to 52% of saline levels ($p<0.01$; **Fig 2.3A**). However, this suppression was reversed by concomitant β-EP treatment to 83% of saline levels ($p<0.05$ *vs.* MSH, p=0.23 *vs.* saline; **Fig 2.3A**). By 4 h cumulative refeeding was suppressed in the MSH group to 64% of saline levels ($p<0.05$; **Fig 2.3A**), however this suppression was still reversed by β-EP treatment (82% of saline) such that this group was not significantly different from saline. At this timepoint, the saline group gained 9.2±1.7g while the MSH group lost 0.8±1.4g ($p<0.001$ *vs.* saline, **Fig 2.3B**). This effect was partly reversed in the MSH+β-EP group that gained 5.5±1.2g, which was significantly different from the MSH group ($p<0.01$) but not from the saline group (p=0.08). By 8 h, cumulative refeeding and body weight gain were still suppressed by MSH, however this was no longer reversed by β-EP (**Fig 2.3A,B**). After 24 h, there were no significant differences in food intake or body weight gain between groups (data not shown).

Experiment 3b: We next sought to investigate if β-EP $_{1-27}$ can antagonize MSH's anorexic and metabolic effects. Four groups of rats were fasted overnight and were injected icv with 1µg of each peptide: NDP-MSH, NDP-MSH+β-EP $_{1-31}$, NDP-MSH+β-EP $_{1-27}$ or saline the next morning and rats were refed 1h later. After 2 h, MSH suppressed food intake to 55% of saline levels ($p<0.001$, **Fig 2.4A**). Concomitant β-EP $_{1-31}$ injection partly reversed this effect so that food

intake was only suppressed to 82%, which was significantly different from MSH ($p<0.05$), but not from saline ($p=0.10$). Concomitant β-EP $_{1-27}$ injection did not reverse MSH's suppressive effects; MSH+β-EP $_{1-27}$ suppressed food intake to 67%, of saline ($p<0.01$, **Fig 2.4A**) and was not different from MSH alone ($p=0.22$). At 4 h, food intake was suppressed in the MSH group to 71% of saline ($p<0.05$, **Fig 2.4A)**, and this was partly reversed by β-EP $_{1-31}$ such that food intake was not different from saline (**Fig 2.4A**); however β-EP $_{1-27}$ did not reverse the effects of MSH. Weight gain at this timepoint was suppressed by MSH to 24% of saline levels ($p<0.05$, **Fig 2.4B**). This suppression was partly reversed by β-EP $_{1-31}$ treatment to 74% of saline levels and was not different from saline ($p=0.44$). MSH-induced suppression of weight gain was not reversed by β-EP $_{1-27}$; in fact, weight gain in the MSH+β-EP $_{1-27}$ group was suppressed to 13% of saline levels (**Fig 2.4B**). At both the 8 and 24 h timepoints, cumulative food intake and weight gain were similar between groups (data not shown).

Experiment 3c: To further investigate the effects of β-EP $_{1-27}$ on food intake and body weight gain, we administered a single icv injection of either 5 µg β-EP $_{1-27}$ or saline in the morning to fed rats. Food intake and body weight change were similar between these groups at all timepoints. At 2 h, the β-EP $_{1-27}$ group consumed 1.4±0.5g, while the saline group consumed 2.0±0.6g and at 4 h, the β-EP $_{1-27}$ group consumed 2.6±0.7g, while saline consumed 3.5±0.6g.

Experiment 4: Chronic effects of continuous icv infusion of NDP-MSH alone and in combination with β-EP $_{1-31}$

In this experiment, we sought to investigate if β-EP $_{1-31}$ could chronically antagonize the anorexigenic and metabolic effects of NDP-MSH. Rats received either 2.4 µg /day MSH, 2.4 µg MSH+2.4 µg β-EP/day, or saline for 7 days via an osmotic minipump.

MSH significantly suppressed cumulative food intake during each day of the study. During days 1-3, this suppression was partly reversed by β-EP treatment, so that the MSH+β-EP group was not significantly different from saline (**Fig 2.5A**). During days 4-7, β-EP no longer antagonized the anorexic effects of MSH. At 7 days cumulative food intake was lower in both the MSH and MSH+β-EP groups compared to saline (**Table 2.1**).

MSH significantly suppressed weight gain during each day of the study. During days 1-3, this suppression was partly reversed by β-EP treatment such that the MSH and MSH+β-EP groups tended to be different from one another ($p=0.06$), and the MSH+β-EP and saline groups were not significantly different from one another on days 2 and 3 (**Fig 2.5B**). During Days 4-7, β-EP no longer antagonized the suppressive effects of MSH on body weight gain (**Fig 2.5B**), so that by day 7 weight was equivalent between the MSH and MSH+β-EP groups (**Table 2.1**).

Fat mass, hormones, and hypothalamic mRNA analyses (**Table 2.1**). Total fat pad mass was reduced by 10% and 27% in the MSH ($p=0.36$) and MSH+β-EP ($p<0.05$) groups respectively. Leptin was suppressed in the MSH ($p<0.05$) and MSH+β-EP groups ($p<0.01$) *vs.* saline. Insulin was suppressed in the MSH+β-EP group ($p<0.05$) *vs.* saline. *Agrp* mRNA levels were higher in the MSH+β-EP group versus saline ($p<0.01$) and versus MSH alone ($p<.05$). *Pomc* mRNA levels were similar between groups, however the *Pomc/Agrp* ratio was suppressed to 71 ± 14% of saline in the MSH group ($p<0.05$) and to 68 ± 7.8% in the MSH+β-EP group ($p<0.05$). Furthermore *Prcp* mRNA levels were decreased in the MSH+β-EP group compared to saline ($p<0.05$). *Pcsk1*, *Pcsk2* and *Cpe* mRNA were also measured, but no differences were detected between groups (data not shown).

Discussion

In this study, we confirm the short-term stimulatory effect of β-EP on food intake and show that this can be elicited during both the light and dark cycles. However, this effect was not sustained with chronic icv β-EP infusion. In fact, when infused at high doses, food intake was decreased. Of note, the stimulatory effect on feeding was only observed with full length β-EP and not with C-terminally cleaved β-EP $_{1\text{-}27}$. Most importantly, we showed that a subthreshold dose of β-EP was able to attenuate the effects of α-MSH on food intake and body weight gain in both an acute fasting and refeeding model and during chronic icv infusion for several days. These studies show that different POMC-derived peptide products can interact to regulate energy balance and underscore the importance of understanding how the balance between these peptide products is maintained.

Our studies show that a single icv injection of β-EP stimulates food over a 2-6 h period and that this occurs in the morning in satiated rats as well as in the evening. After the initial stimulation, food intake tended to decline such that cumulative food intake the next morning was not different from controls. It is unclear if this decline is an independent primary or secondary effect of β-EP or solely a compensatory reduction in food intake. However, when higher doses of β-EP were administered during the dark cycle cumulative overnight food intake decreased in the absence of an acute stimulatory effect. This acute stimulatory effect of ß-EP on feeding is consistent with previous reports with central injection of ß-EP and other mu-opioid receptor agonists DAMGO and morphine (256, 260, 269-271). Most studies were performed in freely fed rodents during the light cycle but stimulatory effects have been noted in some studies during the dark cycle (271). Effects were most apparent between 2 and 4 h after opioid administration.

However when morphine was administered to food deprived rats, food intake during refeeding was reduced especially at higher doses of morphine that had been shown to stimulate food intake in fed rats (269). Similarly icv DAMGO stimulated food intake in fasted mice at low doses but inhibited intake at higher doses (260). When we administered 1 µg of ß-EP to fasted rats food intake was not stimulated, and in fact tended to be lower, in contrast to what was observed at this dose in satiated rats. Thus the effects of opioids on food intake appear to be dependent on feeding state. This is further illustrated in another model showing that ß-EP null mice work significantly less than wild-type mice for food reinforcers under nondeprived conditions, but there was no difference when the mice were tested in a food deprived state (63).

In our study no effects on feeding or body weight gain were detected with chronic low dose infusion of β-EP, however, with higher doses food intake decreased by 3-4 days. This may be due to possible sedating or other behavioral effects associated with infusing a pharmacological dose of β-EP into the lateral ventricle (272, 273). However, although behavior was not formally assessed, we did not observe any obvious signs of sedation or behavioral change. Another potential explanation for the decrease in food intake during chronic ß-EP infusion is the suppression in *Agrp* mRNA levels observed in rats infused with the higher dose of β-EP for 4 days. This decrease was noted despite a fall in leptin and insulin levels which is usually associated with an increase in *Agrp* mRNA (274, 275). These data suggest that β-EP can suppress AgRP leading to deceased food intake. AgRP neurons express mu opioid receptors and recent electrophysiological data show that POMC neurons release an opioid that inhibits AgRP neuronal activity (260, 276). Furthermore there is evidence that opioids can mediate some of the downstream orexigenic effects of AgRP (277) which could be enhanced with acute opioid

injection but reduced if opioid tolerance develops with chronic icv infusion. In addition, expression of PRCP, an enzyme that inactivates α-MSH, also tended to be suppressed after chronic icv β-EP infusion; this could lead to increased α-MSH and contribute to the decreased food intake observed (99).

POMC neurons also express mu opioid receptors that exert well established inhibitory effects on POMC neuronal activity and gene expression (177, 278-280). However more recently presynaptic opioid receptors have been described that colocalize with GABAergic terminals onto POMC neurons that could lead to disinhibition of POMC neurons at low opioid concentrations (280). In addition it appears that postsynaptic receptors are desensitized by opioids while presynaptic receptors are not. Thus feedback regulation of POMC by β-EP is potentially quite complicated and these mechanisms could explain the differential effects observed with various opioid administration protocols. In our study we did not detect a change in *Pomc* gene expression after 4 days of icv β-EP infusion but did detect a change in *Agrp* expression. Thus the effects of icv β-EP on food intake appear to be biphasic, with acute stimulatory effects and longer-term inhibitory effects. The acute stimulatory effects are consistent with prior reports demonstrating acute stimulation of food intake with opioid agonists and inhibition with opioid antagonists (256, 281). The inhibitory effects of chronic β-EP infusion are consistent with effects reported with chronic morphine infusion in the rat and also with genetic studies showing that selective ablation of only the β-EP portion of *Pomc* yields mice that tend to be hyperphagic and obese (64, 282).

The effects of icv β-EP injection were observed only with full length β-EP $_{1\text{-}31}$ and not with β-EP $_{1\text{-}27}$, which possesses markedly reduced opioid activity and can even antagonize the analgesic

effects of β-EP $_{1-31}$ (261). Although there are no other studies of β-EP $_{1-27}$ on feeding in mammalian models, high doses of β-EP $_{1-27}$ did stimulate food intake in the chick, but was much less potent than β-EP $_{1-31}$ and was also able to antagonize β-EP $_{1-31}$ induced stimulation of food intake (66). Although β-EP $_{1-31}$ is the predominant form of β-EP in the hypothalamus, a significant portion is normally cleaved to β-EP $_{1-27}$ and β-EP $_{1-26}$ (261, 283). There is evidence that the processing enzymes, prohormone convertase 2 and carboxypeptidase E (CPE), may be responsible for the C-terminal processing of β-EP and that this process may be regulated with respect to food intake. For example, CPE deficient mice are obese and have little C-terminal processing of β-EP (95). In addition, mice with ablation of the transcription factor FoxO1 in POMC neurons have a lean phenotype and were found to have increased CPE expression in the arcuate with relatively more processing of β-EP to the C-terminal forms (171). Our studies further underscore the importance of C-terminal processing of β-EP as injection of β-EP $_{1-27}$ did not impact energy balance.

Despite the fact that α-MSH and β-EP $_{1-31}$ are cleaved from the same precursor peptide, their biological effects are quite different and in some cases even antagonistic. This has been shown with respect to analgesia, sexual behavior and neuroendocrine function (263-268). We therefore sought to determine how these peptides interact with respect to energy balance in both acute and chronic infusion models. We show that a subthreshold dose of β-EP, that did not increase food intake or body weight when administered alone, was able to attenuate the suppressive effects of NDP-MSH on food intake and body weight gain in both an acute fasting and refeeding model and during chronic icv infusion for several days. This was accomplished with a molar dose of β-EP that was approximately half of the molar dose of NDP-MSH that was administered. While a

previous study showed that pretreatment with the melanocortin-receptor agonist MTII blocked the acute orexigenic effects of a relatively large dose of icv β-EP (60), this study shows that a relatively low dose of β-EP can attenuate the negative effects of MSH on energy balance, which are the predominant effects of the *Pomc* gene on energy balance. β-EP $_{1-31}$ (but not β-EP $_{1-27}$) attenuated the suppressive effects of MSH on feeding and body weight gain after an overnight fast. When infused chronically over a 7 day period, MSH suppressed food intake and body weight gain during the entire study. These suppressive effects were partially reversed by concomitant β-EP infusion for the first 3 days. However, after 3 days, β-EP no longer antagonized the effects of MSH. In fact at the time of sacrifice the β-EP+MSH group had the lowest fat pad mass and leptin levels as well an increase in *Agrp* expression consistent with the weight loss and lower leptin levels induced primarily by the MSH infusion. This is in contrast to the suppressive effects of β-EP infusion alone on *AgRP* expression in *Exp 2*. It is unclear why the antagonism of MSH by β-EP cannot be sustained after 3 days but this may result from opioid receptor downregulation and opioid tolerance which has been demonstrated to occur with chronic icv β-EP infusion (272, 273). In addition, it is possible that the development of opioid tolerance leads to enhanced sensitivity to the effects of MSH infusion.

POMC is synthesized in the arcuate nucleus and processed to both α-MSH and β-EP which are presumably released together onto downstream neurons that regulate energy balance. Although each of these peptides binds to its own distinct melanocortin or opioid receptor respectively, there is evidence that functional antagonism may occur via a post-receptor mechanism. Melanocortin and opioid receptors are both G-protein coupled but the former is coupled to stimulatory G-proteins while the later is coupled to inhibitory G-proteins, resulting in increased

or decreased production of cAMP respectively (255, 284). Depending on the relative amounts of α-MSH and β-EP that are released, differential effects on neuronal activity and gene expression would be expected. At present there is no evidence for differential sorting of α-MSH and β-EP into different secretory granules. However, the first cleavage step of POMC by PC1 yields pro-ACTH and β-LPH, which could potentially be sorted differently (285). In the pituitary there is evidence for a novel subcellular localization of β-LPH and β-EP (but not ACTH and α-MSH) in peroxisomes however the mechanism by which this occurs is unclear (286). Furthermore in the hypothalamus there is evidence for differential sorting of prothyrotropin-releasing hormone peptide products (287). Regardless of whether or not there is differential sorting of β-EP and α-MSH, there are clearly distinct mechanisms by which these peptides can be inactivated, thus altering the balance between these two peptides. Wallingford and colleagues have shown that the enzyme PRCP inactivates α-MSH by removing the C-terminal valine residue (99). They show that *Prcp* null mice have a lean phenotype with elevated levels of α-MSH in the hypothalamus and that inhibition of PRCP activity decreases food intake. PRCP is highly expressed in the lateral hypothalamus and dorsomedial nucleus and to a lesser extent in the arcuate (99). Thus PRCP could serve to modulate the strength of the α-MSH signal at melanocortin receptors on second order neurons without affecting β-EP signaling. In contrast, C-terminal cleavage of β-EP would decrease β-EP signaling at opioid receptors on second order neurons without affecting MSH signaling.

In summary these studies show that the POMC-derived peptides α-MSH and β-EP can interact to regulate food intake and body weight. While MSH has sustained negative effects on food intake and body weight gain, the short-term stimulatory effects of β-EP injection were not sustained.

However a subthreshold dose of β-EP was able to attenuate the effects of MSH on food intake and body weight gain in both an acute fasting and refeeding model and during chronic icv infusion for several days. This study highlights the importance of understanding how the balance between α-MSH and β-EP is maintained and the potential role of differential POMC processing in regulating energy balance.

TABLE 2.1: *Effects of chronic NDP-MSH, NDP-MSH+β-EP 1-31, or saline infusion*

	Saline	MSH	MSH+β-EP
	(g)		
Food intake	196.9±4.9	166.0±10.0**	165.1±7.1**
Weight	387.3±4.6	360.8±10.0*	360.1±6.7*
Fat pads	**(g)**		
Inguinal	3.2±0.2	3.0±0.2	2.6±0.2*
RP	2.5±0.2	2.2±0.4	1.6±0.2*
Hormones	**(ng/ml)**		
Insulin	1.7±0.2	1.6±0.2	1.1±0.1*
Leptin	2.9±0.2	2.4±0.1*	1.8±0.1**†
mRNA	**(AU)**		
Pomc	1.00±0.08	0.82±0.25	1.01±0.17
Agrp	1.00±0.06	1.06±0.12	1.47±0.08**†
Prcp	1.00±0.02	0.97±0.04	0.88±0.05*

Values are mean ± SE.
**p<.01,*p<.05 vs. saline; †p<.05 vs. MSH
RP, Retroperitoneal; AU, Arbitrary Units

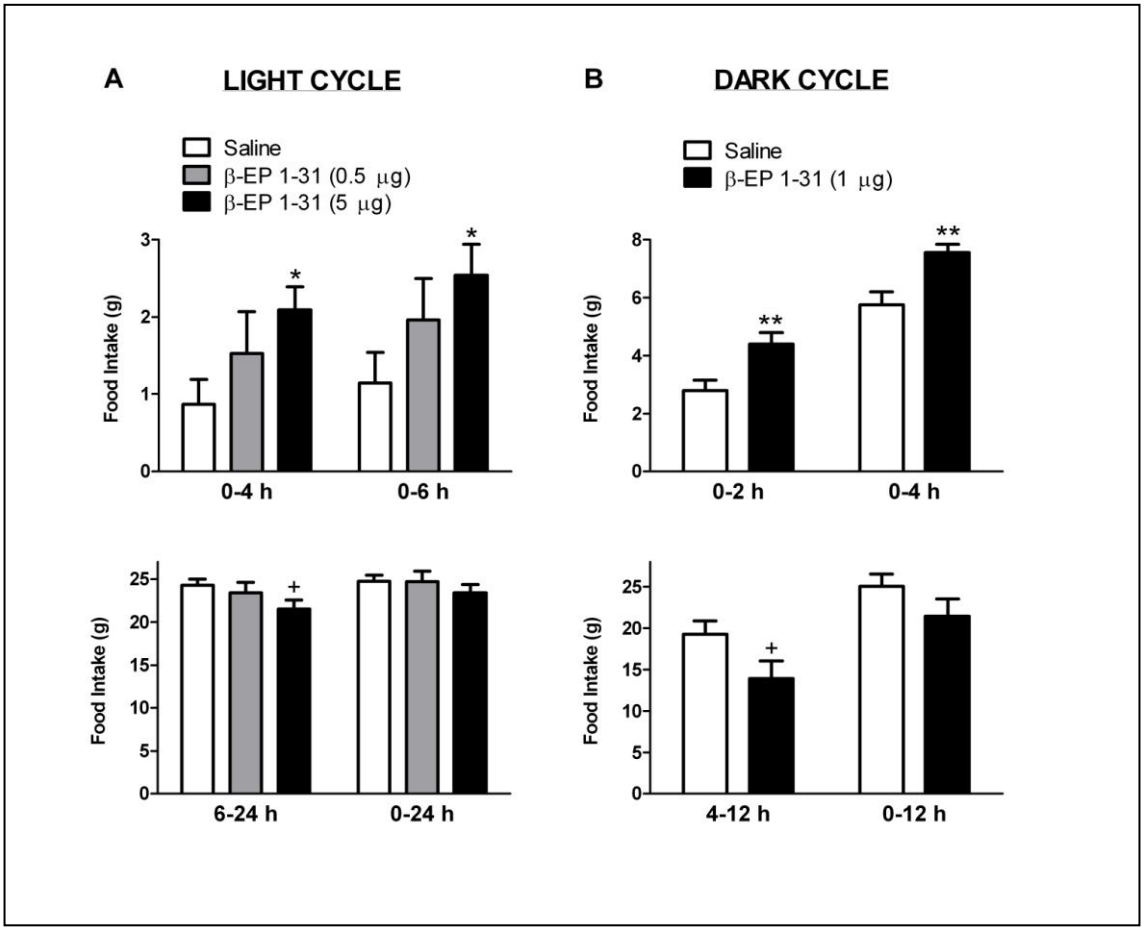
A
LIGHT CYCLE
Saline
β-EP 1-31 (0.5 μg)
β-EP 1-31 (5 μg)
Food Intake (g)
0-4 h
0-6 h
6-24 h
0-24 h
B
DARK CYCLE
Saline
β-EP 1-31 (1 μg)
Food Intake (g)
0-2 h
0-4 h
4-12 h
0-12 h

Figure 2.1 Effects of a single icv injection of β-EP 1-31 on food intake during the light (A) and dark (B) cycle. (A) 5 μg β-EP 1-31 stimulated cumulative food intake at 4 and 6 h during the light cycle; the stimulation with 0.5 μg β-EP was not significant (*n*=9/group) (*top*). However, between 6-24 h, the 5 μg dose tended to decrease food intake compared to saline. Cumulative food intake was similar between all groups at 24 h (*bottom*). **(B)** 1 μg β-EP stimulated cumulative food intake at 2 and 4 h during the dark cycle (*n*=8/group) (*top*). However, between 4-12 h, β-EP tended to decrease food intake. Food intake was similar between groups at 12 h (*bottom*). *p<0.05 , **p<0.01, +p=0.06 *vs.* saline.

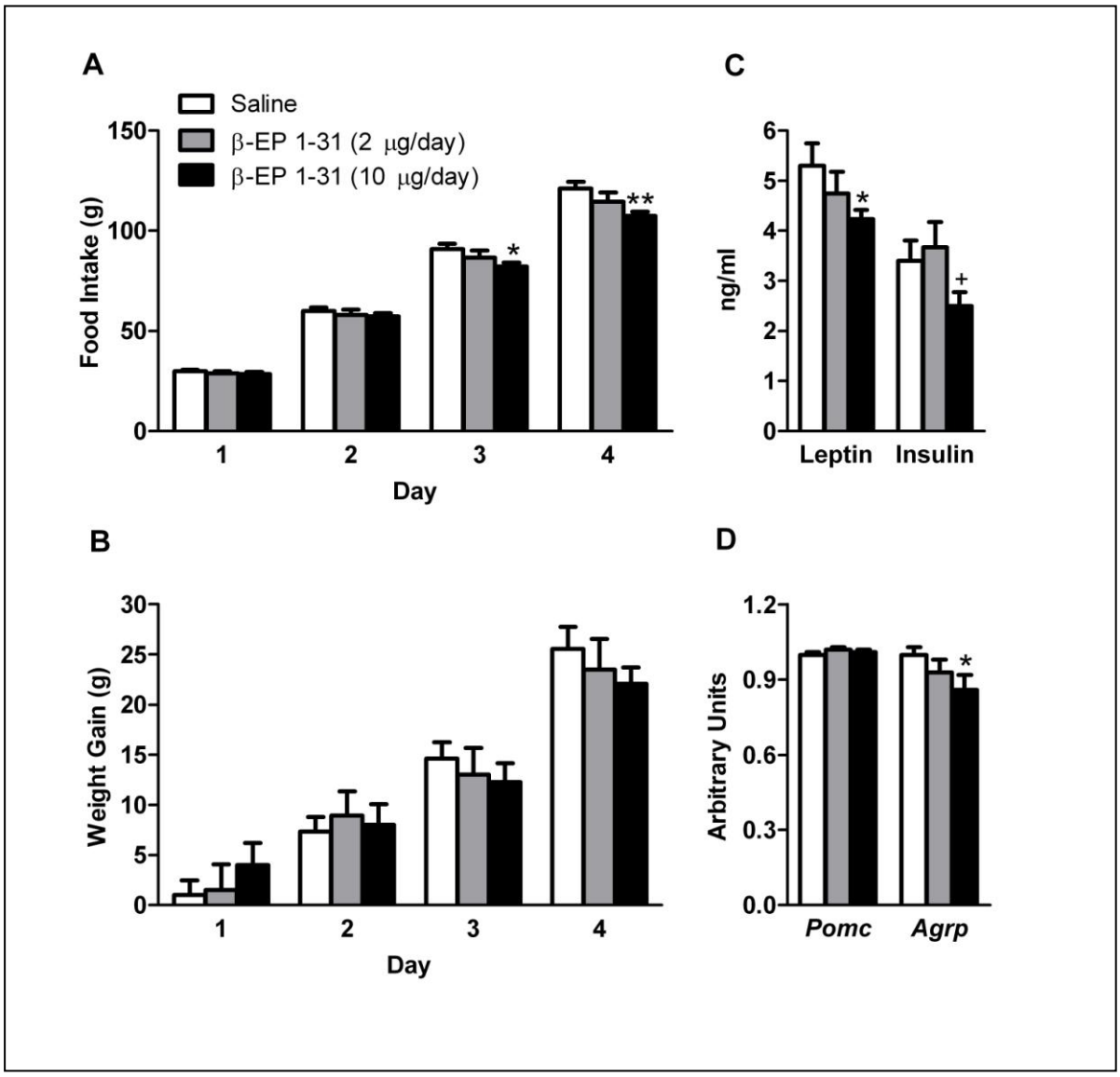
A
Saline
β-EP 1-31 (2 μg/day)
β-EP 1-31 (10 μg/day)
Food Intake (g)
150
100
50
0
1
2
3
4
Day
*
**
C
ng/ml
6
5
4
3
2
1
0
Leptin
Insulin
*
+
B
Weight Gain (g)
30
25
20
15
10
5
0
1
2
3
4
Day
D
Arbitrary Units
1.2
0.9
0.6
0.3
0.0
Pomc
Agrp
*

Figure 2.2 Effects of a 4-day icv β-EP 1-31 infusion on metabolic parameters. No significant increases in cumulative food intake **(A)** or weight gain **(B)** were detected with chronic β-EP 1-31 treatment at either 2 or 10 µg/day (*n*=7-9/group). However, on days 3 and 4, cumulative food intake was lower in the 10 µg/day β-EP group, but not in the 2 µg/day group, compared to saline. **(C)** Leptin levels were significantly lower and insulin levels (p=.10) tended to be lower in the 10 µg/day group compared to saline. **(D)** *Agrp* mRNA was lower in the 10 µg/day β-EP group. *p<0.05, **p<0.01 *vs.* saline; +p<.05 *vs.* 2 µg/day.

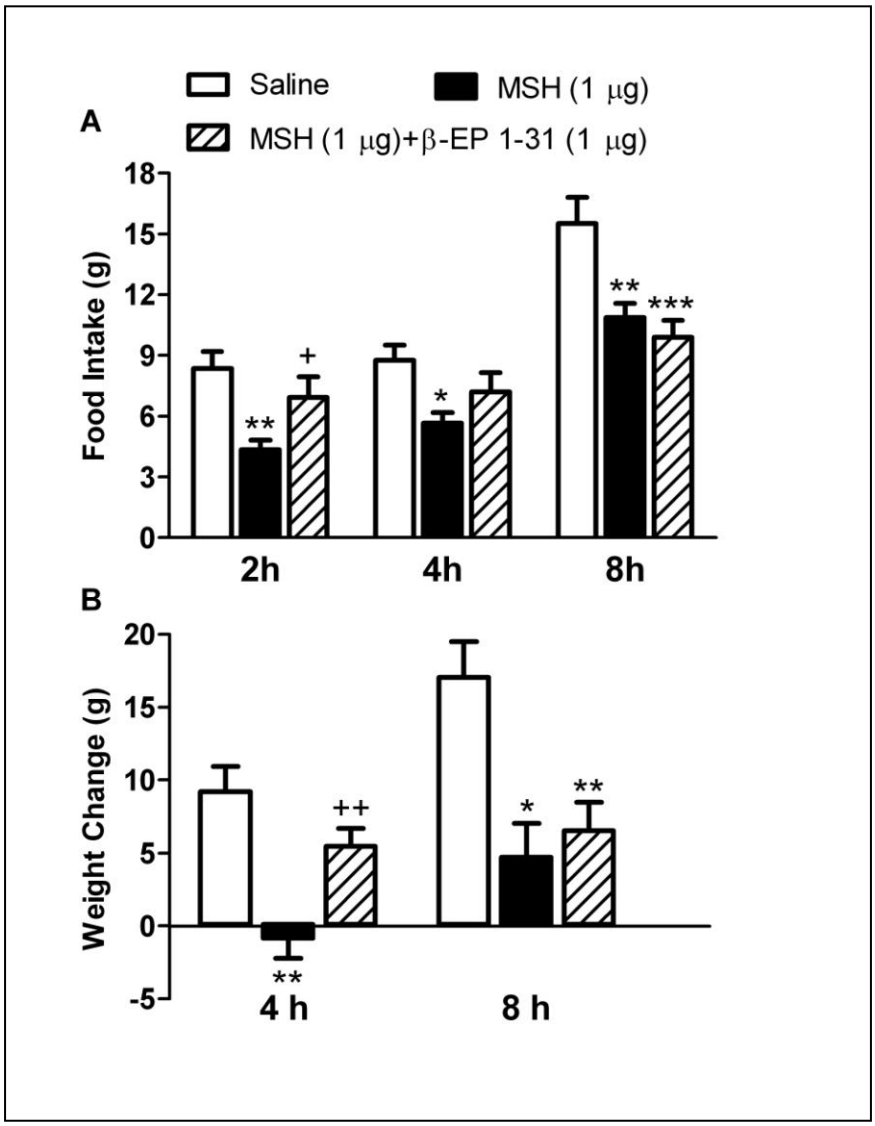
Saline
MSH (1 μg)
MSH (1 μg)+β-EP 1-31 (1 μg)
A
Food Intake (g)
18
15
12
9
6
3
0
**
+
*
**
***
2h
4h
8h
B
Weight Change (g)
20
15
10
5
0
-5
**
++
*
**
4 h
8 h

Figure 2.3 Effects of a single icv injection of β-EP 1-31 on NDP-MSH-induced suppression of feeding and body weight gain. After an overnight fast, (**A**) cumulative refeeding was significantly suppressed by a single icv injection of NDP-MSH at 2, 4 and 8 h (*n*=6-7/group). Concomitant β-EP 1-31 injection reversed this suppression at 2 and 4 h. At 2 h the MSH and MSH+β-EP groups were significantly different from one another. At 8 h β-EP no longer antagonized MSH's effects. **(B)** The MSH group gained significantly less weight at 4 and 8 h compared to the saline group. This effect was reversed by β-EP at 4 h but was no longer evident at 8 h. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs.* saline; +$p<0.05$, ++$p<0.01$ *vs.* MSH.

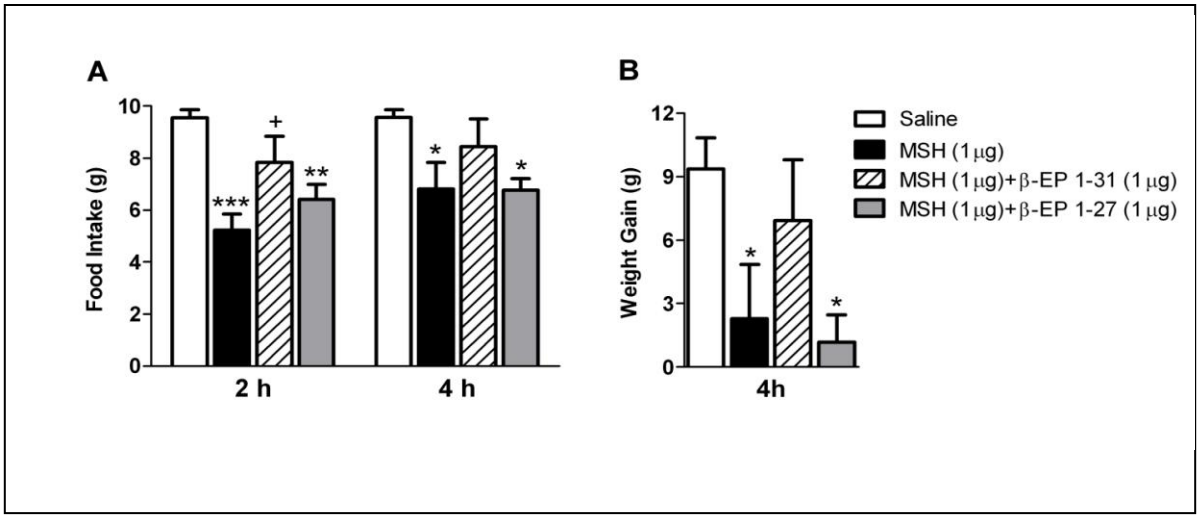

Figure 2.4 Effects of a single icv injection of β-EP 1-27 or β-EP 1-31 on NDP-MSH-induced suppression of feeding and body weight gain. After an overnight fast **(A)** cumulative refeeding was significantly suppressed by a single icv injection of NDP-MSH at 2 and 4 h (*n*=6-8/group); this suppression was significantly reversed by concomitant β-EP 1-31 injection, but not by β-EP 1-27 injection. At 2 h, the MSH and MSH+β-EP 1-31 groups were significantly different from one another but the MSH and MSH+β-EP 1-27 groups were not. **(B)** MSH significantly suppressed weight gain at 4 h and this was reversed by concomitant β-EP 1-31 injection, but not by β-EP 1-27. *p<0.05, **p<0.01, ***p<0.001 *vs.* saline; +p<0.05 *vs*. MSH.

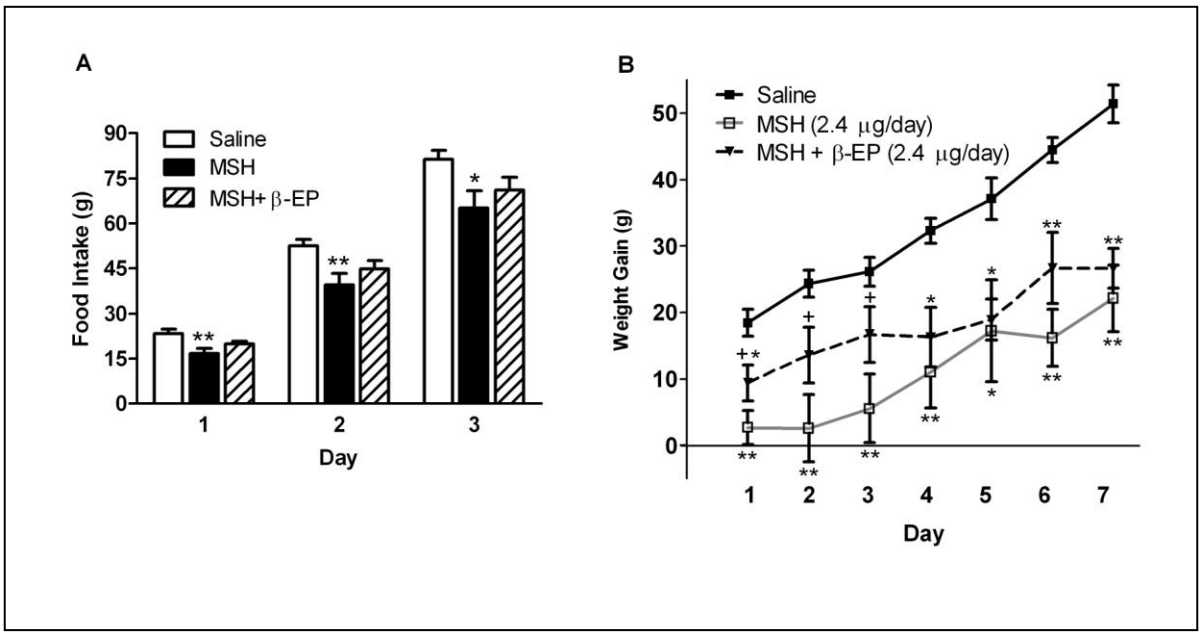

Figure 2.5 Effects of a 7-day icv β-EP 1-31 infusion on NDP-MSH-induced suppression of feeding and body weight gain. **(A)** Chronic icv MSH infusion (2.4 μg/day) significantly suppressed cumulative food intake during the entire study. Concomitant β-EP infusion (2.4μg/day) partly reversed this suppression during the first 3 days of the experiment (n=6-8/group). **(B)** Chronic icv MSH infusion significantly suppressed cumulative body weight gain during the entire study. Concomitant β-EP infusion partly reversed this suppression during the first 3 days of the experiment. However, during days 4-7, β-EP no longer antagonized MSH's effects on body weight. **p<0.01, *p<0.05 *vs.* saline; +p=0.06 *vs.* MSH.

CHAPTER 3: EFFECTS OF AGRP INHIBITION ON ENERGY BALANCE AND METABOLISM

Abstract

Activation of brain melanocortin-4 receptors (MC4-R) by α-melanocyte-stimulating hormone (MSH) or inhibition by agouti-related protein (AgRP) regulates food intake and energy expenditure (EE) and can modulate neuroendocrine responses to changes in energy balance. To examine the effects of AgRP inhibition on energy balance, a small molecule non-peptide compound, TTP2515, developed by TransTech Pharma, Inc., was studied. *In vitro*, TransTech Pharma Inc. showed that the AgRP inhibitor prevented AgRP from antagonizing α-MSH-induced increases in cAMP in MC4-R-expressing cells. When administered to rats by oral gavage, the AgRP inhibitor TTP2515 blocked icv AgRP-induced increases in food intake, weight gain and adiposity and suppression of T4 levels. In both diet-induced obese (DIO) and leptin-deficient mice, TTP2515 decreased food intake, weight gain, adiposity and respiratory quotient (RQ). TTP2515 potently suppressed food intake and weight gain in lean mice immediately after initiation of a high fat diet (HFD) but had no effect on these parameters in lean chow-fed mice. However, when tested in AgRP KO mice, TTP2515 also suppressed food intake and weight gain during HFD feeding. In several studies TTP2515 increased T4 but not T3 levels, however this was also observed in AgRP KO mice. TTP2515 also attenuated refeeding and weight gain after fasting, an effect not evident in AgRP KO mice at moderate doses. This study shows that TTP2515 exerts many effects consistent with AgRP inhibition however experiments in AgRP KO mice indicate some off-target effects of this drug. TTP2515 was particularly effective

during fasting and with leptin deficiency, when AgRP is elevated, as well as during acute and chronic HFD feeding. Thus the usefulness of this drug in treating obesity deserves further exploration, but more studies are needed to define the AgRP dependent and independent mechanisms by which TTP2515 exerts its effects on energy balance.

Introduction

The hypothalamic melanocortin system, comprised of proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons and brain melanocortin receptors (MC-R), is a critical regulator of energy homeostasis in both animals and humans (3). These neurons are situated in the arcuate nucleus of the hypothalamus and project to second-order neurons that express MC-Rs in the hypothalamus and other brain regions, where their peptide products interact to regulate energy balance (3, 19). POMC-derived α-melanocyte-stimulating hormone (MSH) is an agonist for the G-protein coupled MC4-R that produces a reduction in food intake and increase in EE (33, 43, 51, 254). Oppositely, AgRP behaves as a potent melanocortin receptor antagonist, and in some reports, an inverse agonist, that promotes positive energy balance (32, 111, 112, 288). Both neurons are targets for leptin and insulin and can integrate a variety of nutritional and neuronal signals to regulate energy balance (3).

The role of AgRP in the regulation of energy balance has been examined in a number of different rodent models. Studies have shown that overexpression of AgRP induces hyperphagia, hyperinsulinemia and obesity (123). Similarly, intracerebroventricular (icv) AgRP administration increases food intake, body weight gain and adiposity, and decreases energy expenditure (EE) (111-113); increased adiposity and a metabolic phenotype are observed even when hyperphagia is prevented (112). In addition, activation of AgRP neurons using designer receptors rapidly induces voracious feeding, decreases EE and increases adiposity (134). AgRP expression in the arcuate nucleus is increased both during fasting when leptin levels are low (289), and in *ob/ob* mice with genetic leptin deficiency (120); these effects can be normalized

with leptin injection (290). There is also evidence that AgRP mediates some of the neuroendocrine responses to food deprivation including the suppression of the hypothalamic-pituitary-thyroid (HPT) axis (111, 128, 189, 192).

While reduction of hypothalamic AgRP expression by RNA interference has been shown to increase metabolic rate and decrease body weight (129), genetic deletion of AgRP yields only a mild or non-existent phenotype, implicating developmental compensation(127, 128). Postembryonic ablation of AgRP neurons can yield a dramatic phenotype that hinges on the age at the time of neuronal ablation such that starvation occurs if neurons are ablated in adult mice but not in neonatal mice (133). The starvation phenotype appears to be due to the loss of γ-aminobutyric acid (GABA) from AgRP neurons and thus cannot be attributed to the loss of AgRP peptide (137). To date there has been no specific pharmacological inhibitor of AgRP that could be used to help elucidate the extent to which AgRP contributes to energy homeostasis and neuroendocrine responses to food deprivation. Furthermore, an AgRP inhibitor has the potential to be utilized in a therapeutic capacity, particularly under conditions in which AgRP is elevated. The drug development of MSH agonists has been complicated by a number of side effects possibly related to the extensive projection of POMC neurons and wide distribution of brain MC-Rs (291-295). In contrast, AgRP neuronal projections are more limited to brain areas involved with energy balance (296), making AgRP an attractive target for pharmacological inhibition (294, 295).

TransTech Pharma Inc. has developed a series of potent, small molecular weight, non-peptide compounds that selectively inhibit AgRP's antagonist activity. These AgRP inhibitors display

high brain penetration after oral administration and *in vitro* interact with AgRP and prevent it from antagonizing MC4-R signaling. The AgRP inhibitor does not directly interact with the MC4-R, either alone or in the presence of α-MSH. The AgRP inhibitor is specific for AgRP as it does not block the ability of SHU9119, a synthetic MC3/4-R antagonist, to antagonize MC4-R signaling. We utilized the AgRP inhibitor TTP2515 in numerous rodent models to further investigate the contribution of AgRP to energy balance. We initially examined the ability of TTP2515 to inhibit the effects of exogenous icv AgRP administration in the rat. We next investigated the ability of this molecule to inhibit endogenous AgRP in lean and obese mouse models, including diet-induced obese (DIO) and leptin-deficient *ob/ob* mice. As the melanocortin system is known to mediate adaptive responses observed after acutely switching mice to a high fat diet (HFD), we investigated the effects of TTP2515 under these conditions (297). As AgRP is markedly upregulated by fasting, we also studied the metabolic responses to fasting and refeeding (289, 290, 298). Finally, we examined the effects of TTP2515 on the thyroid axis under both fed and fasted conditions in a number of these experiments. AgRP KO mice were employed in these various models to determine which effects of TTP2515 were specifically due to AgRP antagonism.

Materials and Methods

Animals: All animal experiments were approved by the Columbia University Institutional Animal Care and Use Committee.

Rats: Male Sprague-Dawley rats weighing 200–250 g were purchased from Charles River (Wilmington, MA, USA) and used for all rat experiments. Animals had *ad libitum* access to water and LabDiet® Rodent Chow 5001 (13.5% fat; PMI Nutrition International). Rats were acclimatized to a natural light/dark cycle prior to surgery. In all experiments, rats were anesthetized with pentobarbital (50mg/kg) by intraperitoneal (ip.) injection for icv cannula placement. In the acute icv injection rat experiment, a 22-gauge stainless steel cannula was inserted stereotaxically into the right lateral ventricle (coordinates from bregma: lateral 1.3 mm; caudal 0.8 mm; depth from dura 3.5 mm). In the chronic infusion experiment, a 28-gauge stainless steel cannula connected by vinyl catheter tubing to a 7-day osmotic pump (ALZET model 2001, Cupertino, CA, USA) delivering 1 μl/hr of normal saline was inserted stereotaxically into the right lateral ventricle (same coordinates as above). Rats were individually housed and allowed to recover for 5-7 days after cannula placement. During this recovery period, rats receiving icv injections were acclimated to restraining to minimize stress during injections. Before each experiment, rats were divided into treatment groups of equivalent weight and daily food intake. In all experiments peptides were dissolved from lyophilized stock in sterile normal saline immediately before use. Animals exhibiting signs of illness and whose food consumption fell to less than 10 grams per day were excluded from analyses. One rat from *Experiment 1a* was excluded from Day 2 onwards due to illness and decreased food intake.

Mice: Mice were housed under barrier conditions with a 12:12-hr light-dark cycle. Male C57BL/6 mice from Charles River Laboratories and C57BL/6J mice and leptin-deficient mice (Stock #632) from Jackson Laboratories (Bar Harbor, ME, USA) were utilized in the experiments as indicated. The AgRP KO mouse line was obtained from Dr. Van der Ploeg (127). Mice were backcrossed 6 times to a coisogenic C57BL/6J line, the C57BL/6J-$A^{w\text{-}J}$/J strain. AgRP KO and WT mice were generated from homozygous matings and male mice were used in the experiments as indicated. Ablation of AgRP in the mediobasal hypothalamus was confirmed by genotyping and via both qRTPCR for gene expression (*F5'GCAAAGGCCATGCTGACTGC3', R5'CTTCTTGAGGCCATTCAGAC3'*) and via two separate assays measuring AgRP (Human AgRP Quantikine ELISA, R&D Systems, Inc, Minneapolis, MN; and RIA, using synthetic $AgRP_{83\text{-}132}$ standard and iodinated tracer as described previously (109)).

Mouse body weight, food intake, and body composition: All body weight and food intake measurements were obtained daily from singly housed mice unless otherwise indicated. Food was measured using either the LabMaster Calorimetry System feeding baskets or specialized feeding chambers that fit into traditional homecages. Body composition measurements were obtained by NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany). In all experiments, baseline body weight, food intake and body composition were equivalent between groups, unless otherwise indicated.

Mouse diets: The following diets were used for mouse experiments as indicated: normal chow diet (Lab diet #5053, 13% kcal from fat), breeder chow diet (Lab diet #5058, 22% kcal from fat),

45% high fat diet (Research Diets 12451, 45% kcal from fat), 60% very high fat diet (Research Diets 12492, 60% kcal from fat), and 10% chow diet (Research Diets 12450B, 10% kcal from fat).

Indirect calorimetry: The LabMaster Calorimetry System (TSE Systems, Bad Homburg, Germany) was used to measure oxygen consumption (VO2), carbon dioxide production (VCO2) and activity every 26 min (except for *Experiment 5*, which was every 14 min). Mice were acclimated to the calorimetry system for a minimum of 24 h prior to starting the experiment. After acclimation, baseline measurements were obtained for a minimum of 24 h to appropriately randomize mice. In all experiments, baseline calorimetry parameters were equivalent between groups. Approximately 2 h of measurements were excluded during each dosing session as the mouse was physically removed from the chamber for gavaging. Relative VO2 and energy expenditure (EE) were derived by dividing the mean VO2 or EE of a specific timeperiod (ie-dark cycle) by the total counts of that timeperiod; values are presented as multiplied by 1000.

Drug compound: In all animal experiments, TTP2515 was dissolved in sterile water and administered via oral gavage at the dosage indicated (5-50 mg/kg). The control group received water at the same volume per kg as the treatment group. Animals were acclimated to gavaging before experimental treatment was administered. In the all experiments except *Experiment 2a*, TTP2515 was administered twice daily, with doses 10-12 h apart.

Hormone analyses: Blood was collected from the trunk after decapitation at sacrifice or from the submandibular pouch. Trunk blood was collected into tubes containing EDTA and plasma was stored at -80° C. Submandibular blood samples were collected into tubes at room

temperature and serum was stored at -80° C. Hormones were measured using commercially available RIAs: Leptin and insulin (Linco Research Inc, St Charles, MO, USA) and total T4, free T4 and total T3 (Coat-A-Count ®, Siemens Healthcare Diagnostics, Tarrytown, NY).

Statistical analysis: Statistical analysis was performed with Student's *t* test when only two groups were compared. Analysis of variance (ANOVA) followed by Fisher's protected least squares difference test was used when comparing more than two groups. $P < 0.05$ was considered statistically significant. Results are reported as mean values ± SEM.

Experimental procedures:

Experiment 1: Effects of TTP2515 on the orexigenic and metabolic effects of icv AgRP injection in rats

Experiment 1a: Four groups of rats were studied (n=6-7/group). Half received icv human $AgRP_{83-132}$ (Phoenix Peptides 003-53) with either oral TTP2515 or water and half received ICV saline with either oral TTP2515 or water. On Day 0, between 1730-2030 h, rats received either oral TTP2515 (30 mg/kg) or water and either 0.2 nmol AgRP or saline icv (delivered in a 5 ul bolus over the course of one minute). The following day (Day 1), rats received either TTP2515 (30 mg/kg) or water between 1130-1230 h. On Day 2 rats received a final dose of oral TTP2515 or water and icv AgRP or saline between 1500-1800 h. Measurements were obtained on Days 1-4 between 0900-1000 h and rats were sacrificed on Day 4.

Experiment 1b: Four groups of rats were used to study the effects of TTP 2515 during chronic icv AgRP infusion (n=7-8/group). On Day 0, icv pumps infusing saline were exchanged for new

pumps delivering either saline or 0.64 nmol/day human $AgRP_{83-132.}$ The first dose of water or TTP2515 (30 mg/kg) was administered shortly after the exchange, and water or TTP2515 continued to be administered twice daily between 800-1000 h and 1800-2000 h. Rats were sacrificed on Day 8. Trunk blood was collected for hormone assays and adipose and liver weight were quantified.

Experiment 2: Effects of TTP2515 on metabolic parameters in DIO mice

Experiment 2a: 8-week old C57BL/6 mice received a 45% high fat diet for 16 weeks at which time mean body weight increased from 21.1±0.3g to 39.4±1.0g. Mice were randomized to receive either water or TTP2515 (30 mg/kg) by oral gavage twice daily for 21 days (n=7-8/group). Body weight and food intake were monitored approximately every 3 days. On Day 15, blood was obtained after a 6 h fast. Body composition was assessed before treatment and on Day 20 of treatment. Mice received their last dose of TTP2515 or water at 0800 h on Day 21 and were sacrificed 6 h later.

Experiment 2b: 6-week old C57BL/6J mice received a 45% high fat diet for 15 weeks during which time mean body weight increased from 21.5±0.3g to 34.6±0.5g. Mice were acclimatized to calorimetry chambers and received treatment of either TTP2515 (30 mg/kg) or water twice daily starting during the dark cycle and continuing for 3.5 days (n=8/group).

Experiment 3: Effects of TTP2515 on metabolic parameters in leptin-deficient mice

9-week old leptin-deficient *ob/ob* mice maintained on a normal chow diet were randomized to receive water or TTP2515 twice daily at increasing doses (*Days 1-4*, 5 mg/kg; *Days 5-7*, 15

mg/kg; *Days 8-10,* 30 mg/kg; *Days 11-25*, 50 mg/kg) for a total of 25 days (n=7/group). Mice were placed in calorimetry chambers during Days 1-14. Mice were fasted before the evening of Day 13 and food intake was measured during refeeding the next morning. A second fast was performed before the evening of Day 19 for a glucose tolerance test (GTT). Body composition was assessed before treatment and on Day 24 of treatment. Mice received their last dose of TTP2515 or water on Day 24 at 2200 h and were sacrificed at 1100 h on Day 25.

Experiment 4: Effects of TTP2515 on metabolic parameters in fed and fasted lean BL6 mice

16-week old C57BL6/J mice on a breeder chow diet were administered either TTP2515 (30 mg/kg) or water twice daily in calorimetry chambers (n=8/group). Mice were fed on Days 1-3 and then fasted. Mice received their last dose of TTP2515 or water at 2100 h on Day 4 and were sacrificed at 0800 h on Day 5.

Experiment 5: Effects of TTP2515 on metabolic parameters in AgRP KO and WT mice on a 45% fat diet and calorimetry parameters in AgRP KO mice

Experiment 5a. 20-week old AgRP KO and WT mice maintained on breeder chow were switched to a 45% high fat diet (n=6-7/group). The next day (24 h later, referred to as Day 1) mice started treatment with either water or TTP2515 (30 mg/kg) in the AgRP KO groups or TTP2515 in the WT group, twice daily for 20 days. Body composition was assessed before treatment and on Day 19 of treatment. Mice received their last dose of TTP2515 or water on Day 20 at 0900 h and mice were sacrificed 6 h later.

Experiment 5b: 5-7 month old AgRP KO mice maintained on a breeder chow diet received either TTP2515 at increasing doses (*Days 1-7*, 30 mg and *Days 7-10*, 50 mg/kg) or water for 10 days in calorimetry chambers (n=8/group).

Experiment 6: Effects of TTP2515 on hyperphagia and metabolic parameters after an acute switch to a very high fat diet in C57BL6/J mice and AgRP KO mice

Experiment 6a: 14-week old C57BL6/J mice maintained on a breeder chow diet were switched to a 60% very high fat diet and simultaneously started treatment with either TTP2515 (30 mg/kg) or water twice daily for 5 days (n=8/group). Body composition was assessed on Day 4 of treatment. Mice received their last dose of TTP2515 or water at 0800 h on Day 6 and were sacrificed 4 h later.

Experiment 6b: 6-7 month old AgRP KO mice maintained on a 10% chow diet were switched to a 60% very high fat diet and simultaneously started treatment with either TTP2515 (5, 15 or 30 mg/kg) or water for 7 days (n=7/group). Body composition was assessed at baseline and on Day 4 of treatment. On the evening of Day 4, mice received TTP2515 or water at 2000 h. On Day 5, submandibular pouch blood was collected at 1100 h and mice continued to receive TTP2515 or water twice daily with the last dose at 2200 h on Day 6. Mice were fasted and blood was collected at 0800 h on Day 7.

Experiment 7: Effects of TTP2515 on refeeding and T4 after fasting in AgRP KO and WT mice

Experiment 7a: 5-7 month old AgRP KO and WT mice (n=7-8/group) maintained on a normal chow diet were utilized in three separate fasting and refeeding experiments. In all experiments,

both AgRP KO and WT mice were fasted for 24 h and then started treatment with either TTP2515 (10, 25, or 50 mg/kg) or water. Mice were refed 1 h after the first dose. Food intake and body weight measurements were obtained at the 3, 9, and 20 h timepoints and a second dose of TTP2515 or water was administered at the 9 h timepoint.

Experiment 7b: 3-4 month old AgRP KO and WT mice maintained on normal chow diet were utilized in this experiment (n=7-9/group) to measure T4 levels after fasting. Before intervention, submandibular pouch blood was collected for baseline values. After recovery, mice were fasted and received 3 doses of TTP2515 or water at 2100 h on Day 1, and at 0900 and 2000 h on Day 2 and another blood sample was obtained at 0700 h on Day 3.

Results

Experiment 1: Effects of TTP2515 on the orexigenic and metabolic effects of icv AgRP injection in rats

Experiment 1a: Rats received either a single icv injection of AgRP or saline on Days 0 and 2, and a single oral gavage of TTP2515 (30 mg/kg) or water on Days 0, 1 and 2. On Day 1, after a single icv injection and oral gavage, food intake and body weight gain were significantly increased in the AgRP +Water group compared to the Saline+Water group ($p<.05$, **Fig 3.1A,B**), however these parameters were blunted by concomitant treatment with TTP2515 (food intake $p<.05$, body weight gain $p=.06$). On Day 2, food intake ($p=.05$) and body weight gain ($p<.05$) increased in the AgRP+Water group and these elevations were blocked in the AgRP+TTP2515 group ($p<.05$). On Day 3, food intake and body weight gain were significantly increased in the AgRP+Water group ($p<.05$), however these increases were attenuated in the AgRP+TTP2515 group ($p=.06$ vs. AgRP+Water) so that this group was not different from saline. On Day 4, the increase in food intake persisted in the ICV AgRP+Water group ($p=.05$) and this was partly reversed in the AgRP+TTP2515 group ($p=.11$), so that this group was not different from saline. No significant differences between the Saline+Water and Saline+TTP2515 group were observed during the entire experiment.

Experiment 1b: Rats were continuously infused icv for 8 days with either AgRP or saline and also received twice daily oral gavage with either TTP2515 (30mg/kg) or water. AgRP significantly stimulated food intake during the entire experiment ($p<.05$), and this increase was partly reversed in the AgRP+TTP2515 group ($p<.05$) during days 2-8 **(Fig 3.1C)**. AgRP

treatment also significantly increased body weight gain during days 2-8 ($p<.05$, **Fig 3.1D**) and this increase was partly reversed in the AgRP+TTP2515 group ($p<.05$ vs. AgRP+Water). At sacrifice, liver weight **(Table 3.1)** and fat pad mass (**Fig 3.1E**) were significantly increased in rats receiving AgRP ($p<.0001$), however this increase was attenuated in the AgRP+TTP2515 group ($p<.05$ vs. AgRP+Water). Total T4 levels were significantly suppressed by AgRP+Water treatment ($p<.01$ vs. Saline+Water, **Fig 3.1F**) and this was reversed in the AgRP+TTP2515 group ($p<.05$). Free T4 levels tended to be suppressed in the AgRP+Water group ($p=.08$ vs. Saline+Water, **Fig 3.1G**) and this was reversed in the AgRP+TTP2515 group ($p<.01$). No differences in total T3 levels were detected (**Table 3.1**). Leptin and insulin levels were significantly elevated in both groups receiving icv AgRP, and concomitant TTP2515 treatment tended to decrease levels of these hormones, however this did not reach significance (**Table 3.1**).

Experiment 2: Effects of TTP2515 on metabolic parameters in DIO mice

Experiment 2a: DIO mice were gavaged with TTP2515 (30 mg/kg) or water twice daily for 21 days. Cumulative food intake and body weight gain were significantly decreased compared to water-treated mice from Day 4 onwards (**Fig 3.2A,B**). On Day 20, the TTP2515-treated mice had significantly reduced percent fat mass compared to water-treated mice ($p<.05$, **Fig 3.2C**) and had lost a greater amount of fat during treatment vs. the water group (-2.6±0.4g vs. -0.5±0.2g, $p<.001$), and tended to have lower absolute fat grams after treatment (12.0±0.9g vs. 9.8±0.8g, $p=.08$). No difference in lean mass was observed between groups after treatment (**Table 3.2**). Mice received their last dose of TTP2515 or water at 800 h on Day 21 and were sacrificed 6 h later. Insulin levels tended to be lower in TTP2515-treated mice after a 6 h fast on Day 15 of the

study (p=0.14) and both insulin (p=0.13) and leptin levels (p=0.13) at sacrifice tended to be lower in TTP2515-treated mice (**Table 3.2**). At sacrifice, total T4, but not T3, levels were significantly elevated by TTP2515-treatment (p<.0001, **Fig 3.2D,E**).

Experiment 2b: A separate group of DIO mice were placed in the calorimetry system and received either TTP2515 (30 mg/kg) or water for 3.5 days. Cumulative food intake (Water 10.5±0.5g vs. TTP2515 10.0±0.6g) and body weight change (Water -0.1±0.2g vs. TTP2515 -0.5±0.4g) were not significantly different in DIO mice receiving TTP2515-treatment for 3.5 days. During the first two nights of treatment, VO2, EE and physical activity were reduced in TTP2515-treated mice compared to the water-treated mice. On the first night, this was only a tendency, (**Table 3.3**) however this effect was significant during the night of day 2 (**Table 3.3**, **Fig 3.2F,G**). Yet, these effects were transient, as VO2, EE and activity were not different during the nights of days 3 and 4 (**Table 3.3**). Furthermore, as the observed decrease in VO2 and EE was likely driven by decreased activity, VO2 and EE were divided by activity to produce a "relative" value. Relative VO2 and EE were significantly higher in TTP2515-treated mice during the dark cycle on day 1 (VO2: 374.2±54.4 vs. 231.2± 22.6 ml/hr/kg per counts; EE: 2.1 ±0.3 vs. 1.3± 0.1 Average W kg^{-1} per counts, p<.05) and day 2 (VO2: 428.1±8.3 vs. 227.0±22.2 ml/hr/kg per counts; EE: 2.4±0.5 vs. 1.3±0.1 Average W kg^{-1} per counts, p<.05) but not different between groups on days 3 and 4. Respiratory quotient (RQ) was not different during days 1-2, however during the light cycle of day 3, mean RQ tended to be lower in TTP2515-treated mice compared to the water group (p<.08), and was significantly lower during the first half of the light cycle (p<.05, **Fig 3.2H**). Mice were removed from the calorimetry system in morning of day 4.

Experiment 3: Effects of TTP2515 on metabolic parameters in leptin-deficient mice

Experiment 3: Leptin-deficient *ob/ob* mice maintained on a normal chow received either TTP2515 at increasing doses (5-50 mg/kg) or water treatment for 25 days. During days 1-13, mice were placed in the calorimetry system. In *ob/ob* mice average daily food intake was decreased with 15 mg ($p=0.06$), 30 mg/kg ($p<0.05$), and 50 mg/kg ($p<0.01$) TTP2515 treatment (**Fig 3.3A**) and significant reductions in body weight were observed at the 50 mg/kg dose ($p<0.05$, **Fig 3.3C**). No effects on VO2, EE or activity were observed during any of the dosing periods except transiently at the 15 mg/kg dose, where mean VO2 and mean EE ($p<0.05$) were decreased during the dark cycle of day 6, with a trend for decreased activity ($p=0.10$, **Table 3.3**). However, this decrease in VO2 and EE was not evident if adjusted for activity. Mean RQ was significantly lower in 50 mg/kg TTP2515-treated mice, but this was not noted at the other doses. The decrease in RQ was observed during the light cycle immediately upon starting the 50 mg/kg treatment on day 11 and was again noted on day 12 (**Fig 3.3D)**.

Mice were fasted during the evening of day 13. Fasting did not significantly increase activity in water-treated mice, however TTP2515-treatment during fasting decreased activity (**Table 3.3**, $p<0.01$), VO2 and EE compared to water-treated mice ($p=.05$, **Table 3.3**). If adjusted for activity, VO2 and EE were increased in the TTP2515-treated group (data not shown, $p<.05$). TTP2515-treated mice also lost significantly less weight during the fast versus water-treated mice (Water -2.3±0.1 vs. TTP2515 -1.5±0.2g, $p<0.01$), which may be secondary to the decreased activity observed. Mice were refed the next morning in their homecages outside calorimetry system. Both groups refed to a similar degree at 22 h (Water 4.0±0.5 vs. TTP2515 3.0±0.5g,

p=0.17) and gained a similar percentage of their original body weight during refeeding (Water 42.8±3.4 vs. TTP2515 25.6±15.9%, p=.31).

On day 21, mice were fasted overnight and a GTT performed the next morning revealed no difference in glucose tolerance between groups (data not shown). MRI performed on day 24 showed that TTP2515-treated mice had significantly less fat mass than water-treated mice ($p<.01$, **Fig 3.3B**); lean mass was not different between groups. Mice received their last dose of TTP2515 or water on Day 24 at 2200 h and were sacrificed at 1100 h on Day 25. At sacrifice, liver weight ($p<.05$) was significantly reduced in TTP2515-treated mice (**Table 3.2**), however plasma glucose, insulin, body temperature (**Table 3.2**) and total T4 levels (**Fig 3.3E**) were not different between groups.

Experiment 4: Effects of TTP2515 on metabolic parameters in fed and fasted lean BL6 mice

Experiment 4: Lean mice on a breeder chow diet were treated with either TTP2515 (30 mg/kg) or water for a total of 4 days in the calorimetry system. Mice were fed *ad lib* and then fasted. In fed mice, TTP2515 treatment did not decrease daily food intake (Water 3.0±0.0 vs. TTP2515 3.1±0.2g) or attenuate weight gain, and weight loss was similar between groups during fasting (**Fig 3.4A**). No significant differences in VO2 or EE were noted during days 1 and 2. However, a small but significant effect of TTP2515 on physical activity was noted during the dark cycle on day 1; this was no longer evident during the dark cycle of day 2 ($p<.05$, **Table 3.3**). Although no differences in RQ were observed during the dark cycle, on Day 3 mean RQ was significantly lower during the light cycle in TTP2515-treated mice ($p<0.05$, **Fig 3.4B**). As mice were fasted from the evening of Day 3 onwards, it is unknown if this reduction in RQ persists. During

fasting, a significant increase in activity was noted in the control water mice during the dark cycle that was not observed in TTP2515-treated mice. Activity in the water group during fasting was elevated versus the fed state (day 2) as well as compared to TTP2515-treated mice during fasting ($p<0.05$, **Fig 3.4C,D**). No difference in VO2 or EE was observed between the TTP2515 and water groups during fasting (**Table 3.3**). Total T4 and T3 levels were similar before treatment in these two groups of mice that were subsequently treated with TTP2515 or water and fasted. After fasting, total T4 and T3 levels were significantly suppressed in both groups. However, after fasting, T4 levels were significantly higher in TTP2515-treated mice compared to water-treated mice, while total T3 levels were similar between groups (**$p<.05$, Fig 3.4E,F**).

To further investigate if TTP2515 modulates metabolic parameters in lean mice, we conducted a separate experiment in which 50 mg/kg TTP2515 or water was administered twice daily via oral gavage to lean, breeder-chow fed WT mice (n=7/group) for 7 days. During this time daily and cumulative food intake (WT 21.8±0.6 vs. TTP2515 21.1±0.4g, p=.59) and daily and cumulative body weight gain (Water -0.4±0.2 vs. TTP2515 -0.2±0.3g, p=.62) were similar between groups and both groups had similar fat and lean mass after 7 days of treatment (data not shown). The last dose of TTP2515 or water was administered at 2000 h on Day 6 and a blood sample obtained at 0900 h on Day 7 revealed a significant increase in total T4 levels in the TTP2515 group compared to water (5.3±0.2 vs. 4.3±0.2 ug/dl, $p<.01$).

These experiments demonstrate that TTP2515 does not modulate food intake or body weight gain in lean, chow-fed mice at doses up to 50 mg/kg. However, chronic TTP2515 treatment did

increase total T4 levels in both fasted and fed lean mice. It is also evident that TTP2515 can transiently suppress physical activity and prevent fasting-induced increases in physical activity.

Experiment 5: Effects of TTP2515 on metabolic parameters in AgRP KO and WT mice on a 45% fat diet and calorimetry parameters in AgRP KO mice

Experiment 5a: AgRP KO mice were treated with either TTP2515 (30 mg/kg) or water and WT mice received TTP2515 (30 mg/kg) for a total of 19 days while receiving a 45% high fat diet. At the start of the experiment, AgRP KO TTP2515 (28.8±0.5g) and AgRP KO water (29.3±0.6g) groups weighed significantly less than the AgRP WT TTP2515 group (32.3±1.2g); however, baseline food intake was similar between groups. Cumulative food intake was significantly lower in the WT TTP2515 group during days 1-7 and in the KO TTP2515 group on days 2-5, compared to the KO water group ($p<0.05$, **Fig 3.5A**) . For the remainder of the experiment, cumulative food intake was similar between all three groups. Similarly, both KO and WT TTP2515-treated groups lost a significant amount of weight compared to the KO water group during most of the treatment period **(Figure 3.5C)**. WT TTP215-treated mice appeared lose a greater amount of weight compared to KO TTP2515 mice, however this was not significant. By day 19 of the experiment, the cumulative weight loss in the WT TTP2515 group was -1.35±0.40g vs. a gain of 0.34±.59 g in the KO water group ($p<.05$), while the KO TTP2515 group lost -0.95±.52g ($p=.09$ vs. KO water). After treatment, the WT TTP2515 group gained significantly less fat compared to the KO water group, while the KO TTP2515 group did not ($p=0.12$, **Fig 3.5B**). Lean mass was similar between all groups after treatment. At sacrifice, both

total and free T4 levels were higher in WT and KO TTP2515-treated mice compared to KO water-treated mice ($p<.05$, **Fig 3.5D,E**).

Experiment 5b: Lean AgRP KO mice maintained on breeder chow diet were administered either TTP2515 (30 mg/kg days 1-7, 50 mg/kg days 7-10) or water in the calorimetry system. During the 30 mg/kg treatment period, no significant effects on average daily food intake (Water 3.2±0.2 vs. TTP2515 2.9±0.1) or body weight were observed (Water 27.2±0.8 vs. TTP2515 26.6±0.5g). During the 30 mg/kg dosing period, RQ was transiently lower during the dark cycle on days 1 and 2 ($p<0.05$, data not shown) in the TTP2515 group and was not different during any other point in the experiment (**Fig 3.5H**). On day 3, VO2, EE and activity were transiently lower in TTP2515-treated mice during the dark cycle ($p<0.05$, **Fig 3.5F,G; Table 3.3**); this is similar to what has been observed in lean, DIO and *ob/ob* mice in *Exp 3-5*. The reduction in activity persisted during the light and dark cycle of Day 4 ($p<.05$) and during the dark cycle on Day 6 ($p=.08$), without any change in VO2 or EE (**Table 3.3**). Adjustment for activity showed that relative VO2 and EE were equivalent between groups during the dark cycle on day 4 (data not shown) and tended to be higher in TTP2515-treated mice during the dark cycle on day 6 (data not shown, $p=.05$). No other differences in calorimetry parameters were observed during the 30 mg/kg treatment period. During the 50 mg/kg treatment period, average daily food intake was similar between groups (Water 3.0±0.1 vs. TTP2515 2.8±0.1g, $p=.30$) and body weight was not different (Water 27.3±0.9 vs. 26.5 ±0.5, $p=0.40$), although the TTP2515-treated group tended to gain significantly less weight by day 10 (Water 0.2±0.2 vs. TTP2515 -0.4±0.2g, $p=.05$). At the 50 mg/kg dose, there were no significant differences in any calorimetry parameters except activity, which tended to be lower in TTP2515-treated mice on day 8 during the dark cycle

(p=.08), day 9 during the light (p=.05) and dark cycle (p=.08) and day 10 dark cycle (p<.05), without any change in VO2 or EE (**Table 3.3**). If adjusted for activity, relative VO2 and EE were higher in the TTP2515 group vs. water, or not significantly different between groups on these days (data not shown, Day 8 p<.05, Day 9 p=.05, Day 10 p=.32).

Experiment 6: Effects of TTP2515 on hyperphagia and metabolic parameters after an acute switch to a very high fat diet in C57BL6/J mice and AgRP KO mice

Experiment 6a: Lean, BL6 mice maintained on breeder chow were all switched to a 60% fat diet and simultaneously started treatment with either TTP2515 (30 mg/kg) or water. After switching to a high-fat diet, water-treated mice significantly increased their caloric intake (p<.0001, baseline vs. day 1) while the TTP2515-treated mice significantly decreased their caloric intake (p<.05, baseline vs. day 1). Furthermore, daily food was significantly lower in TTP2515-treated mice compared to water-treated mice during days 1-2 (p<.001), however by day 3 daily food intake was similar between groups. Cumulative caloric intake was significantly lower in TTP2515-treated mice versus water-treated mice during the entire treatment period (p<.01, **Fig 3.6A**). Cumulatively, water-treated mice gained weight after switching to the higher-fat diet; oppositely, TTP2515-treated mice lost weight (p<.01, **Fig 3.6B**). A body composition measurement on day 4 revealed that TTP2515-treated mice had significantly lower fat mass compared to water-treated mice (p<.0001, **Fig 3.6C**), while lean mass was equivalent between groups (Water 19.6±0.5 vs. TTP2515 19.7±0.4g). At sacrifice, leptin (p=0.12) and insulin levels were not significantly different between groups, however total T4 levels were higher (p<.0001) in TTP2515-treated mice (**Fig 3.6D-F**).

Experiment 6b: AgRP KO mice were all switched from a 10% fat chow diet to a 60% very high fat diet and simultaneously started treatment with either water or TTP2515 at 5, 15, or 30 mg/kg. On day 1, after switching AgRP KO mice to the high fat diet, the water group's caloric intake increased slightly, although not significantly. In contrast, the 30 mg/kg TTP2515 treatment group consumed significantly fewer calories vs. baseline ($p<.001$ vs. baseline values). On day 1, the 15 mg/kg and 30 mg/kg TTP2515 groups dose-dependently consumed fewer calories than the water group ($p<0.05$ water vs. both 15 and 30 mg/kg groups, $p<0.05$ 15 mg/kg vs. 30 mg/kg dose) (**Fig 3.6G**). Daily caloric intake was also significantly lower on days 2 and 3 in the 15 and 30 mg/kg groups ($p<0.01$) versus saline. Cumulative food intake was significantly lower in 15 mg/kg and 30 mg/kg TTP2515 groups compared to water during the entire study ($p<0.05$, **Fig 3.6G**). The 5 mg/kg group also tended to have lower cumulative food intake by day 5 ($p=0.07$). Cumulatively, water-treated mice gained weight after switching to the higher-fat diet; oppositely, TTP2515-treated mice lost weight ($p<0.01$, **Fig 3.6H**). MRI on day 5 revealed that fat mass was significantly lower in the 15 and 30 mg/kg groups compared to water ($p<.01$, **Fig 3.6I**) and the 5 mg/kg group tended ($p=.05$) have lower fat mass vs. water. Lean mass was similar between groups (data not shown). After 5 days of treatment, total T4 levels were not different between groups; however fasting with treatment revealed a significant and dose-dependent increase in total T4 levels in TTP2515-treated mice (**Fig 3.6J**).

These experiments indicate the TTP2515 treatment yields a potent, anorexic effect in both AgRP KO and WT mice that are simultaneously switched to a high fat diet. The anorexic effects persist for approximately 2-3 days, after which time daily food intake was equivalent between the TTP2515 and water groups. However, cumulative food intake and weight gain continued to

be suppressed in TTP2515-treated mice demonstrating that these mice do not catch-up to their water-treated counterparts.

Experiment 7: Effects of TTP2515 on refeeding and T4 after fasting in AgRP KO and WT mice

Experiment 7a: In all experiments, baseline food intake was similar between groups, however AgRP WT mice were approximately 3 g heavier than AgRP KO mice ($p<.001$). In three separate experiments, mice were fasted for 24 h and received a single dose of TTP2515 (10, 25, or 50 mg/kg) or water the next morning and were refed 1 h later. Food intake and body weight measurements were obtained at 3, 9 and 20 h after refeeding and a second dose of TTP2515 or water was administered at the 9 h timepoint. The 10 mg/kg dose did not attenuate refeeding or body weight gain in either KO or WT TTP2515-treated mice compared to their respective water counterparts at any of the timepoints measured (**Fig 3.7A,B**). At the 25 mg/kg dose, no significant differences in refeeding or body weight gain were observed at the 3 h timepoint. However, at the 9 h timepoint WT TTP2515-treated mice gained significantly less weight than WT water-treated mice ($p<.01$, **Fig 3.7C,D**), but no difference between KO water-treated and KO TTP2515-treated mice was observed. There were no significant differences in food intake at the 9 h timepoint. At the 20 h timepoint, WT TTP2515 mice refed significantly less than WT water-treated mice ($p<0.05$), however this effect was not observed between KO water and KO TTP2515-treated mice ($p=0.32$). No significant differences in body weight gain were noted at the 25 mg/kg dose. At the 50 mg/kg dose, at both the 3 and 9 h timepoints, no significant differences in food intake or body weight gain were observed. However, at the 9 h timepoint, there was a trend for WT TTP2515-treated mice to refeed less than WT water-treated mice

($p=0.05$, **Fig 3.7E,F**). At the 20 h timepoint, both WT and KO TTP2515-treated mice refed significantly less than their respective water counterparts ($p<0.05$). At this time WT TTP2515 treated mice also gained significantly less weight than WT water treated mice ($p<0.05$) while KO water and TTP2515 groups ($p=0.21$) gained similar amounts of body weight.

Thus the 25 mg/kg TTP2515 dose attenuated refeeding specifically in WT, but not AgRP KO mice without any significant effect on weight gain. At the 50 mg/kg dose, TTP2515-treatment attenuated refeeding in both AgRP KO and WT mice and only significantly attenuated weight gain in the WT but not the AgRP KO mice.

Experiment 7b: AgRP KO and WT mice were fasted and received either TTP2515 at 50 mg/kg or water during this time. The last dose of TTP2515 was administered at 2000 h and blood samples were collected at 0700 h the next day. Baseline total T4 levels before fasting and treatment were not significantly different between groups. After fasting and TTP2515 treatment, total T4 levels were not significantly different between groups (**Fig 3.7G**).

Discussion

TransTech Pharma Inc. showed that an AgRP inhibitor was able to prevent AgRP-induced suppression of cAMP in an *in vitro* model. *In vivo,* we showed that the AgRP inhibitor TTP2515 was able to attenuate the effects of centrally infused AgRP when administered to rats by oral gavage. TTP2515 blocked AgRP-induced increases in food intake, weight gain and adiposity, as well as suppression of total T4 levels. The effects on food intake and body weight gain were observed in two different experiments using either single icv AgRP injections or continuous icv AgRP infusion. These effects were not observed in icv saline-treated rats in either experiment and animals receiving TTP2515 appeared to consume food and behave normally.

Subsequent experiments utilized TTP2515 in mouse models to investigate its effects on endogenous AgRP. Although TTP2515 did not reduce food intake, body weight gain or adiposity in lean wild-type mice at either the 30 mg/kg or 50 mg/kg dose, in DIO mice, TTP2515 effectively reduced these parameters. This is consistent with the expected actions of an AgRP inhibitor resulting in increased MC4-R activity. These effects are similar to what has been reported in DIO mice overexpressing MSH (51). However, the effects of TTP2515 on food intake and body weight could not be attributed to AgRP inhibition as an attenuation in these parameters was also observed in DIO AgRP KO mice treated with TTP2515. There was a tendency for WT TTP2515-treated mice to lose more weight than KO TTP2515-treated mice but this was not significant. However, WT TTP2515-treated mice gained significantly less fat mass during the experiment compared to KO water-treated mice, while the KO TTP2515-treated mice and water treated mice gained more similar amounts, suggesting that TTP2515 is somewhat

more effective in WT mice in preventing fat mass accretion, compared to KO DIO mice. Furthermore, a significant decrease in RQ during the light cycle was also noted in TTP2515-treated DIO mice, consistent with predicted changes in adiposity and energy partitioning that might be expected with AgRP inhibition. The effects of TTP2515 on RQ noted in WT mice during the light cycle were not observed when KO mice were treated with either 30 mg/kg or 50 mg/kg TTP2515. This data suggests that the effects of TTP2515 on energy partitioning may be specific for AgRP antagonism and is consistent with findings from a report on AgRP KO mice (128). In contrast to what would be predicted, TTP2515-treated DIO mice displayed a transient decrease in VO2 and EE which could be explained by the concomitant unexpected decrease in physical activity observed. In fact, if adjusted for activity, VO2 and EE were no longer suppressed in TTP2515-treated mice. These transient effects on VO2, EE and activity were also evident in TTP2515-treated AgRP KO mice and thus could not be attributed to AgRP antagonism. Thus, TTP2515 treatment reduced food intake, body weight gain and adiposity in both AgRP KO and WT mice on a high-fat diet, but not in mice on a chow diet and reductions in adiposity and RQ observed in high-fat diet fed mice appeared to be more pronounced in WT than KO mice.

TTP2515 was extremely effective in preventing weight gain in obese leptin-deficient mice on a chow diet that have increased hypothalamic AgRP expression (120). Significant, dose-dependent reductions in food intake and body weight gain as well as decreases in adiposity and RQ were noted. It is unknown if these effects are specific for AgRP antagonism as we did not study leptin-deficient AgRP KO mice. However, the same 50 mg/kg dose that potently reduced food intake, weight gain, and fat mass accretion in *ob/ob* mice failed to modulate any of these

parameters in lean wild-type mice on a chow diet. These data suggest that the effects observed in *ob/ob* mice could be due to AgRP antagonism.

TTP2515 was quite effective under fasting conditions where AgRP expression is increased in the hypothalamus (289, 290, 298). Dose response studies show that TTP2515 can attenuate rebound feeding in mice at both a 25 mg/kg and 50 mg/kg dose. At 25 mg/kg TTP2515 attenuated rebound feeding in WT mice, but not in KO mice, and at the 50 mg/kg dose, TTP2515 attenuated body weight gain in WT mice, but not KO mice, indicating that these effects appear to be relatively specific for AgRP antagonism. As attenuated rebound feeding at the 50 mg/kg dose was observed in both KO and WT mice, this suggests that higher doses may yield non-specific effects. Furthermore in lean BL6 mice, TTP2515 treatment attenuated fasting-induced increases in activity. This is in agreement with previous data that has shown that activating the AgRP neuron drives food-seeking behavior (134). Unfortunately, activity was not studied in AgRP KO mice during fasting.

Several lines of evidence demonstrate a role for the melanocortin system in modulating the hyperphagic response initially observed after switching mice to a high diet. First, switching BL6 mice from a normal chow diet to a higher fat diet (42-45%) produces an initial hyperphagic response that is quickly downregulated (297, 299); however MC4R knockout mice are unable to downregulate their food intake after initiation of the high fat diet (297, 299). Secondly, AgRP increases fat intake (300, 301), while MTII decreases preferential fat consumption (301-303). Furthermore, A^y mice and MC4-R KO rats have an increased preference for fat (143, 304). In addition, *Agrp* mRNA levels decline after mice are switched to a high fat diet (305). We

therefore hypothesized that TTP2515 would be particularly effective in attenuating the acute effects after initiation of a high fat diet. Introduction of the 60% high fat diet to mice that simultaneously started TTP2515-treatment significantly reduced caloric intake; this reduction was significant compared to both baseline chow diet intake and versus water treatment. Mice treated with TTP2515 recover after approximately 3 days and resume normal daily food intake, however lower cumulative food intake and body weight gain persist in TTP2515-treated mice after 5 days. This effect is quite remarkable given that mice maintained on a breeder chow diet and administered TTP2515-treatment are unaffected by the compound. This data suggests that TTP2515 has a potent effect on fat intake. However, surprisingly, TTP2515 was equally effective when the same experiment was conducted in AgRP KO mice. Thus this effect is clearly not due to AgRP antagonism. The dose-responsive effect observed in KO mice suggests that TTP2515 may bind to a similar, perhaps yet unknown, target which strongly promotes fat intake. As the effect on fat intake is initiated so rapidly and potently, it suggests that TTP2515 may even have a local effect in the gut in the presence of increased dietary fat that leads to the suppression of food intake. Regardless of the mechanism, these results are of interest as compounds that promote decreased dietary fat intake may have clinical utility.

It was also predicted that an AgRP inhibitor would increase thyroid hormone levels under certain conditions given the role that AgRP plays in regulating the thyroid axis and mediating the fasting induced suppression of T4 and T3 levels (111, 189, 192). Additionally, in one study, AgRP KO mice were reported to have higher total T4 and T3 levels (128). In the current study icv AgRP suppressed plasma T4 levels (as expected), but this was prevented by TTP2515 treatment. TTP2515 also increased total and free T4 levels in lean and DIO mice when the animals were

treated for at least 4 days. T4 was also increased after TTP2515 treatment in fasted mice, however this was only observed in an experiment when the drug was administered for 4 days and not when it was administered for 1 ½ days. Of note, TTP2515 had no effect on T3 levels at any time in any experiment. Given that T4 measurements are notoriously subject to technical problems with a variety of assays, especially in the presence of drugs that can affect binding proteins and assay performance, the validity of the T4 measurements comes into question. In any case, the effects of TTP2515 on T4 levels are not specific for AgRP antagonism. Both TTP2515-treated KO and WT mice had elevated free and total T4 levels compared to KO mice receiving water.

Previous studies have utilized genetic models of AgRP deletion or AgRP neuronal ablation to uncover AgRP's effects on energy balance and metabolism. However, the interpretation of these studies is complicated by developmental compensation and the loss of other neurotransmitters from AgRP neurons (127, 128, 137). This is the first report of a pharmacological inhibitor of the AgRP peptide that could be used to help elucidate the role of AgRP in regulating energy homeostasis. The effects of TTP2515 are most prominent under conditions in which AgRP is known to be elevated such as during fasting and with leptin deficiency (120, 289, 290, 298). TTP2515 also affected feeding and metabolic responses after acute or chronic exposure to a high fat diet, conditions that can be modulated by the melanocortin system. Although TTP2515 demonstrated some clear off-target effects in AgRP KO mice, there was some relative specificity to the effects on adiposity and RQ as well as on re-feeding after fasting that are consistent with AgRP's orexigenic and metabolic function. The cause of the off-target effects are at present unclear. It is of interest that most of the non-AgRP mediated effects observed are still consistent

with what would be expected with stimulation of the melanocortin system. It may be that TTP2515 also binds to a peptide of similar function to AgRP that modulates food intake, energy balance and preference for fat intake. Although Agouti is a potential candidate as it possesses 25% sequence homology to AgRP (120), in mice it is expressed in the skin and functions in a paracrine manner and is not known to regulate energy balance unless ectopically expressed as in mice with the A^y mutation (306). However interactions with other related peptides or non-peptides is clearly possible. Future studies should be directed at uncovering the mechanism by which TTP2515 causes such potent effects favoring negative energy balance even in the absence of AgRP.

The rationale for using an AgRP inhibitor rather than an MC4-R agonist to increase melanocortin signaling relates to the fact that AgRP neuronal projections are more restricted to brain areas regulating energy balance and will affect only a subset of MC-Rs compared to the wide distribution of brain MC4-Rs that would be affected by an MC4-R agonist (292, 293). Although MSH agonists are effective, they have been associated with a number of side effects, including increased blood pressure, due to the extensive distribution of brain MC-Rs (295, 296). Furthermore, an AgRP inhibitor has the potential to be utilized in a therapeutic capacity, particularly under conditions in which AgRP is elevated. In humans, this might be utilized optimally during diet and weight loss or during weight maintenance after weight loss has been achieved. Additionally, the potent effect of TTP2515 on fat consumption suggests that this peptide could also be advantageous before consuming a fat-rich meal to attenuate high-fat diet food intake.

In summary, when administered orally TTP2515 exerts numerous metabolic effects in rats and mice. Many of these effects are consistent with AgRP inhibition, yet in some cases are evident in AgRP null mice indicating off target effects of this drug. TTP2515 is most effective in obese models including DIO and *ob/ob* mice, as well as during consumption of a high fat diet and does not cause weight loss in lean mice on a low fat diet. Thus the usefulness of this drug in treating human obesity deserves further exploration. Future studies are needed to define the AgRP dependent and independent mechanisms by which this drug exerts its effects on energy balance.

TABLE 3.1: *Effects of TTP2515 on hormone levels and liver weight in rats receiving icv AgRP infusion*

	Saline+Water	**AgRP+Water**	**AgRP+TTP2515**	**Saline+TTP2515**
Leptin (ng/ml)	2.7±0.4	21.9±0.8*	18.5±4.2*	2.3±0.3
Insulin (ng/ml)	2.7±0.6	12.6±2.0*	9.7±2.2*	1.6±0.3
Plasma Glucose (mg/dl)	162.1±4.5	170±7.0	159.3±8.0	150.6±4.5
Liver Weight (g)	14.1±0.7	25.0±1.1*	21.8±1.7*†	13.7±0.5
Total T3 (ng/dl)	49.4±3.2	40.5±4.0	40.1±4.6	44.7±4.5

All measurements reported at sacrifice in *ad lib* fed rats. Values are mean ± SE. *p<.05 all groups; †p<.05 vs. AgRP+Water

TABLE 3.2: *Effects of TTP2515 in DIO and leptin-deficient mice*

	Water	**TTP2515**
Exp 3: DIO mice		
Lean Mass (Day 20) (g)	19.8±0.4	19.9±0.6
Glucose-Day 15 (ng/dl)	161.0±4.4	164.0±3.9
Insulin-Day 15 (ng/ml)	2.4±0.5	1.6±0.2
Insulin (ng/ml)	2.0±0.3	2.1±0.7
Leptin (ng/ml)	25.0±2.6	23.3±3.8
Liver Weight (g)	1.3±0.1	1.3±0.1
Liver Weight as a % of Body Weight (%)	3.5±0.1	3.8±0.1
Exp 4: Leptin-deficient mice		
Glucose (ng/dl)	452.9±34.6	479.7±34.5
Insulin (ng/ml)	6.1±1.3	6.6±0.7
Liver weight (g)	2.9±0.1	2.3±0.2*
Liver Weight as a % of Body Weight (%)	5.7±0.2	5.3±0.3
Body Temperature (ºC)	32.8±0.3	32.6±0.2

All measurements reported at sacrifice in *ad lib* fed mice unless otherwise indicated. Values are mean ± SE. *p<.05 vs. water

TABLE 3.3: *VO2, EE and activity during the dark cycle in calorimetry experiments*

	Average VO2 *(ml/kg/hr)*		**Average EE** *(W kg^{-1})*		**Locomotor activity** *(total counts)*	
Exp 3b: DIO mice	*Water*	*TTP2515*	*Water*	*TTP2515*	*Water*	*TTP2515*
Day 1: Dark Cycle	3277±112	3096±80	18.3±0.6	17.3±0.5	15462±1952	10094±1858
Day 2: Dark Cycle	3335±103	2992±92*	18.6±0.6	16.7±0.5*	15746±1571	9646±2366*
Day 3: Dark Cycle	3170±119	3058±81	17.7±0.7	17.1±0.5	13773±1541	12710±2671
Day 4: Dark Cycle	3208±89	3232±119	17.9±0.5	18.0±0.7	13561±1924	12181±2593
Exp 4: Leptin-deficient mice						
Day 6 Dark Cycle	2291±110	2007±68*	13.2±0.6	11.5±0.4*	4271±620	2809±539
Day 12 Dark Cycle	2072±51	1924±74	11.8±0.3	11.0±0.4	4517±964	3029±413
Day 13 Dark Cycle (Fasting)	1406±36	1262±57†	7.7±0.2	6.9±0.3†	5328±916	1852±298**
Exp 5: Fed/fasted lean BL6 mice						
Day 1 Dark Cycle	2963±63	2824±46	17.1±0.4	16.3±0.3	10625±1772	6249±811*
Day 2 Dark Cycle	2979±72	2894±28	17.1±0.4	16.7±0.2	8592±1264	7223±983
Day 3 Dark Cycle	2440 ±106	2420 ±52	13.5±0.6	13.3±0.3	19086±4939	10664±2218
Day 4 Dark Cycle	2589±108	2450±44	14.3±0.6	13.5±0.3	22196±5323	9410±1773*
Exp 6b: AgRP KO mice						
Day 3 Dark Cycle	3232±127	2936±50*	18.7±0.8	16.9±0.3*	18331±4147	8137±1410*
Day 4 Dark Cycle	3141±140	3008±74	18.2±0.8	17.4±0.4	19671±4254	8634±2317*
Day 6 Dark Cycle	3111±134	2961±83	18.0±0.8	17.1±0.5	20756±6871	7769±1131
Day 8 Dark Cycle	3002±107	2917±83	18.0±0.7	17.4±0.5	18542± 3809	10767±1770
Day 9 Dark Cycle	3069±119	2913±63	17.7±0.7	16.8±0.4	21404±7107	8209±1377
Day 10 Dark Cycle	3037±99	2945±81	17.5±0.6	17.0±0.5	16181± 3614	7462±1357*

In all VO2 and EE measurements above, adjustment for activity rendered VO2 and EE either significantly higher in the TTP2515 group or equivalent between groups. W, Watts; Values are mean ± SE. **p<.01, *p<.05, †p=.05 vs. water

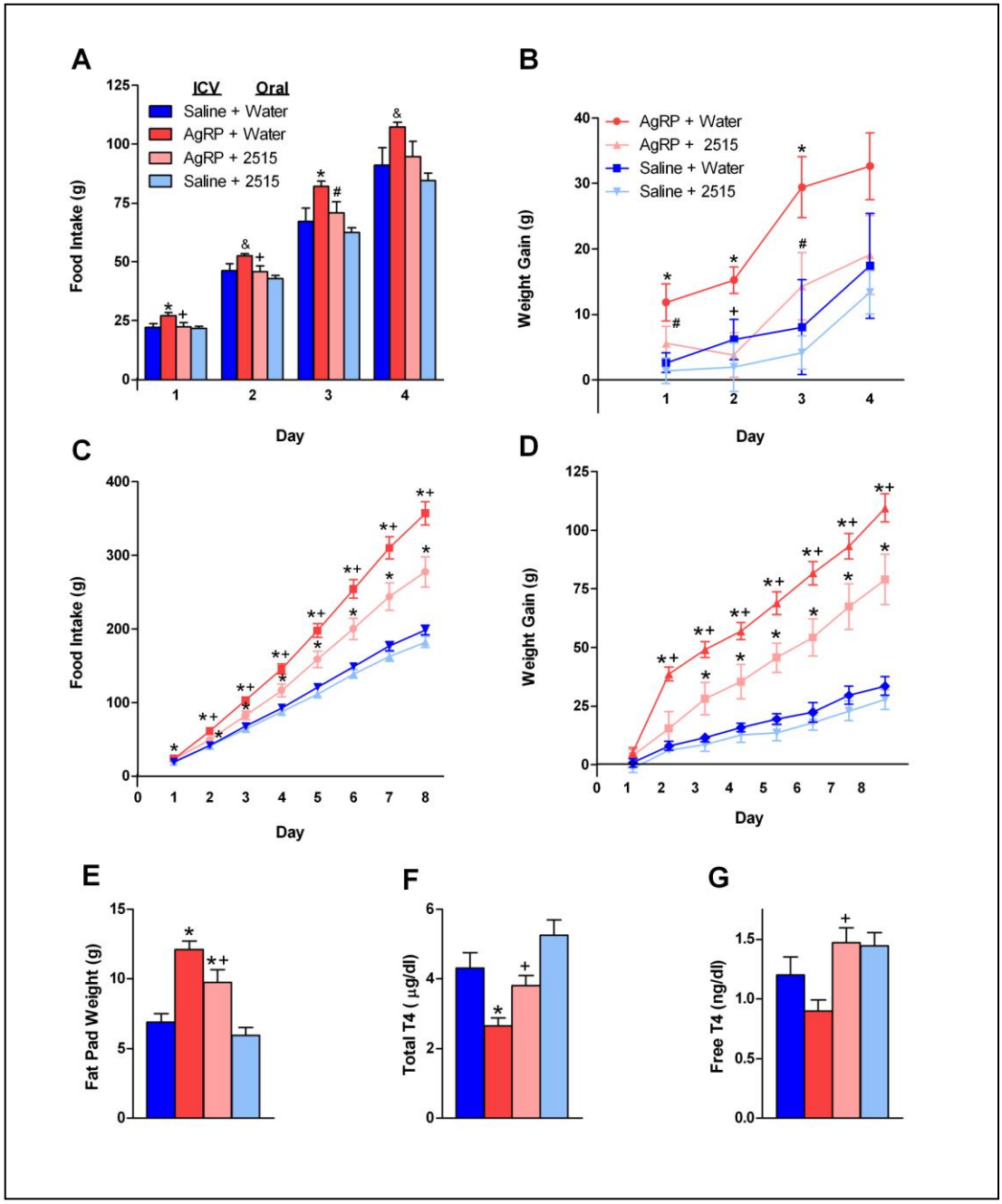
A
ICV
Oral
Saline + Water
AgRP + Water
AgRP + 2515
Saline + 2515
Food Intake (g)
Day
B
AgRP + Water
AgRP + 2515
Saline + Water
Saline + 2515
Weight Gain (g)
Day
C
Food Intake (g)
Day
D
Weight Gain (g)
Day
E
Fat Pad Weight (g)
F
Total T4 (µg/dl)
G
Free T4 (ng/dl)

Figure 3.1. **Effects of TTP2515 on body weight and food intake in rats receiving icv AgRP**. **(A-B)** Rats received a single icv injection of 0.2 nmol AgRP or saline on Days 0 and 2 and received 30 mg/kg TTP2515 or water via oral gavage once per day during Days 0-2 (n=6-7/group). **(A)** AgRP+Water increased food intake and this was reversed in the AgRP+TTP2515 group. **(B)** AgRP+Water increased weight gain during days 1-3 and this was reversed in the AgRP+TTP2515 group. **(C-G)** In a separate experiment, rats were continuously infused icv with either 0.64 nmol/day AgRP or saline and gavaged twice daily with either water or TTP2515 at 30 mg/kg (n=7-8/group). **(C)** AgRP significantly increased food intake during the entire treatment period and this was partly blocked in the AgRP+TTP2515 group during days 2-8. **(D)** AgRP significantly increased weight gain during days 2-8 and this was partly blocked in the AgRP+TTP2515 group. **(E)** Fat pad mass was significantly increased by AgRP and this was partly blocked in the AgRP+TTP2515 group. **(F)** Total T4 levels were significantly suppressed in the AgRP+Water group and this suppression was almost entirely reversed in the AgRP+TTP2515 group. **(G)** Free T4 levels tended to be suppressed in the AgRP+Water group (p=.08 vs Saline+Water) and this suppression was significantly reversed in the AgRP+TTP2515 group.

&p=.05, *p<.05 vs Saline+Water; #p=.06, +p<.05 AgRP+Water *vs* AgRP+TTP2515

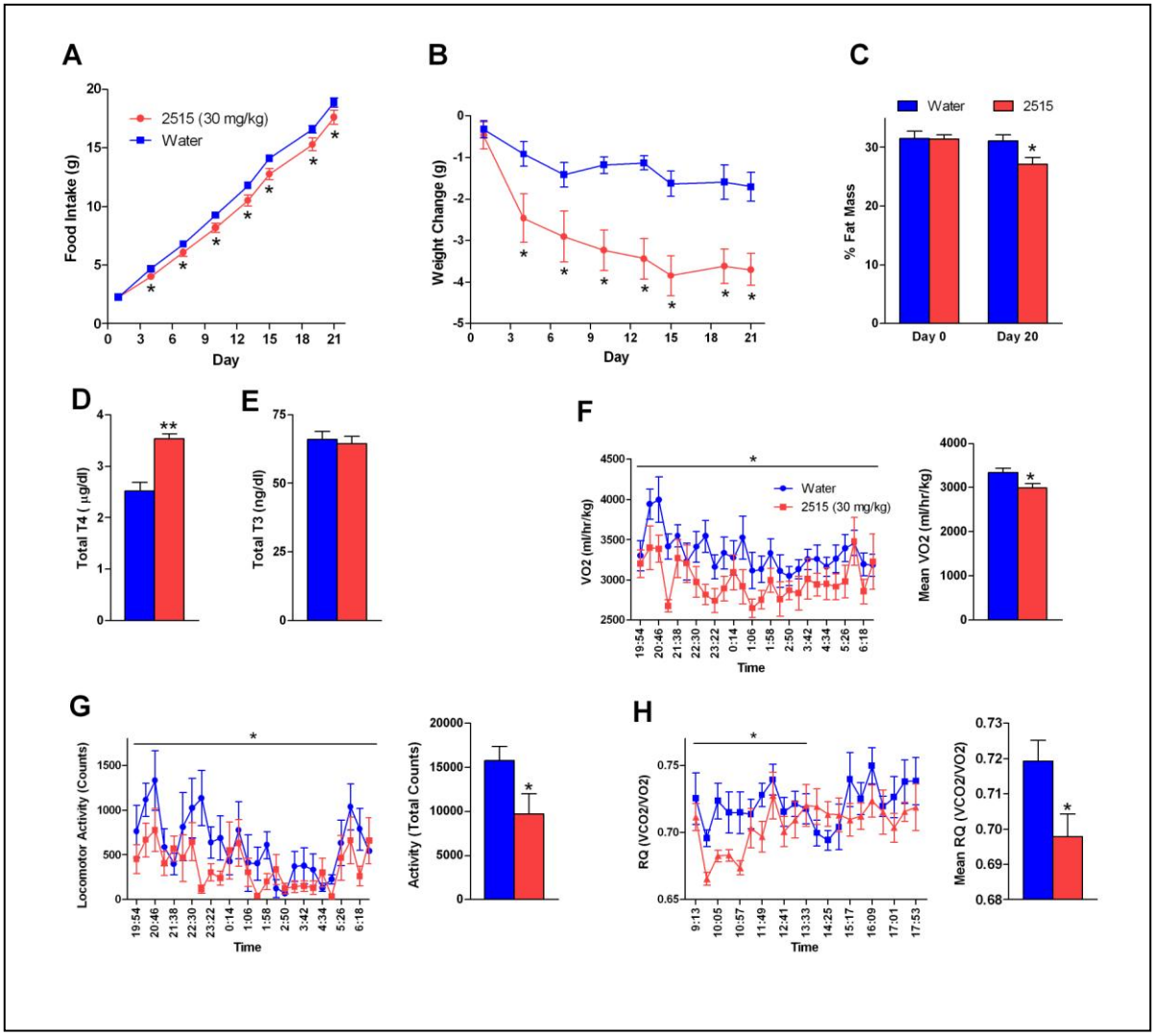
A
Food Intake (g)
2515 (30 mg/kg)
Water
Day
B
Weight Change (g)
Day
C
% Fat Mass
Water
2515
Day 0
Day 20
D
Total T4 (µg/dl)
E
Total T3 (ng/dl)
F
VO2 (ml/hr/kg)
Water
2515 (30 mg/kg)
Time
Mean VO2 (ml/hr/kg)
G
Locomotor Activity (Counts)
Time
Activity (Total Counts)
H
RQ (VCO2/VO2)
Time
Mean RQ (VCO2/VO2)

Figure 3.2. Effects of TTP2515 on metabolic and calorimetry parameters in DIO mice. (A-E) DIO mice (16 weeks on 45% fat diet) received TTP2515 (30 mg/kg) or water twice daily via oral gavage (n=7-8/group). **(A)** TTP2515 treatment significantly decreased food intake from day 4 onwards. **(B)** TTP2515-treated mice lost significantly more weight from day 4 onwards. **(C)** Percent fat mass was significantly lower in TTP2515-treated mice at the end of the study. **(D)** TTP2515-treated mice had elevated total T4 levels. **(E)** Total T3 levels were similar between groups. **(F-H)** In a separate experiment DIO mice (45% fat diet for 15 weeks) received TTP2515 (30 mg/kg) or water twice daily via oral gavage (n=8/group). **(F,G)** Mean oxygen consumption and total activity were significantly decreased during the dark cycle of day 2 in TTP2515-treated mice. **(H)** Mean respiratory quotient was significantly decreased during the first half of the light cycle of day 3 in TTP2515-treated mice. **p<.0001, *p<.05 vs. water

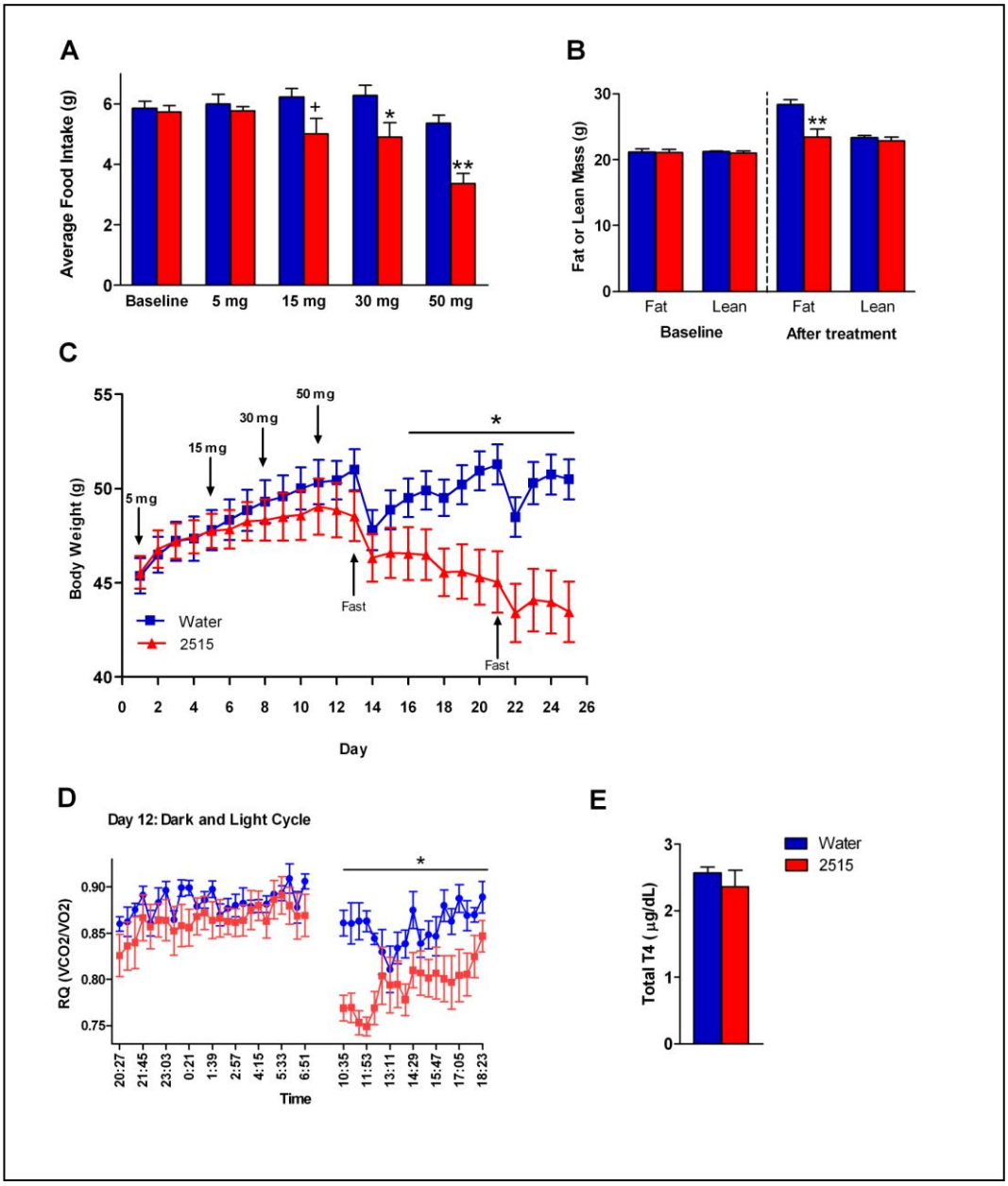
A
Average Food Intake (g)
Baseline
5 mg
15 mg
30 mg
50 mg
B
Fat or Lean Mass (g)
Fat
Lean
Fat
Lean
Baseline
After treatment
C
Body Weight (g)
5 mg
15 mg
30 mg
50 mg
Fast
Fast
Water
2515
Day
D
Day 12: Dark and Light Cycle
RQ (VCO2/VO2)
Time
E
Water
2515
Total T4 (µg/dL)

Figure 3.3. **Effects of TTP2515 on metabolic and calorimetry parameters in leptin-deficient mice.** Leptin-deficient mice received either TTP2515 at increasing doses (5-50 mg/kg) or water twice daily via oral gavage (n=7/group). **(A)** Food intake tended to be lower in TTP2515-treated mice during the 15 mg/kg treatment period and was significantly lower during the 30 and 50 mg/kg treatment periods. **(B)** Fat mass was significantly decreased after 24 days of TTP2515 treatment with no difference in lean mass. **(C)** Body weight gain was significantly lower in TTP2515-treated mice on the 50 mg/kg dose. **(D)** Immediately upon starting the 50 mg/kg dose, mean respiratory quotient was significantly decreased in TTP2515-treated mice during the light cycle (day 12). **(E)** Total T4 levels at sacrifice were unchanged between groups. +p=.06, *p<.05, **p<.01 vs water

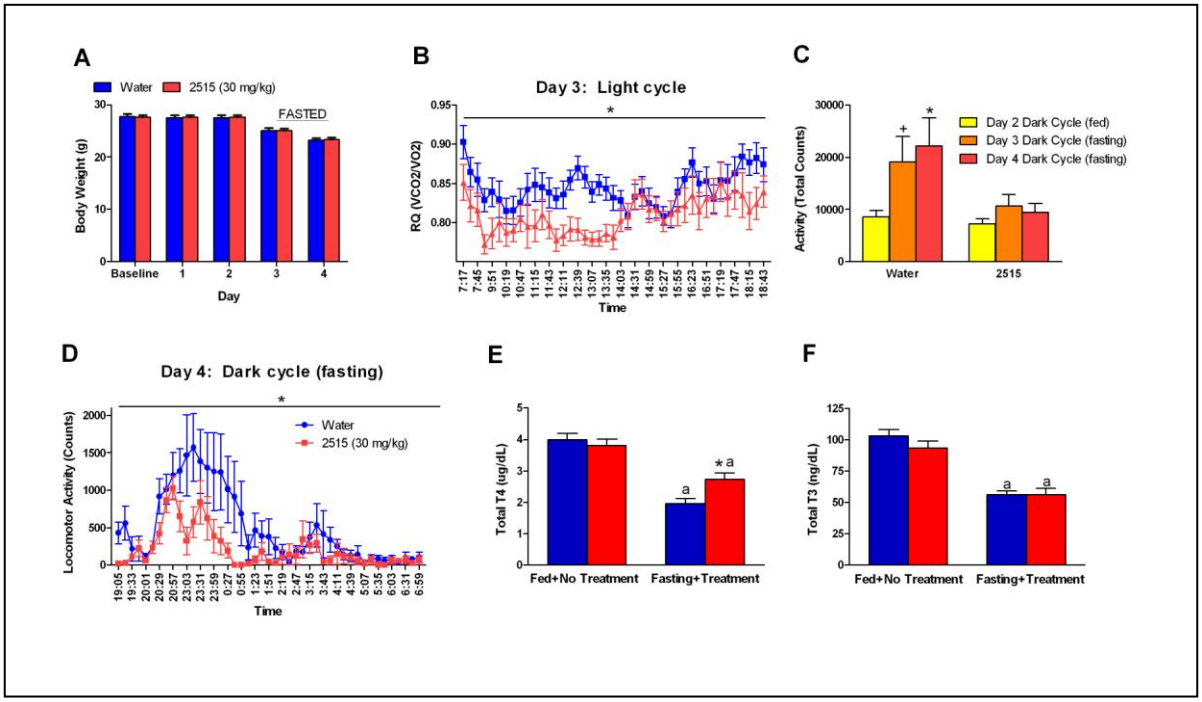
A
Water
2515 (30 mg/kg)
FASTED
Body Weight (g)
Baseline
1
2
3
4
Day
B
Day 3: Light cycle
RQ (VCO2/VO2)
Time
C
Day 2 Dark Cycle (fed)
Day 3 Dark Cycle (fasting)
Day 4 Dark Cycle (fasting)
Activity (Total Counts)
Water
2515
D
Day 4: Dark cycle (fasting)
Water
2515 (30 mg/kg)
Locomotor Activity (Counts)
Time
E
Total T4 (ug/dL)
Fed+No Treatment
Fasting+Treatment
F
Total T3 (ng/dL)
Fed+No Treatment
Fasting+Treatment

Figure 3.4. Effects of TTP2515 on metabolic and calorimetry parameters in lean fed and fasted mice. Lean, chow-fed mice received either TTP2515 (30 mg/kg) or water twice daily via oral gavage during both *ad lib* feeding and fasting (n=8/group). **(A)** Body weight was not different between groups during either the fed or fasted periods. **(B)** During the light cycle of day 3, mean RQ was significantly suppressed in TTP2515-treated mice. **(C)** During fasting, total activity was elevated in water-treated, but not TTP2515-treated mice during the dark cycle. **(D)** During fasting, total activity was significantly lower in TTP2515-treated mice compared to the water group during the dark cycle. **(E)** After fasting, total T4 levels were significantly suppressed in both groups, however total T4 levels were significantly higher in TTP2515-treated mice. **(F)** Total T3 levels were suppressed by fasting and fasted levels were similar between TTP2515 and water-treated mice.

+p=.06 vs respective Day 2 Dark Cycle; *p<.05 vs water, ap<.05 vs respective fed treatment

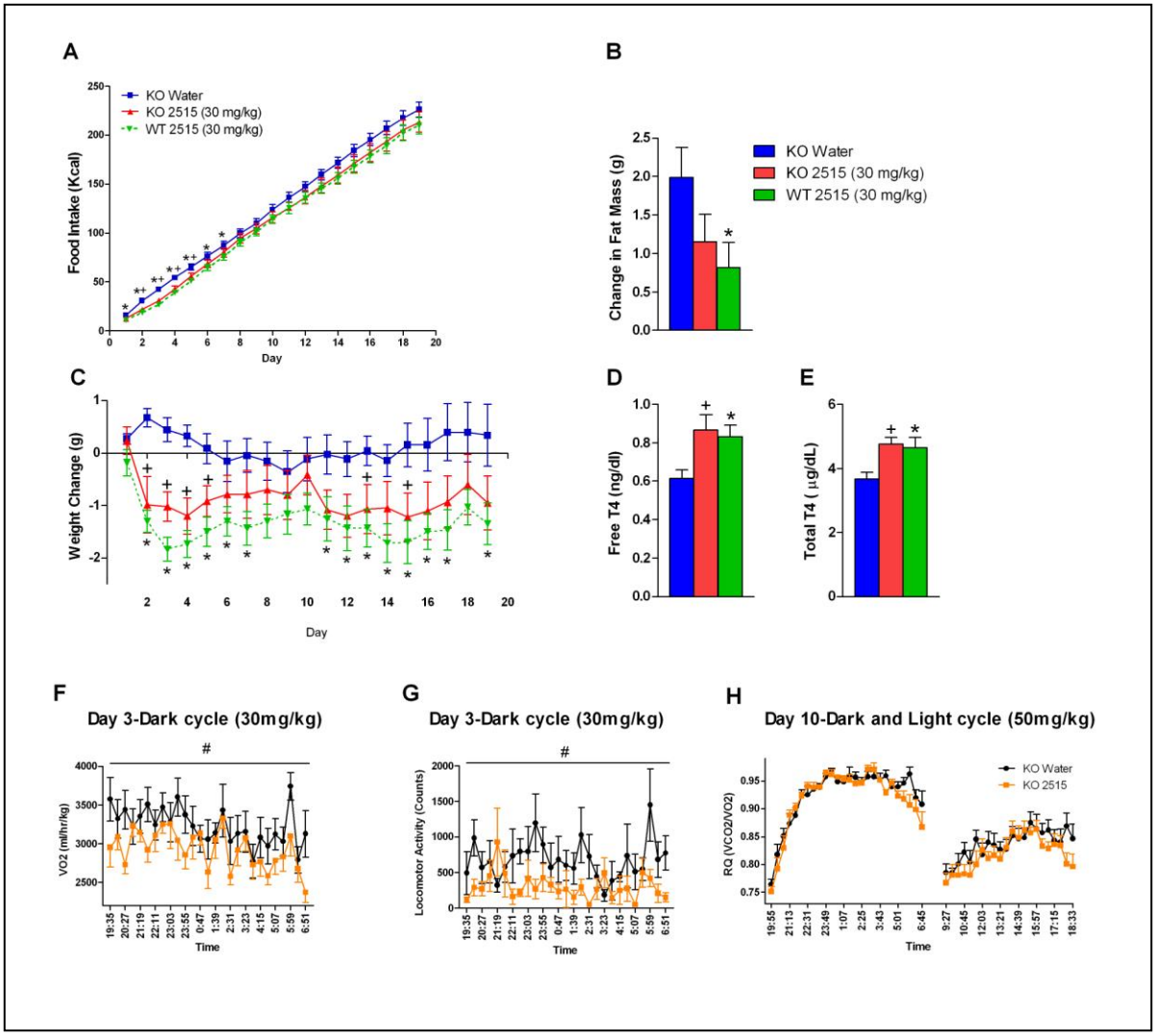
A
KO Water
KO 2515 (30 mg/kg)
WT 2515 (30 mg/kg)
Food Intake (Kcal)
Day
B
KO Water
KO 2515 (30 mg/kg)
WT 2515 (30 mg/kg)
Change in Fat Mass (g)
C
Weight Change (g)
Day
D
Free T4 (ng/dl)
E
Total T4 (µg/dL)
F
Day 3-Dark cycle (30mg/kg)
VO2 (ml/hr/kg)
Time
G
Day 3-Dark cycle (30mg/kg)
Locomotor Activity (Counts)
Time
H
Day 10-Dark and Light cycle (50mg/kg)
KO Water
KO 2515
RQ (VCO2/VO2)
Time

Figure 3.5. **Effects of TTP2515 on metabolic and calorimetry parameters in AgRP KO and WT mice. (A-F)** AgRP KO and WT mice on a 45% high fat diet received either TTP2515 (30 mg/kg) or water twice daily via oral gavage (n=6-7/group). **(A)** Cumulative food intake was lower in WT TTP2515-treated mice during days 1-7 and lower in KO TTP2515 mice during days 2-5 versus KO water-treated mice. **(B)** Cumulative body weight change was intermittently lower in both the KO and WT TTP2515 groups compared to the KO water group; however the WT TTP2515 group more frequently reached significance. **(C)** WT TTP2515-treated mice gained significantly less fat mass during treatment versus the KO water group, however, the KO TTP2515 group did not (p=.12). **(E,F)** Total and free T4 levels were elevated in both TTP2515 treatment groups compared to the KO water group. **(G-I)** AgRP KO mice were administered either TTP2515 (30 mg/kg days 1-7; 50 mg/kg days 7-10) or water twice daily (n=8/group). **(G,H)** On day 3 during the dark cycle, VO2 and activity were significantly lower in TTP2515-treated mice. (**I**) RQ was not different between groups during the light or dark cycle at the 50 mg/kg dose (showing Day 10, representative graph of this timeperiod).

+p<0.05 KO Water vs KO TTP2515; *p<.05 KO Water vs WT TTP2515; #p<.05 vs water

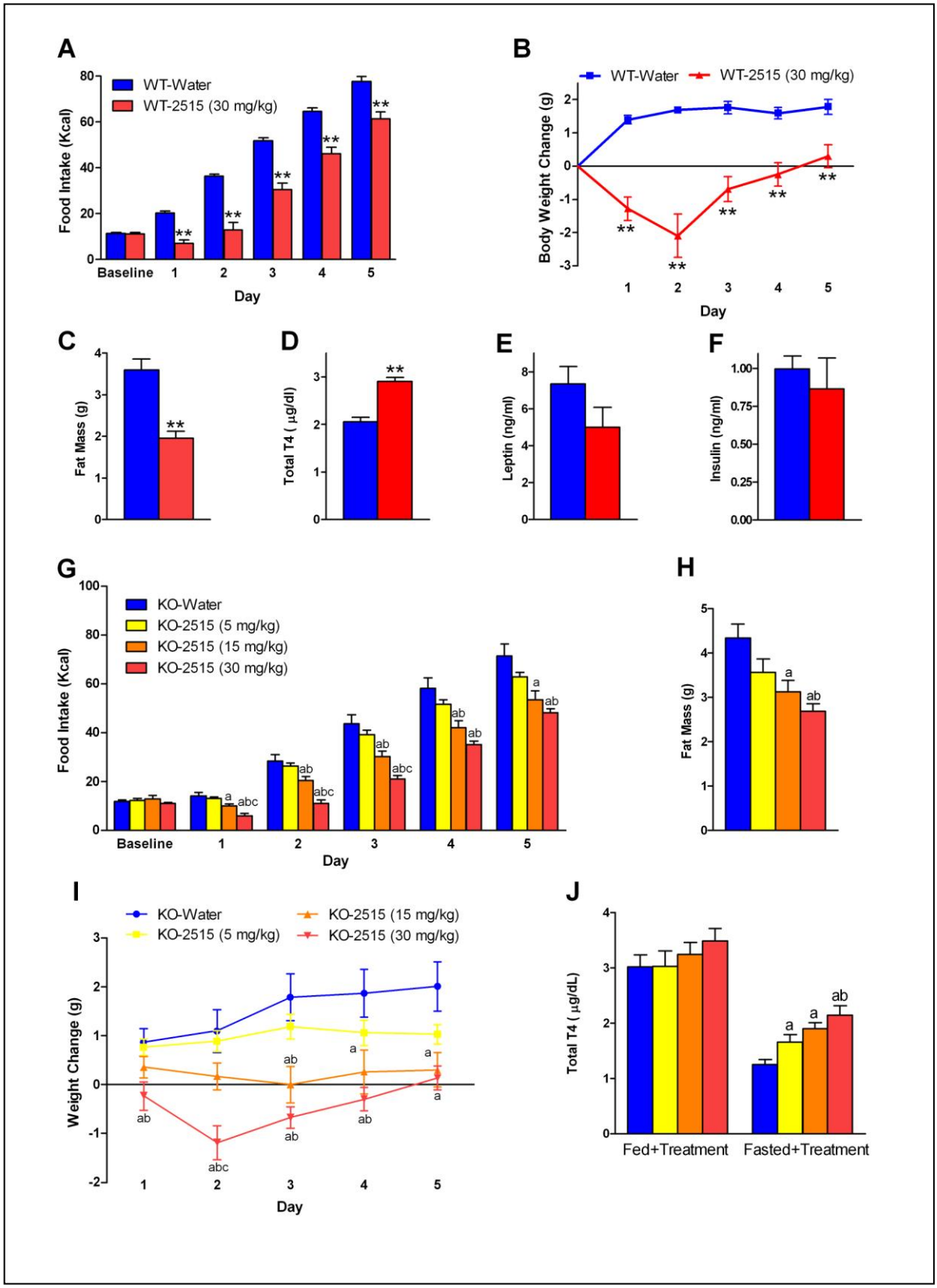
A
Food Intake (Kcal)
WT-Water
WT-2515 (30 mg/kg)
Baseline
1
2
3
4
5
Day
B
WT-Water
WT-2515 (30 mg/kg)
Body Weight Change (g)
Day
C
Fat Mass (g)
D
Total T4 (µg/dl)
E
Leptin (ng/ml)
F
Insulin (ng/ml)
G
KO-Water
KO-2515 (5 mg/kg)
KO-2515 (15 mg/kg)
KO-2515 (30 mg/kg)
Food Intake (Kcal)
Baseline
Day
H
Fat Mass (g)
I
KO-Water
KO-2515 (5 mg/kg)
KO-2515 (15 mg/kg)
KO-2515 (30 mg/kg)
Weight Change (g)
Day
J
Total T4 (µg/dL)
Fed+Treatment
Fasted+Treatment

Figure 3.6. Effects of TTP2515 in WT or AgRP KO mice acutely switched to a high-fat diet. (A-F) BL6 mice were switched from a breeder chow diet to a 60% fat diet and simultaneously started receiving treatment with TTP2515 (30 mg/kg) or water twice daily (n=8/group). **(A-C)** Cumulative caloric intake, cumulative weight gain and fat mass were all significantly lower in TTP2515-treated mice. **(D)** Total T4 levels were significantly higher in TTP2515-treated mice. **(E)** Leptin levels tended to be lower in TTP2515-treated mice (p=.12). **(F)** Insulin levels were similar between groups. **(G-J)** In a separate experiment, lean AgRP KO mice on a 10% fat chow diet were switched to a 60% fat diet and simultaneously starting receiving treatment with TTP2515 (5, 15, or 30 mg/kg) or water twice daily (n=7/group). **(G)** Cumulative caloric intake was dose-dependently lower in TTP2515-treated mice at the 15 and 30 mg/kg dose compared to the water group. **(H)** Cumulative weight gain was lower in TTP2515-treated mice at the 30 mg/kg dose during the entire study and at the 15 mg/kg dose on days 3-5 vs. water. **(I)** Fat mass was lower in TTP2515-treated mice at both the 15 and 30 mg/kg doses vs. water. The 5 mg/kg group tended (p=.05) to have lower fat mass than the water group. **(J)** After 5 days of treatment, total T4 levels were equivalent between groups, however fasting and continued treatment revealed an increase in total T4 levels in fasted mice. *p<.05, **p<.01 vs water; *a*p<.05 vs water, *b*p<.05 vs 5 mg/kg, *c*p<.05 vs 15 mg/kg.

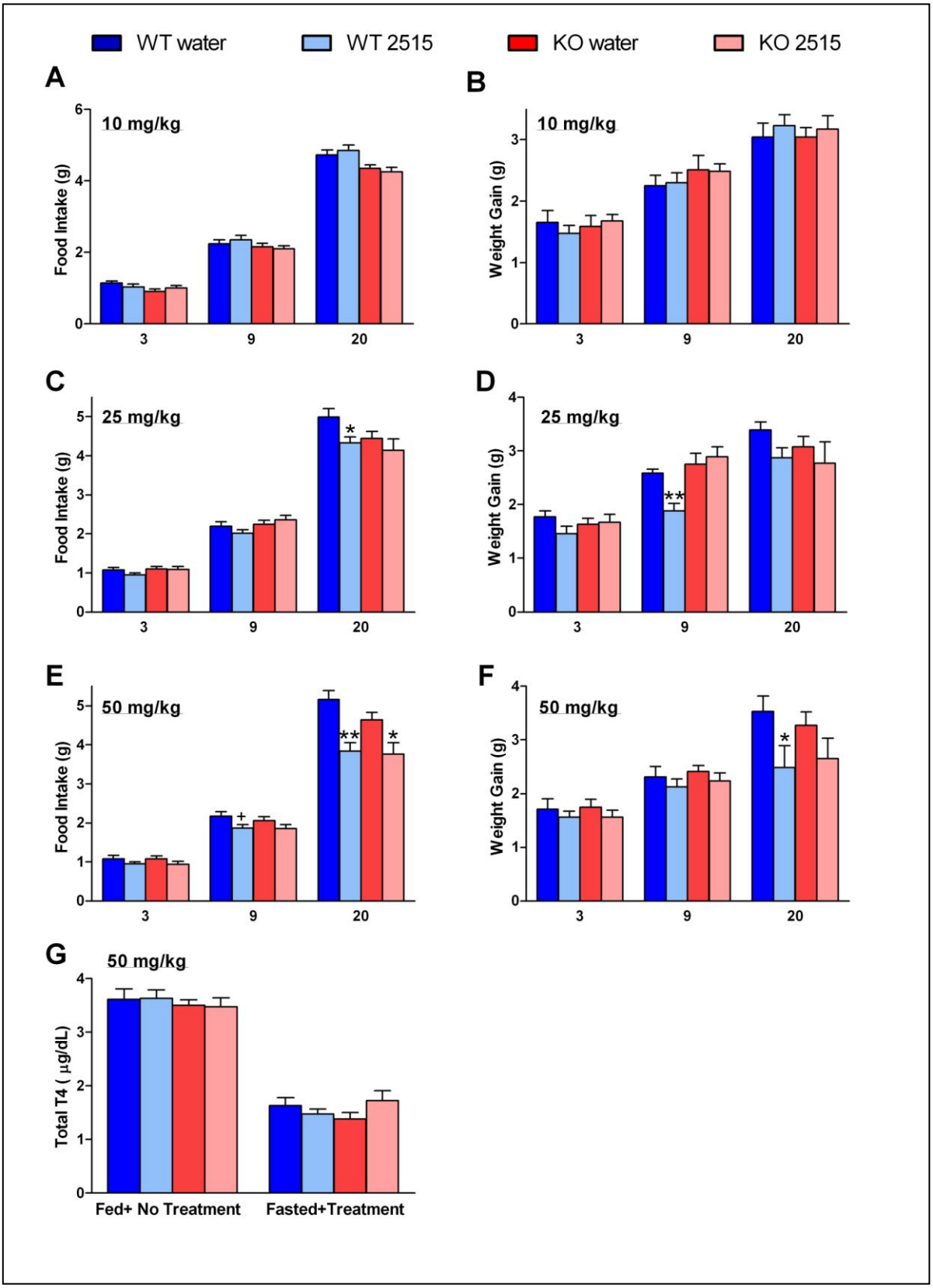
WT water
WT 2515
KO water
KO 2515
A
10 mg/kg
Food Intake (g)
B
10 mg/kg
Weight Gain (g)
C
25 mg/kg
Food Intake (g)
D
25 mg/kg
Weight Gain (g)
E
50 mg/kg
Food Intake (g)
F
50 mg/kg
Weight Gain (g)
G
50 mg/kg
Total T4 (µg/dL)
Fed+ No Treatment
Fasted+Treatment

Figure 3.7. Effects of TTP2515 on refeeding and T4 after fasting in AgRP KO and WT mice. AgRP KO and WT mice maintained on normal chow were fasted for 24 h and received either water or TTP2515 at 10, 25, or 50 mg/kg in three different experiments (n=7-8/group). **(A,B)** At the 10 mg/kg dose, no differences in food intake or body weight gain were observed. **(C)** At the 25 mg/kg dose, food intake was significantly attenuated at the 20 h timepoint in the WT TTP2515 group compared to the WT water group. **(D)** At the 25 mg/kg dose, weight gain was significantly attenuated at the 9 h timepoint in the WT TTP2515 group compared to the WT water group. **(E)** At the 50 mg/kg dose, the WT TTP2515 group tended to refeed less at the 9 h timepoint (p=.05). At the 20 h timepoint, both TTP2515 groups refed less than their respective water groups. **(F)** At the 20 h timepoint, the WT TTP2515 group gained significantly less weight than the WT water group. **(G)** A separate group of mice was fasted and blood samples were obtained before and after treatment (n=7-9/group). No difference in total T4 levels was observed before or after treatment. **p<.01, *p<.05, +p=.05 vs respective water control

CHAPTER 4: REGULATION OF PROLACTIN IN MICE WITH ALTERED HYPOTHALAMIC MELANOCORTIN ACTIVITY

Abstract

This study used two mouse models with genetic manipulations of the melanocortin system to investigate prolactin regulation. Mice with overexpression of the melanocortin receptor (MC-R) agonist, α-melanocyte-stimulating hormone (Tg-MSH) or deletion of the MC-R antagonist agouti-related protein (AgRP KO) were studied. Male Tg-MSH had lower blood prolactin levels at baseline (2.9±0.3 vs. 4.7±0.7 ng/ml) and after restraint stress (68 ±6.5 vs. 117±22 ng/ml) compared to WT mice ($p<0.05$). Pituitary prolactin content was not different. Blood prolactin was also decreased in male AgRP KO mice at baseline (4.2±0.5 vs. 7.6±1.3 ng/ml) and after stress (60±4.5 vs. 86.1±5.7 ng/ml) compared to WT mice ($p <0.001$). Pituitary prolactin content was also decreased in male AgRP KO mice vs. WT (4.3±0.3 vs. 6.7±0.5 μg/pituitary, $p<0.001$). No differences in blood or pituitary prolactin levels were observed in female AgRP KO mice vs. WT mice. Hypothalamic dopamine activity was assessed as the potential mechanism responsible for changes in prolactin levels. *Tyrosine hydroxylase* mRNA, the rate-limiting step in dopamine synthesis, was measured in the mediobasal hypothalamus (MBH) in both genetic models and was not significantly different. Hypothalamic dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) content were also measured in male AgRP KO and WT mice and no differences were detected. However these results do not preclude changes in dopamine activity as dopamine turnover was not directly investigated. This is the first study to show that baseline and stress-induced prolactin release and pituitary prolactin content are reduced in mice with genetic

alterations of the melanocortin system and suggests that changes in hypothalamic melanocortin activity may be reflected in measurements of serum prolactin levels.

Introduction

Prolactin is a lactogenic hormone secreted by the anterior pituitary that normally increases during pregnancy and lactation (307). Although a number of different factors have been shown to modulate prolactin release, the most critical factor is dopamine produced by tuberoinfundibular (TIDA) neurons in the arcuate nucleus of the hypothalamus (218). These neurons project to the median eminence where dopamine is released into hypophyseal portal blood and subsequently binds to dopamine-type 2 (D2) receptors in lactotrophs and inhibits prolactin synthesis and release (218). Although prolactin releasing factors do exist, prolactin secretion is primarily under tonic inhibitory dopamine control such that antagonism of D2 receptors causes a robust stimulation of prolactin release (218). Prolactin is also regulated by a short-loop feedback system, in which prolactin inhibits its own release by binding to prolactin receptors on TIDA neurons (308, 309). Many factors can regulate prolactin release via modulation of the TIDA dopamine system. The hypothalamic neuropeptides, α-MSH and AgRP are part of the brain melanocortin system and have been shown to inhibit and stimulate prolactin release respectively when injected icv in rodents and primates (211-215, 310, 311). These peptides are produced by POMC and AgRP neurons in the arcuate nucleus and play a critical role in regulating energy balance and metabolism (3). Proopiomelanocortin (POMC) is post-translationally processed to α-MSH which is the agonist for MC3-R and MC4-Rs in the brain and promotes negative energy balance, while AgRP is the antagonist for these receptors and promotes positive energy balance (3). This system also has a number of neuroendocrine effects on the thyroid, gonadal and adrenal axes (181, 184, 312). With respect to prolactin secretion, acute central injection of α-MSH suppresses basal blood prolactin levels as well as blunts

increases in prolactin due to stress, interleukin 1-α administration, estrogen treatment, or the surge on the day of proestrus (211-213, 311). Oppositely, AgRP has been shown to increase prolactin release and AgRP-induced increases in prolactin are blocked by α-MSH (215). The effects of α-MSH on prolactin release have been shown to be mediated by dopamine as α-MSH cannot block dopamine-receptor antagonist induced increases in prolactin levels (211, 213). Furthermore, icv α-MSH increases DOPAC and 3,4-dihydroxyphenylalanine (DOPA) content specifically in the median eminence (219).

There is evidence that endogenous α-MSH may play a physiological role in prolactin regulation as icv injection of an α-MSH antiserum has been shown to enhance basal and stress-induced prolactin release in the rat (214). There is also evidence of an anatomical connection between POMC and TIDA neurons as revealed by electron microscopy showing synaptic connections between POMC immunoreactive axon terminals and tyrosine hydroxylase-immunopositive cell bodies and dendrites (223). However, in contrast there are some reports of stimulatory effects of α-MSH on prolactin including stimulation from cultured mouse pituitary cells (313-315).

It was unknown how chronic changes in hypothalamic melanocortin activity would affect pituitary prolactin content and release. Therefore, in this study, genetic models of increased melanocortin signaling were employed to determine if chronic activation of the melanocortin system by either increasing α-MSH or eliminating AgRP signaling would suppresses prolactin levels. We used mice with overexpression of an N-terminal POMC transgene (Tg-MSH) that includes α- and γ_3-MSH (but not ACTH or β-EP) which were previously reported to possess a

leaner phenotype, and mice with selective AgRP deletion, which were reported to possess a metabolic phenotype similar to WT counterparts (50, 51, 127). Although the metabolic phenotypes have been carefully described, the effects on prolactin regulation are unknown. In both mouse models, we investigated blood prolactin levels under both unstressed and stressed conditions and measured pituitary prolactin content. Male mice were used in most experiments but these parameters were investigated in female AgRP KO mice as well. For comparison, basal and stress-induced corticosterone levels were measured in parallel in several of these experiments. To investigate the mechanism responsible for alterations in prolactin levels we measured mediobasal hypothalamic (MBH) tyrosine hydroxylase activity and dopamine and DOPAC content, as well as the effects of functional dopamine receptor antagonism.

Materials and Methods

Animals and treatment protocols: All animals were housed under barrier conditions with a 12-h light, 12-h dark cycle. Animals had *ad libitum* access to water and rodent chow. Mice were handled regularly to limit any stress while procedures were performed. All protocols were approved by the Columbia University Institutional Animal Care and Use Committee and were conducted in accordance with the NIH guide for the care and use of laboratory animals.

Tg-MSH: Transgenic mice were generated as described previously to overexpress NH2-terminal POMC under the control of the cytomegalovirus (CMV) promoter (50). The transgene contained part of the 5'UTR, the signal sequence, the sorting sequence, γ3-MSH, the joining peptide, and α-MSH, including the COOH-terminal glycine necessary for amidation. The transgene was expressed in multiple tissues, including the hypothalamus and pituitary. Mice were backcrossed to a coisogenic C57BL/6J line, C57BL/6J-A^{wJ}strain for six generations, and used for the current studies. This is a white-bellied agouti-colored line, which was used instead of black mice to visualize the darkening effect of MSH on coat color. Studies were performed in transgenic homozygous mice with control mice being wild-type (WT) animals generated from the backcross at the N6 generation.

AgRP KO: The AgRP KO mouse line was obtained from Dr. Van der Ploeg (127). Mice were backcrossed 7 times to a coisogenic C57BL/6J line, C57BL/6J-$A^{w\text{-}J}$/J, strain for seven generations. AgRP KO and WT mice were generated from homozygous matings and mice were used in the experiments as indicated. Mice were genotyped using the following primers: *F1:*

5'GCTTCTTCAATGCCTTTTGC3', F2: 5'GCCAGAGGCCACTTGTGTAG 3', R: 5'GTTTCGGAGCCAAATGGTTA3'.

Stress-experiments: Animals were placed under light restraint stress for 5 or 15 minutes using TV-150 (Braintree Scientific, Braintree, MA) and 25 ul of blood was obtained after stress.

Experiment 1: Effects of transgenic MSH overexpression on blood and pituitary prolactin levels

Experiment 1a: A trunk blood sample was obtained at sacrifice from non-stressed male Tg-MSH and WT mice (n=40 or 43/group).

Experiment 1b: A trunk blood sample was obtained at sacrifice in male Tg-MSH and WT mice (n=9-10/group) after 5 minutes of restraint stress.

Experiment 1c: At sacrifice, pituitary was collected from male Tg-MSH and WT mice (n=8 or 10).

Experiment 2: Effects of AgRP deletion on blood and pituitary prolactin levels

Experiment 2a: A submandibular blood sample was obtained from non-stressed male AgRP KO and WT mice (n=17 or 20/group) acclimated to handling.

Experiment 2b: Trunk blood was obtained at sacrifice from non-stressed male AgRP KO and WT mice (n=8 or 11/group)

Experiment 2c: Mice from *Experiment 2a* recovered and were subjected to restraint stress. Measurements were obtained 5 and 15 minutes after stress (n=17-18/group).

Experiment 2d: Male AgRP KO and WT mice (n=16/group) were sacrificed and the pituitary was collected.

Experiment 2e: Female AgRP KO and WT mice (n=9-10/group) were utilized in this experiment. The stage of the estrus cycle was monitored by daily vaginal smear. Blood samples were obtained on metestrus to minimize cyclic fluctuations in prolactin (316). After 5 and 15 minutes of restraint stress, a blood sample was obtained. Mice were sacrificed approximately 1.5 h later and the pituitary was collected.

Experiment 3: Effects of transgenic MSH overexpression or AgRP deletion on MBH tyrosine hydroxylase mRNA levels.

Experiment 3a: Male Tg-MSH and WT mice (n=8/group) were sacrificed and a 3 mm MBH dissection was obtained for RNA analyses.

Experiment 3b: Male AgRP KO and WT mice (n=7-8/group) were sacrificed and a 2 mm MBH dissection was obtained for RNA analyses.

Experiment 4: Effects of AgRP deletion on MBH dopamine and DOPAC content.

Male AgRP KO and WT mice (n=7/group) were sacrificed and a 2 mm MBH dissection (~10 mg tissue) was collected for high-performance liquid chromatography (HPLC) analyses. Samples were homogenized in 0.1 N perchloric acid (PCA) with 50 ng/mL of 3,4 dihydroxybenzylamine (DHBA), which was utilized as an internal standard.

Experiment 5: Effects of AgRP deletion on serum prolactin levels after dopamine receptor antagonism

Male AgRP KO and WT mice (n=9 or 11/group) were utilized in this experiment. A submandibular blood sample was obtained from non-stressed mice. After two weeks, mice received a subcutaneous metoclopramide injection (2 μg/g body weight), and a blood sample was obtained after 30 minutes.

Measurement of hypothalamic RNA levels: RNA isolation was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen USA, Valencia, CA) in conjunction with the RNase-Free DNase set (Qiagen USA) under the manufacturer's instructions and total RNA was quantified using spectrophotometry. cDNA was synthesized using the Superscript III First-Strand cDNA Synthesis Kit (Life Technologies Corporation/Invitrogen, Grand Island, NY) and was analyzed using quantitative RT- PCR performed with Lightcycler 480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN) in the Lightcycler 480 Real-Time PCR system (Roche Applied Science). Samples were normalized to β-actin. Primer sequence: Tyrosine Hydroxylase: *F5'CGAGGTGCCCAGTGGCGACC 3' R 5'GGTCCAGGTCCAGGTCAGGG 3'.*

Blood and pituitary hormone measurements: Blood was collected as plasma or serum and stored at -80° C. The pituitary was homogenized in 0.1 N HCl and used for analyses. Prolactin levels were measured by a double antibody RIA with an antiserum to mouse prolactin and reference preparations provided by the National Hormone and Pituitary Program. Purified mouse prolactin was iodinated with I-125 by the lactoperoxidase method for use as tracer. Corticosterone levels

were measured using a sensitive double antibody RIA from MP Biomedicals (Irvine, CA USA) (317).

HPLC analyses: Dopamine and DOPAC content were determined by HPLC-EC and normalized to protein content in each sample as previously described (318).

Statistical analysis: Statistical analysis was performed with Student's *t* test. $P < 0.05$ was considered statistically significant. Results are reported as mean values ± SEM.

Results

Experiment 1: Effects of transgenic MSH overexpression on blood and pituitary prolactin levels

Three different groups of mice were used in this experiment. In the first group, male Tg-MSH mice were found to have significantly lower baseline plasma prolactin levels at sacrifice compared to WT mice ($p<0.05$, **Fig 4.1A**). A second set of mice was exposed to restraint stress for 5 minutes and then sacrificed; plasma prolactin levels were also found to be lower in these Tg-MSH mice versus WT mice ($p<0.05$, **Fig 4.1B**). Notably, plasma corticosterone levels were not different in these mice (WT 22.8±2.4 vs. Tg-MSH 20.7±1.0 ug/dl, $p=0.40$). In another cohort of mice, pituitary prolactin content was found to be similar between Tg-MSH and WT mice (**Fig 4.1C**).

Experiment 2: Effects of AgRP deletion on blood and pituitary prolactin levels

Experiments 2a,b: Baseline serum prolactin levels obtained from a submandibular bleed were significantly lower in male AgRP KO mice ($p<0.05$, **Fig 4.2A**). Serum corticosterone levels were also measured in these mice and were found to be similar between groups (WT 1.9±0.3 vs. AgRP KO 1.6±0.3 ug/dl, $p=0.64$). In a separate cohort of AgRP KO mice, plasma prolactin levels at sacrifice were also significantly lower (KO 3.4±0.5 vs. WT 7.9±1.6 ng/ml, $p<0.05$).

Experiment 2c: Mice from *Experiment 2a* were subjected to restraint stress which increased serum prolactin levels; however stress-induced prolactin levels were still significantly lower in AgRP KO versus WT mice at both the 5 and 15 minute timepoints ($p<0.001$, **Fig 4.2B**). Serum corticosterone levels were analyzed in a subset of these mice after stress (n=9 or 11/group) and it

was found that stress-induced corticosterone levels were not significantly lower in AgRP KO mice at either the 5 or 15 minute timepoints (data not shown).

Experiment 2e: In another cohort of male mice, pituitary prolactin content was found to be significantly lower in AgRP KO vs. WT mice ($p<0.001$, **Fig 4.2C**).

Experiment 2f: The estrous cycle of female AgRP KO and WT mice was monitored daily and measurements were obtained on metestrus. Stress-induced serum prolactin levels were not different between AgRP KO and WT mice after either 5 or 15 minutes of restraint stress. Pituitary prolactin content tended to be lower in AgRP KO *vs.* WT mice, however this did not reach significance ($p=0.23$) (**Fig 4.3A,B**).

Experiment 3: Effects of transgenic MSH overexpression or AgRP deletion on MBH tyrosine hydroxylase mRNA levels.

As tyrosine hydroxylase is the rate-limiting step in dopamine synthesis (319), we sought to investigate mRNA levels in the MBH of AgRP KO and WT mice and Tg-MSH and WT mice. Both the AgRP KO ($p=0.29$) and Tg-MSH ($p=0.08$) mice tended to have higher tyrosine hydroxylase mRNA expression compared to their respective WT counterparts, however this did not reach significance (**Fig 4.4A,B**).

Experiment 4: Effects of AgRP deletion on MBH dopamine and DOPAC content.

MBH dopamine and DOPAC content were measured by HPLC from AgRP KO and WT mice. Analyses revealed that dopamine and DOPAC content were not different between groups (**Fig 4.5**).

Experiment 5: Effects of AgRP deletion on serum prolactin levels after dopamine receptor antagonism

Baseline serum prolactin levels were significantly lower in AgRP KO vs. WT mice ($p<0.05$, **Fig 4.6A**). Mice were injected subcutaneously with metoclopramide and prolactin levels were measured 30 minutes later. Serum prolactin levels increased with metoclopramide treatment, however these levels were still significantly lower in AgRP KO mice ($p<0.001$, **Fig 4.6B**). The fold-increase in prolactin levels from baseline was also similar between groups (**Fig 4.6C**).

Discussion

This study demonstrates that prolactin levels are lower with two different genetic manipulations of the melanocortin system resulting in either overexpression of the MC-R agonist α-MSH, or deletion of the antagonist AgRP. In male Tg-MSH and AgRP KO mice, both baseline and stress-induced blood prolactin levels were significantly lower compared to WT mice, and further, pituitary prolactin content was lower in male AgRP KO mice. However, no differences in serum or pituitary prolactin levels were detected in female AgRP KO mice. As dopamine is well recognized to inhibit prolactin secretion (218), dopamine metabolism was also investigated. Tyrosine hydroxylase mRNA, the rate-limiting step in dopamine synthesis (319), was measured in the MBH of both mouse models, and although a trend for increased levels was evident, it did not reach significance in either group. Hypothalamic dopamine and DOPAC content were also examined in male AgRP KO and WT mice, however no differences were detected. Finally, we used the D2 receptor antagonist metoclopramide to investigate functional dopamine receptor antagonism and found that prolactin levels were similarly increased in both groups after treatment.

Previous studies have shown that the melanocortin system can acutely regulate prolactin secretion. Central injections of α-MSH can suppress prolactin levels (211-213). Oppositely icv AgRP increases prolactin levels and these increases can be blocked by α-MSH (215). Furthermore, the mu-opioid receptor agonist β-EP, which is not part of the melanocortin system *per se*, is also derived from POMC and interacts with α-MSH to regulate prolactin secretion by modulating hypothalamic dopamine turnover (220-222). There is evidence that endogenous α-

MSH may play a physiological role in prolactin regulation as icv injection of an α-MSH antiserum has been shown to enhance basal and stress-induced prolactin release in the rat (211, 310). In contrast, there are several reports demonstrating stimulatory effects of α-MSH on prolactin. α-MSH has been shown to stimulate prolactin release and DNA replication in cultured mouse lactotrophs and to sensitize these cells to other secretagogues (314, 320). This appears to be via stimulation of the MC3-R that is expressed by lactotrophs (314). There is also a report that an α-MSH antiserum attenuates stimulation of prolactin by leptin (313). Thus α-MSH may modulate prolactin release at both the pituitary and hypothalamic level. Most studies have only investigated the relatively acute effects of α-MSH on prolactin release. Our study is the first to demonstrate that chronic altered melanocortin signaling can affect prolactin levels.

Several lines of evidence support a role for hypothalamic dopamine in mediating the effects of α-MSH on prolactin secretion. For example, icv α-MSH can no longer inhibit prolactin secretion in the presence of a dopamine-receptor antagonist (211, 213). Anatomic studies have also shown that POMC-immunoreactive terminals contact tyrosine hydroxylase-positive cell bodies and dendrites in the hypothalamus of rats (223), showing a physical interaction between these neurons. Although the MC3-R is highly expressed in the arcuate where TIDA neurons are localized, it is at present unclear which MC-R mediates effects of α-MSH on TIDA activity. In this study, we were unable to show that increased dopaminergic activity was responsible for the suppression of prolactin levels in AgRP KO mice. We investigated tyrosine hydroxylase gene expression and dopamine and DOPAC content in the MBH of AgRP KO and WT mice. It should be noted that the failure to detect changes in static measurements of dopamine and DOPAC content does not preclude a role for dopamine in

mediating the observed effects on prolactin, as dopamine turnover was not directly assessed. We also used a D2R antagonist metoclopramide to investigate if blocking this receptor would yield a greater fold increase in prolactin release in AgRP KO mice compared to WT mice. However, we found that prolactin levels were elevated to a similar degree after D2R antagonism in AgRP KO and WT mice. It is possible that the chronically lower pituitary prolactin content in these mice affected their response to metoclopramide.

It is of interest that despite similarly lower serum prolactin levels in male AgRP KO and Tg-MSH mice, pituitary prolactin content was only lower in the AgRP KO mice. It should be noted that Tg-MSH mice express the transgene in the pituitary as well and have higher circulating levels of α-MSH (50). It is possible that α-MSH acting at the pituitary level prevented the fall in pituitary prolactin content in the Tg-MSH mice. This would be consistent with the stimulatory effect of α-MSH on prolactin release reported *in vitro* in cultured lactotrophs (313). However given that basal and stress-induced prolactin release is suppressed in Tg-MSH mice, the effects of α-MSH on the dopaminergic regulation of prolactin appears to predominate.

In several experiments, we observed that while prolactin levels were suppressed at either baseline or after stress in AgRP KO or MSH-Tg mice, corticosterone measured in the same mice were not different between groups. This is important as it shows that the mechanisms responsible for stress-induced prolactin and corticosterone release in these models are distinct and only prolactin release was affected by these genetic manipulations. A sexually dimorphic effect of AgRP deletion on prolactin levels was also noted in this study. Although reproductive

function was not formally assessed, AgRP KO and WT mice appeared to reproduce with equal frequency and yield similar litter sizes. As prolactin levels are critical for reproduction and nursing including maternal behavior, it is possible that there was developmental compensation in females and hence lower prolactin levels were not evident in female mice as they were in male mice (307). Indeed, there is evidence for developmental compensation in AgRP KO mice with respect to energy balance (127). In this study, only stress-induced prolactin release and pituitary content were measured in female AgRP KO mice, however there could be changes under other conditions such as pregnancy or suckling that were not examined.

In addition to the data showing that α-MSH may regulate dopamine turnover in the hypothalamus, there is evidence that that dopamine may regulate POMC in the hypothalamus (321-323). Furthermore, a positive correlation was reported between *Pomc* mRNA and tyrosine hydroxylase mRNA in the MBH of the rat suggesting coregulation or functional interaction between these two neuronal systems (324). Thus there is evidence for bidirectional interactions between the hypothalamic dopamine system and melanocortin pathways which both play a critical role in maintaining energy homeostasis (325-329).

This study provides additional evidence to support functional interactions between the hypothalamic melanocortin and dopaminergic systems that may be reflected by plasma prolactin levels. Prolactin is known to affect energy balance and fuel metabolism and has also been shown to stimulate AgRP (330-334). There is some evidence that prolactin is elevated in human obesity and it has been postulated that the elevations in prolactin may result from decreased inhibitory input from hypothalamic dopaminergic neurons (335, 336). It may thus be that elevated prolactin levels are a marker for decreased brain dopaminergic activity which in turn reflects a

decrease in hypothalamic melanocortin activity. The current study shows that prolactin levels are reduced in genetic models of increased melanocortin signaling and suggests that melanocortin induced changes in dopamine turnover may be reflected in measurements of serum prolactin levels. The hypothesis that circulating prolactin levels may serve as a biomarker of central melanocortinergic and dopaminergic tone deserves further study.

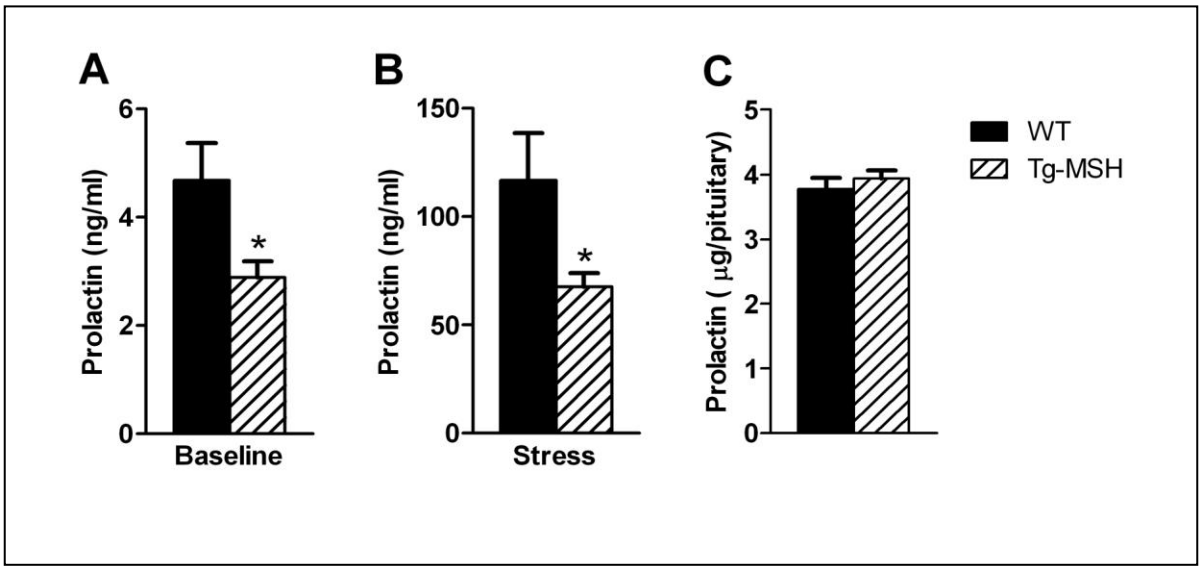

Figure 4.1: Effects of transgenic MSH overexpression on blood and pituitary prolactin levels. (A) Baseline plasma prolactin levels were significantly lower in Tg-MSH mice versus WT mice. **(B)** After 5 minutes of restraint stress, prolactin levels were significantly lower in Tg-MSH mice. **(C)** Pituitary prolactin content was similar between Tg-MSH and WT mice. *p<.05

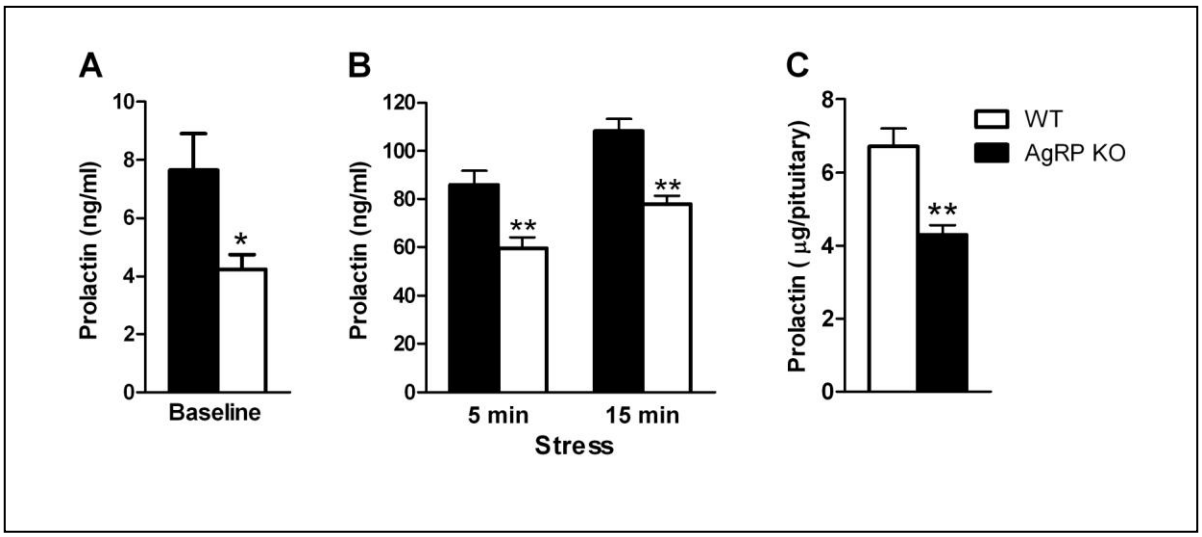

Figure 4.2: Effects of AgRP deletion on blood and pituitary prolactin levels in male mice. (A) Baseline prolactin levels were significantly lower in male AgRP KO mice. **(B)** After 5 and 15 minutes of restraint stress, prolactin levels were significantly lower in AgRP KO male mice **(C)** Pituitary prolactin content was significantly lower in male AgRP KO mice. *p<.05, **p<.001

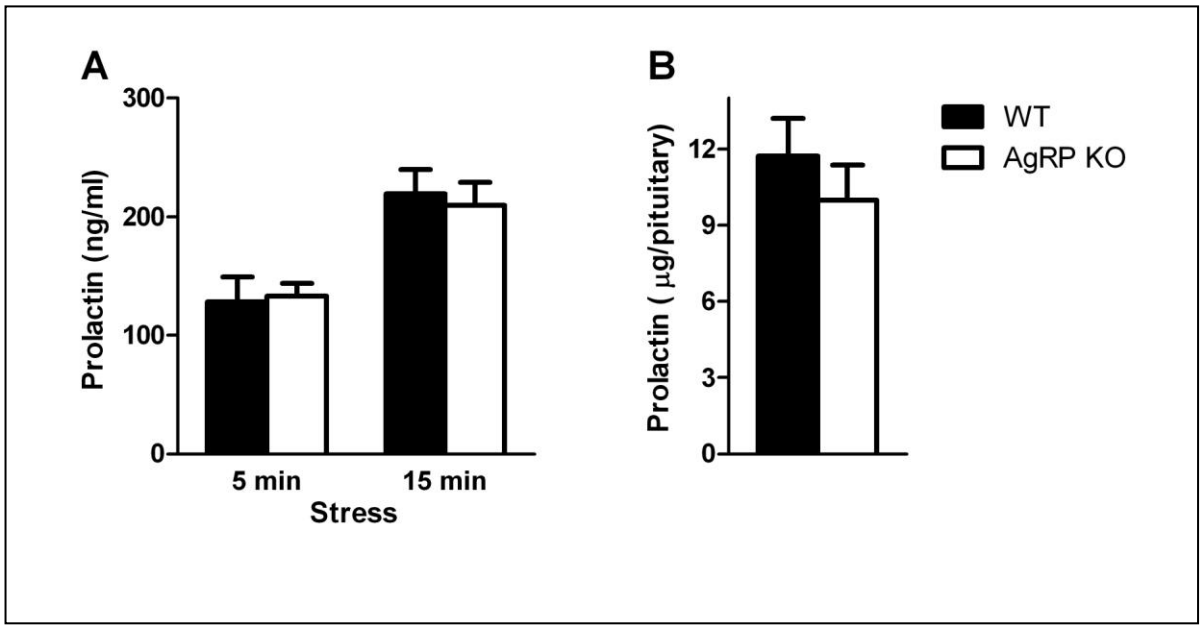

Figure 4.3. Effects of AgRP deletion on blood and pituitary prolactin levels in female mice. **(A)** Stress-induced prolactin levels were similar in female AgRP KO mice compared to WT mice after both 5 and 15 minutes of restraint stress. **(B)** Pituitary prolactin content was not significantly different between female AgRP KO mice versus WT mice (p=.23).

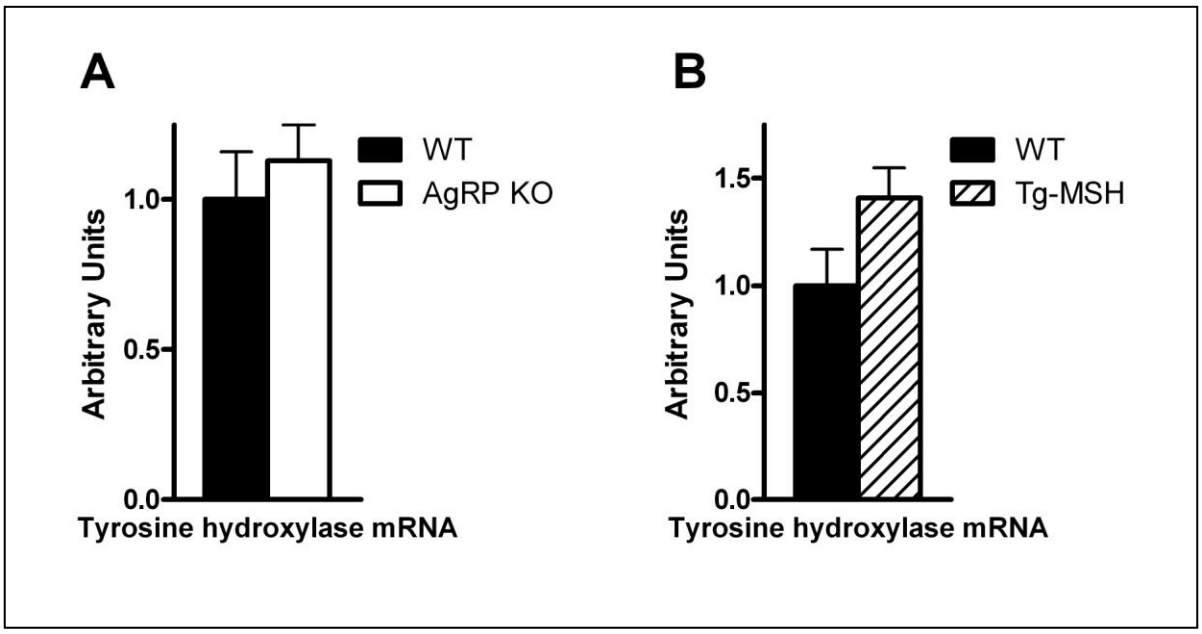

Figure 4.4 Effects of transgenic MSH overexpression or AgRP deletion on hypothalamic *tyrosine hydroxylase* mRNA expression. (A) *Tyrosine hydroxylase* mRNA expression was not different between AgRP KO versus WT mice (p=.29). **(B)** *Tyrosine hydroxylase* mRNA expression was not significantly different between Tg-MSH versus WT mice, although a tendency was evident (p=.08).

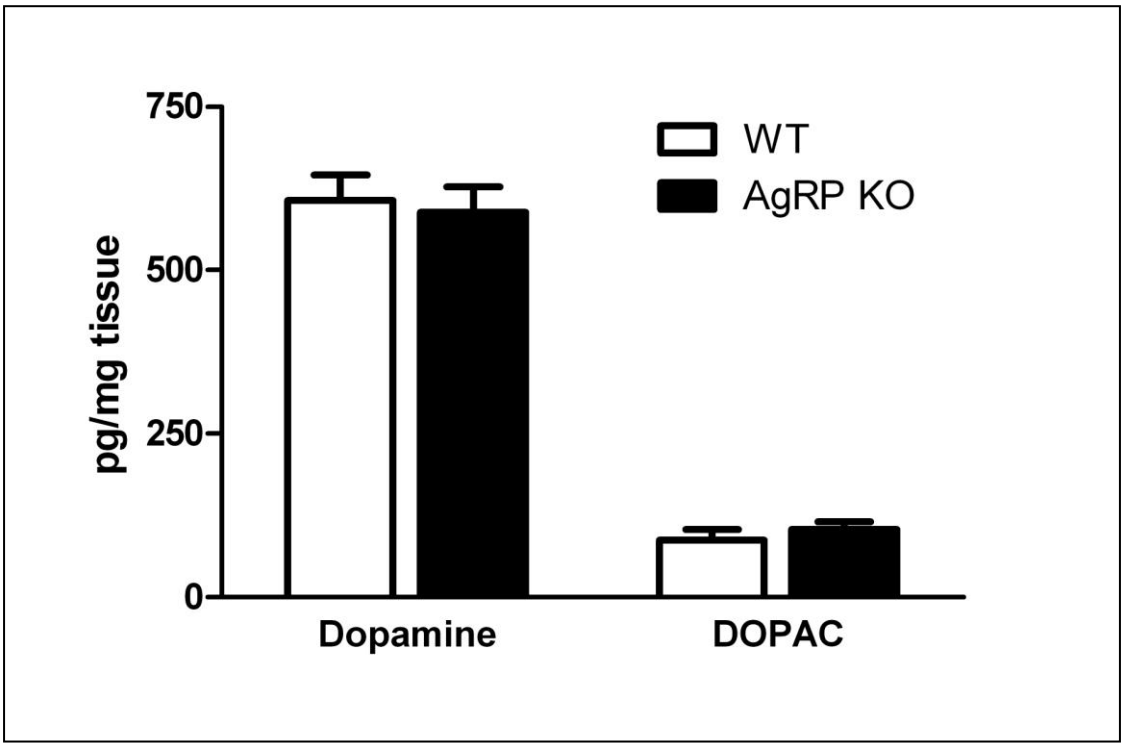

Figure 4.5. Effects of AgRP deletion on mediobasal hypothalamic dopamine and DOPAC levels. Dopamine and DOPAC levels were measured in AgRP KO and WT mice and were found to be similar between groups.

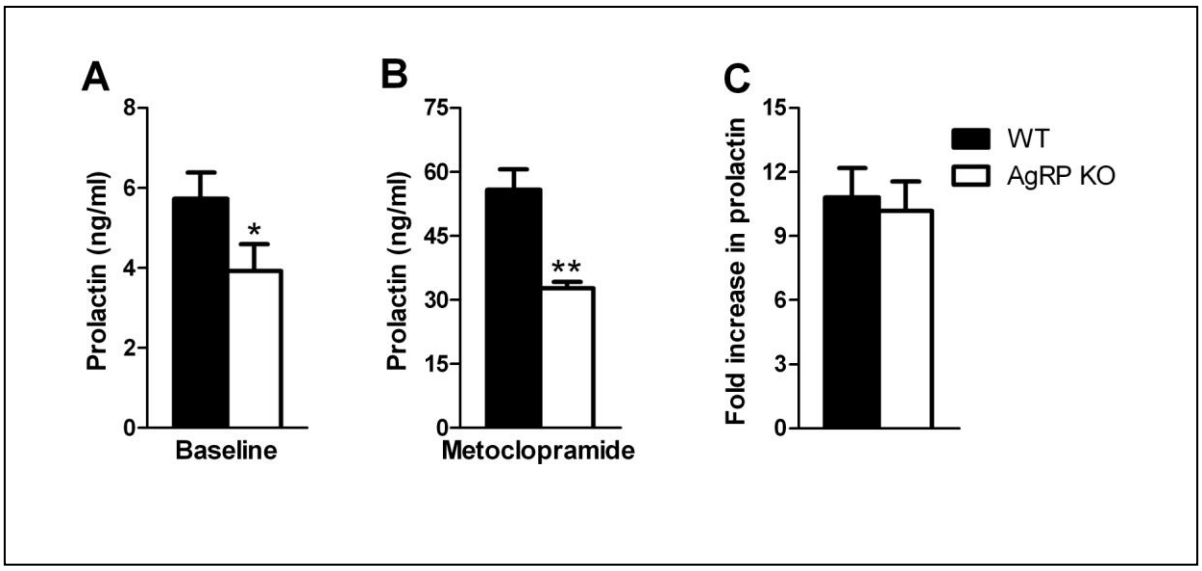

Figure 4.6. Effects of AgRP deletion on serum prolactin levels after dopamine receptor antagonism. Serum prolactin measurements were obtained from AgRP KO and WT mice before and after metoclopramide treatment. **(A)** Baseline prolactin levels were lower in AgRP KO vs WT mice. **(B)** Thirty minutes after metoclopramide injection prolactin levels were still lower in AgRP KO vs WT mice. **(C)** The fold-increase in prolactin levels after metoclopramide treatment was similar between groups.*p<.05, **p<.001

CHAPTER 5: EFFECTS OF THE HIGH FAT DIET ON MELANOCORTIN GENE AND PEPTIDE EXPRESSION

Note: The data in this chapter is largely preliminary.

Abstract

The high fat diet (HFD) is well described to contribute to obesity and there is evidence that the melanocortin system can mediate some of the adaptive responses to the initiation of high fat feeding including attenuation of hyperphagia and weight gain. Although previous studies have shown that HFD feeding consistently suppresses Agouti-related peptide (*Agrp)* mRNA, effects on Proopiomelanocortin (*Pomc)* mRNA are unclear, and effects on POMC precursor, POMC-derived peptides α-melanocyte stimulating hormone (α-MSH) and β-Endorphin (β-EP), and AgRP peptide as well as levels of enzymes responsible for post-translational POMC processing are unknown. We hypothesized that exposure to the 60% HFD would modulate melanocortin gene and peptide expression as well as expression of enzymes responsible for post-translational processing of POMC. In these studies, we sought to investigate the effects of different durations of 60% HFD feeding (versus a 10% control diet) on these parameters after 3 days, 7 days and 8 weeks. Quantitative real time polymerase chain reaction (qRTPCR) was utilized to investigate hypothalamic expression of *Pomc* and *Agrp* as well as levels POMC- processing enzymes proconvertase 1 (*Pcsk1)*, *Pcsk2*, carboxypeptidase E (*Cpe)* and prolylcarboxypeptidase (*Prcp)*. We also utilized a novel enzyme-linked immunosorbent assay (ELISA) to measure POMC precursor peptide as well as radioimmunoassay (RIA) to measure AgRP, α-MSH and β-EP peptide. In *Experiment 1*, 3 days of HFD feeding stimulated *Pomc* mRNA and increased POMC

precursor peptide, α-MSH and β-EP levels versus control mice. No effects on AgRP mRNA or peptide levels were observed at this timepoint. In *Experiment 1*, measurements were obtained in the mediobasal hypothalamus (MBH), including the arcuate, an area of Pomc and Agrp synthesis, and anterior hypothalamus (AH), including the paraventricular nucleus (PVN), an area of POMC and AgRP projection with dense melanocortin 4 receptor (MC4-R) expression. Independent of HFD feeding, we observed that the MBH section contained approximately 4-times greater POMC precursor peptide versus the AH, while distribution of α-MSH and β-EP peptides was fairly similar between sections. Thus it appears that the POMC-processed peptides are found in equal distribution in areas of synthesis and utilization, while the POMC precursor peptide is found mostly in the area of POMC synthesis. In *Experiment 2*, mice were placed on the HFD for 7 days and hypothalamic analyses revealed that POMC peptide levels were increased, however no change in α-MSH or β-EP were observed compared to control mice. β-EP processing was also measured after 7 days of HFD feeding, however no differences in the ratio of β-EP 1-31 to C-terminally cleaved peptides, β-EP 1-27 and 1-26, which have reduced opioid activity, were observed. Gene expression and AgRP peptide levels were not assessed at this timepoint. In *Experiment 3*, mice were placed on a HFD for 8 weeks and high fat feeding increased POMC peptide levels without any increase in *Pomc* mRNA compared to control mice. α-MSH and β-EP levels were also not different between groups but AgRP mRNA and peptide levels were significantly suppressed by the chronic HFD. As rodents have been shown to have variable susceptibility to HFD-induced obesity, mice were stratified into diet-resistant (DR) and diet-induced obese (DIO) groups. After stratification, in the MBH, although absolute α-MSH and β-EP levels were not different, the α-MSH/β-EP ratio was elevated in the DR mice. This

corresponded with a suppression in *Prcp* mRNA levels. PRCP is an enzyme that inactivates α-MSH and hence this could increase α-MSH activity and protect from HFD-induced weight gain. No consistent effects in POMC processing enzymes *Pcsk1*, *Pcsk2*, *Cpe*, or *Prcp* were detected after 3 days and 8 weeks of HFD feeding aside from the change in *Prcp* reported. However, as these enzymes are widely expressed, the relatively large dissections may have precluded the detection of significant changes. These data show that the HFD can modulate activity of the melanocortin system with distinct time related changes in POMC and AgRP. The most consistent finding at all time points was an increase in POMC precursor peptide that persisted even without an increase in mRNA levels. These studies suggest that posttranslational regulation of POMC sorting and processing may play a role in modulating the metabolic response to a HFD. Future studies should focus on peptide and processing enzyme expression in more anatomically discrete hypothalamic nuclei including the arcuate and PVN as well focus on dynamic studies of peptide release.

Introduction

Evidence suggests that a HFD can contribute to obesity in both rodents and humans (337). Although countless studies have used the HFD to study the obese model, limited information exists on the effect of high fat feeding on the melanocortin system; this is surprising considering the critical role it plays in the regulation of energy balance. POMC is post-translationally processed to a number of different peptides including α-MSH, which functions as an agonist for the MC3/4-Rs in the brain and reduces food intake, body weight gain and adiposity, and increases EE and lipid utilization (33-37). AgRP functions as an antagonist at the MC3/4-R and has the opposite effect on energy balance (32, 111, 112). It has been reported that initiation of a HFD in BL6 mice leads to hyperphagia that persists for approximately 2 days and is subsequently downregulated (297). However, this compensation mechanism which downregulates hyperphagia is not evident in mice with MC4-R deletion, suggesting that the melanocortin system may mediate the adaptive response after HFD feeding (297). Numerous studies report that both acute and chronic HFD feeding suppresses *Agrp* mRNA levels (305, 338-341). Oppositely, *Pomc* mRNA levels after initiation of the HFD are unclear; levels are reported to be increased (305, 342), decreased (341, 343, 344) and unchanged (338, 339, 345, 346). Furthermore, very few studies have reported on hypothalamic peptide levels. This is a critical oversight as POMC is not only processed to α-MSH which decreases food intake, but to β-EP, an agonist for the MOR that acutely stimulates food intake (59-62). Reports on the effects of the HFD on peptide processing have been limited to rat models and have not reported any consistent findings (106, 347-349). Furthermore, there is limited information on the effects of the HFD on enzymes involved in POMC post-translational processing. One study reported no difference in

proconvertase 1 (PC1) and PC2 protein levels in whole hypothalamus after 2 or 5 weeks of HFD-feeding (350) and chronic HFD exposure was shown to suppress *Cpe* mRNA levels in the arcuate (171). Since differential processing of POMC-derived peptides by cleavage enzymes can contribute to the regulation of energy balance, further investigation of the HFD on expression of these enzymes is warranted. Furthermore, the contribution of hormones such as leptin and insulin, that are known to modulate melanocortin system activity, should also be evaluated in relation to HFD feeding (149, 150, 158).

It is now well-established in rodents that different strains are more susceptible to DIO, and that within-strain differences in susceptibility also exist (351, 352). Evidence from DR rodents suggests that the melanocortin system may mediate this susceptibility. For example, HFD exposed DR Sprague-Dawley rats were reported to have an increased number of α-MSH positive neurons in the arcuate versus DIO rats, and the DR A/J mouse strain was shown to have decreased weight and increased *Pomc* mRNA levels compared to BL6 mice after exposure to the HFD (353, 354). However, these studies do not provide a complete picture as only certain endpoints (ie-gene or peptide expression) were measured. Further investigation of this potential protective effect of the melanocortin system on weight gain is warranted.

This study sought to investigate the effects of the HFD on melanocortin gene and peptide expression as well as expression of enzymes responsible for post-translational POMC processing. To gain a sense of an appropriate timeframe to study, we first investigated gross hypothalamic changes in peptide expression over an acute timeframe ranging from 36 h-14 days. These preliminary experiments suggested that POMC peptide changes may be evident during the

2-3 day timeframe and this timeframe also coincided with when WT mice downregulate food intake after introduction to a HFD. Hence, the first experiment investigated the effects of 3 days of HFD-feeding (versus a 10% control diet) on hypothalamic peptide and gene expression changes in both the MBH and AH as well as changes in POMC-processing enzymes. We next investigated the effects of 7 days of HFD feeding on POMC and POMC-derived peptides as well as β-EP 1-31 processing as preliminary studies suggested that β-EP 1-31 processing may be increased during this timepoint. Finally, the effects of chronic (8 week) HFD administration on gene expression changes in the MBH and peptide expression changes in the MBH and AH were determined. The HFD group was further subcategorized to DIO and DR to investigate if the melanocortin response to the HFD was different between these two groups.

Materials and Methods

Animals: All animal experiments were approved by the Columbia University Institutional Animal Care and Use Committee. Male C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) for all experiments and were housed under barrier conditions with a 12:12-hr light-dark cycle. In all experiments, body weight was monitored before initiation of the HFD and at sacrifice. In *Experiment 4*, a body composition measurement (NMR Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) was obtained before and after initiation of the HFD.

Mouse diets: Mice were either maintained on a 10% fat control diet (Research Diets 12450B) or received a 60% very high fat diet (Research Diets D12492).

Experimental procedures

Experiment 1: Effects of 3 days of HFD feeding on hypothalamic melanocortin gene and peptide expression

Experiment 1a: 10 week old C57BL6/J male mice were maintained on a 10% control diet for 4 weeks. Half of the mice were switched to a 60% fat diet and the rest remained on the 10% diet for 2.5 days starting during the dark cycle (60 h) (n=9-10/group). Mice were sacrificed on the morning of Day 3. Blood was collected on EDTA and a 2 mm MBH and 1 mm AH section was placed in 0.1 N HCl and processed for peptide analysis.

Experiment 1b: 10 week old C57BL6/J mice were maintained on a 10% control diet for 8 weeks. Half of the mice were switched to a 60% fat diet and the rest remained on the 10% diet

for 2.5 days starting during the dark cycle (60 h) (n=9-10/group). Mice were sacrificed on the morning of Day 3. Blood was collected on EDTA and a 2 mm MBH and 1 mm AH section was frozen in liquid nitrogen for ribonucleic acid (RNA) analyses.

Experiment 2: Effects of 7 days of HFD feeding on hypothalamic melanocortin peptide expression

10 week old C57BL6/J mice were maintained on a 10% control diet for 3 weeks. Half of the mice were switched to a 60% fat diet and the rest remained on the 10% diet for 7 days starting during the dark cycle (n=6-8/group for peptides and n=4/group for HPLC). Mice were sacrificed the morning of Day 7. Blood was collected on EDTA and a 3 mm hypothalamic dissection including the MBH and AH was placed in 0.1 N HCl and processed for peptide analysis.

Experiment 3: Effects of 8 weeks of HFD feeding on hypothalamic melanocortin gene and peptide expression

10 week old C57BL6/J mice were maintained on 10% control diet for 2 weeks. Two-thirds of the mice were switched to a 60% fat diet and the rest remained on the 10% diet for 8 weeks starting during the dark cycle (n=9 or 16). Mice were sacrificed in the morning after 8 weeks of HFD feeding. Blood was collected on EDTA and a 1 mm AH dissection was placed in 0.1 N HCl and processed for peptide analysis. A 2 mm MBH dissection was processed for both RNA and peptide as previously described with some modifications (355). The MBH was homogenized in 500 ul cold sterile AT buffer (10 mM Tris pH 8.0, 3 mM $CaCl_2$, 2mM $MgCl_2$, 0.5 mM DTT, 0.15% Triton-X-100 and 40 units RNasin® Plus RNase Inhibitor (Promega Corporation, Madison, WI, USA); 250 ul of the homogenate was placed into 500 ul 0.2N HCl and this

portion was processed for peptide analyses. The remaining 250 ul homogenate was added to 1 mL Qiazol lysis buffer and processed for RNA as described below. Mice were stratified into high (DIO) and low weight (DR) gainers based on a natural separation between the HFD group (n=7 or 9).

Peptide assays: Brain sections were sonicated in 0.1 N or 0.2 N HCl as indicated above. As previously described, α-MSH, β-EP and $AgRP_{83-132}$ content were detected by RIA (109, 355). $AgRP_{83-132}$ will be referred to as AgRP in the text. HPLC for β-EP content was performed as previously described (356).

α-MSH RIA: Antiserum raised in this laboratory which crossreacts fully with des-acetyl α-MSH; no crossreactivity with adrenocorticotropin hormone (ACTH), corticotropin-like intermediate peptide (CLIP) or POMC. The antiserum does not cross-react with the free acid form of α-MSH, which has not been amidated. *β-EP RIA:* Antiserum to human β-EP, raised in this laboratory, directed at β-EP 18-25; crossreacts fully with rat β-EP, N-acetyl β-EP, β-EP 1-27 and 1-26. Possesses 2% crossreactivity with POMC. *AgRP RIA:* AgRP antiserum provided by Dr. Barsh, raised against human AgRP and directed at the C-terminal end of the molecule. Human AgRP (83-132)-NH2 is used for the standard. This possesses 20% crossreactivity with full length AgRP. *POMC precursor ELISA*. POMC precursor was assayed by a two-site ELISA set up in our laboratory using antibodies provided by Dr. Anne White: Capture monoclonal antibody directed against ACTH 10-18; detection antibody directed against γ3-MSH; there is 100% crossreactivity with 22K pro-ACTH but none with ACTH, α-MSH, γ3-MSH or β-EP. Affinity purified human 31K POMC is used as a standard (357).

Plasma insulin and leptin: In all experiments, trunk blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) immediately after decapitation, and centrifuged at 4° C to separate plasma that was then stored at −80 °C. Plasma insulin and leptin concentrations were measured using RIA kits (Millipore Corporation, Billerica, MA).

Measurement of hypothalamic RNA levels: RNA isolation was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen USA, Valencia, CA) in conjunction with the RNase-Free DNase set (Qiagen USA) under the manufacturer's instructions and total RNA was quantified using spectrophotometry. cDNA was synthesized using the Superscript III First-Strand cDNA Synthesis Kit (Life Technologies Corporation/Invitrogen, Grand Island, NY) and was analyzed using quantitative RT- PCR performed with Lightcycler 480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN) in the Lightcycler 480 Real-Time PCR system (Roche Applied Science). Samples were normalized to β-actin. Primer sequences are as follows:

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Pomc	CAGTGCCAGGACCTCACCACGG	CGGTCCCAGCGGAAGTGACCC
Agrp	GCAAAGGCCATGCTGACTGC	CTTCTTGAGGCCATTCAGAC
Prcp	GCTTCTGCCCCTATCTGGCAGC	GGGCCAAGCAGGCAAAGGCT
Cpe	GCAACGCCCAGGGAATAGAT	GTCTCCTCCGTGCAGATTGG
Pcsk2	CCAGGCCATGGCTGATGGCGTG	CGTAGCTGCCACCGTCCCCAG
Pcsk1	CGTTCAGTTCAAAAAGACTC	GGCAGAGCTGCAGTCATTCT

Statistical analysis: Statistical analysis was performed with Student's *t* test when two groups were compared. Analysis of variance (ANOVA) followed by Fisher's protected least squares difference test was used when comparing more than two groups. $P < 0.05$ was considered statistically significant. Results are reported as mean values ± SEM.

Results

Experiment 1: Effects of 3 days of HFD feeding on hypothalamic melanocortin gene and peptide expression

Experiment 1a: Body weight, insulin, leptin: Body weight (29.4±0.4 g vs. 26.8±0.5 g, p<.001), body weight change, leptin and insulin levels were all significantly higher in 3 day HFD-fed mice compared to the control diet (p<.001, **Fig 5.1A,B**).

Peptides: POMC precursor peptide was significantly elevated in the MBH of the 3 day HFD group (p<.05, **Fig 5.1C**); furthermore POMC peptide content was ~4 fold lower in the AH section compared to the MBH. No difference in AgRP (p=.51, **Fig 5.1D**) or the POMC/AgRP peptide ratio was observed (p=.16, **Fig 5.1E**). α-MSH and β-EP were elevated in both the MBH (α-MSH p<.05, β-EP p=.12) and AH (α-MSH p=.31, β-EP p=.05) sections of the 3 day HFD mice (**Fig 5.1F,G**). The α-MSH/β-EP ratio was also suppressed in the AH (p<.05), but not different in the MBH (**Fig 5.1H**). In the MBH, there were no significant differences between groups with respect to the ratios of POMC/AgRP (p=.16), POMC/α-MSH (p=.74), POMC/β-EP (p=.81) or AgRP/α-MSH (p=.13) (**Table 5.1**). Similarly, the ratio of POMC/α-MSH and POMC/β-EP was not different between groups in the AH (data not shown).

Experiment 1b: Body weight, insulin, leptin: Although absolute body weight was not significantly different between groups (Normal chow 30.6±0.7 vs. 3 day HFD 30.8±0.5), body weight change (p<.001, **Fig 5.2A**), leptin (p<.05, **Fig 5.2B**) and insulin (p<.05, **Fig 5.2B**) levels were significantly higher in the 3-day HFD-fed mice.

RNA: In the MBH, *Pomc* mRNA levels tended to be elevated after 3 days of the HFD (p=.07, **Fig 5.2C**), without any change in *Agrp* mRNA levels (p=.32); the Pomc/Agrp ratio was significantly elevated after 3 days on the HFD (p<.01, **Fig 5.2D**). *Prcp* mRNA tended to be suppressed in the AH (p=.05) although no changes were observed in the MBH, and no other changes in POMC processing enzymes such as *Pcsk1, Pcsk2* and *Cpe* (**Fig 5.2F,G**) were observed.

Experiment 2: Effects of 7 days of high fat feeding on hypothalamic melanocortin peptide expression

Body weight, insulin, leptin: Absolute body weight was not different at sacrifice (Normal chow 30.5±0.5 vs. 7 day HFD 30.3±0.8g), however the 7-day HFD group gained 2.7±0.3g and leptin levels were significantly elevated (p<.05, **Fig 5.3A**), without any change in insulin.

Peptides: POMC peptide levels were elevated (p=.05, **Fig 5.3B**), without any significant change in α-MSH (p=.11, **Fig 5.3C**) or β-EP (p=.63, **Fig 5.3C**). There was no significant change in the POMC/α-MSH ratio (p=.77, **Table 5.1**), but the POMC/β-EP (p=.08, **Table 5.1**) and α-MSH/β-EP ratio (p=.08, **Fig 5.3D**) tended to be elevated in HFD mice. HPLC analyses revealed no significant difference in β-EP processing (p=.26, **Fig 5.3E**).

Experiment 3: Effects of 8 weeks of HFD feeding on hypothalamic melanocortin gene and peptide expression

Body weight, body composition, leptin: Body weight (**Fig 5.4A**), body weight gain, fat mass, gain in fat mass (**Table 5.2**) and leptin levels (**Fig 5.4B**) were significantly elevated after 8

weeks on the HFD ($p<.0001$); lean mass tended to be higher but did not reach significance ($p=.06$), however the increase in lean mass was greater in the HFD group (**Table 5.2**, $p<.05$). When the HFD group was divided into DIO (n=9) vs. DR (n=7) by a natural separation between the groups, all three groups were significantly different from one another with respect to body weight ($p<.001$), body weight gain ($p<.0001$), fat mass ($p<.0001$), gain in fat mass ($p<.0001$), and leptin ($p<.001$) (**Table 5.2**).

mRNA: *Pomc* mRNA levels were unchanged, however *Agrp* levels were significantly suppressed by the HFD ($p<.0001$, **Fig 5.4C**) and this did not change when the HFD group was separated into DIO and DR (data not shown, $p<.001$). The *Pomc/Agrp* ratio was significantly elevated to 146±4.0% of control diet values in the HFD group ($p<.0001$) and when the HFD group was divided the *Pomc/Agrp* ratio was still elevated in both the DIO (155±6%) and DR (136±3%) groups compared to the control diet ($p<.001$). Levels of processing enzymes responsible for cleaving POMC including *Pcsk*1 ($p=.13$), *Pcsk2* ($p=.23$), *Cpe* ($p=.14$), and *Prcp* ($p=.08$) in the MBH all tended to be lower on the HFD (**Fig 5.4J**). These reductions appeared to be driven by the DR mice. When stratified, Pcsk1 ($p=.08$), Pcsk2 ($p=.15$), Cpe ($p<.05$) and Prcp ($p<.05$) levels were lower or tended to be lower in the DR mice compared to normal chow mice (**Table 5.1**).

Peptides: POMC precursor peptide levels ($p=.05$, **Fig 5.4D**) were elevated and AgRP C-terminal peptide levels ($p<.05$, **Fig 5.4E**) were suppressed in the 8 week HFD group. If stratified into DIO and DR, increased POMC peptide levels tended to be driven by the DR group ($p=.07$ vs. control), although the DIO group was also higher ($p=.14$ vs. control, **Table 5.1**). α-MSH and β-

EP levels (**Fig 5.4G,H**) were unchanged between groups in both the AH and MBH sections and separation into DIO and DR groups did not change this (**Table 5.1**), except that β-EP in the AH was significantly higher in the DIO group versus the DR group ($p<.05$, **Table 5.1**). The α-MSH/β-EP ratio in the MBH tended ($p=.08$, **Fig 5.4I**) to be elevated by the HFD, and this appeared to be driven by the DR mice ($p=.05$ vs. control diet, **Table 5.1**).

Discussion

As the HFD is well described to contribute to obesity and it has been reported that the melanocortin system can mediate some of the effects of adaptation to HFD feeding including downregulation of hyperphagia and increase in activity after initiation of a HFD (297), this study sought to directly investigate the effects of HFD exposure on hypothalamic melanocortin gene and peptide expression, as well as expression of enzymes involved in post-translational processing of the POMC peptide. In the first experiment, 3 days of HFD feeding increased *Pomc* mRNA, POMC precursor peptide levels, and α-MSH and β-EP levels compared to mice maintained on a 10% fat control diet. At this time, no effects on AgRP mRNA or peptide levels were observed. After 3 days an increase in body weight, leptin and insulin levels was also observed. These hormones could contribute to the increase in POMC mRNA and peptide levels observed as both hormones are shown to stimulate POMC (57, 151). However, other nutrients in the HFD could contribute as well. We did not observe any significant changes in enzymes responsible for post-translational POMC processing including Pcsk1, Pcsk2, Cpe and Prcp. Interpretation of these results is complex as these enzymes are widely expressed and responsible for post-translational processing of a number of different peptides (77, 91-93). Future studies should isolate the arcuate, the site of Agrp and Pomc synthesis, and the PVN, an area of dense POMC and AgRP projection and MC4-R expression, to determine if enzyme changes in these regions are reflective of HFD-induced changes in POMC peptide processing. Furthermore protein levels as well as measurements of enzyme activity should be conducted as previous studies have reported changes in processing enzyme protein levels without changes in mRNA (78).

The second study investigated the effects of 7 days of HFD-feeding on expression of POMC and POMC-derived peptides and found that POMC precursor peptide levels were elevated, however there was no significant difference in α-MSH and β-EP. We also investigated β-EP processing in this experiment as previous reports have suggested that C-terminal β-EP processing can contribute to the metabolic phenotype of the mouse (95, 171); however we did not observe any difference in β-EP processing after 7 days of HFD feeding. As *Cpe* levels are reported to be suppressed by chronic HFD feeding (171), it would be worthwhile in future studies to determine the form of β-EP present in mice on a chronic HFD.

The third study investigated more chronic effects (8 weeks) of HFD feeding. Chronic HFD-feeding for 8 weeks did not increase *Pomc* mRNA levels, but POMC precursor peptide levels were elevated. There are a number of potential explanations for this observation. Firstly, it could be due to decreased processing of the POMC peptide as we observed a tendency for a decrease in processing enzymes. This appears unlikely as after 3 days of HFD feeding we observed an increase in α-MSH and β-EP along with an the increase in Pomc mRNA, although the chronic effects of HFD feeding could be different. Alternatively, the increase in POMC precursor could be due to altered sorting. POMC is targeted to the secretory pathway by a number of sorting signals including Cpe; in fact, Cpe deficiency misroutes POMC to the constitutive, instead of the secretory, pathway (97). This could affect peptide levels if targeting to the constitutive pathway increases degradation. The fact that increased POMC was not associated with increased α-MSH and β-EP levels could also be due to increased release of the processed peptides. This has been shown with naltrexone, which increases POMC levels with an associated decrease in α-MSH and β-EP levels which has been shown to be due to increased release of these processed peptides

(174, 356). *In vitro* studies using hypothalamic explants could be used in this model to determine effects on peptide release. When chronic HFD fed mice were separated into DR vs. DIO based on a natural separation in body weight between the groups, *Pomc* mRNA levels were still not different between groups, however the increase in POMC peptide levels observed with HFD-feeding tended to be higher in the DR vs. the control group and this increase was not as evident in the DIO group. This is interesting as leptin levels in the DIO group were significantly higher than in the DR group and as leptin is well established to stimulate POMC (158). α-MSH and β-EP levels were not different after chronic HFD-feeding, however when the HFD group was stratified into DIO and DR, β-EP levels in the AH of the DIO group were significantly higher than the DR group. Furthermore, the α-MSH/β-EP ratio, which trended higher when all mice were included in the HFD group, appeared to be driven by the DR mice. Levels of enzymes responsible for processing the POMC precursor peptide were not different between the 8 week HFD and control group, but were found to reach significance when the DIO and DR group were stratified. The DR group had significantly lower levels of *Cpe* and *Prcp* versus the control diet, while this was not evident in the DIO mice. The reduction in *Prcp* corroborates with the increased α-MSH/β-EP ratio observed in the DR mice. PRCP is highly expressed in the DMH and LH, two hypothalamic nuclei included in this MBH dissection (99). PRCP cleaves the C-terminal valine from α-MSH 1-13 rendering it inactive and deletion of PRCP in mice yields a lean phenotype (99). These data support that increased melanocortin activity may be protective against HFD-induced weight gain. This idea is also supported by the literature. It has been shown that DR rats have increased α-MSH positive neurons compared to DIO rats after 14 weeks of HFD-feeding (353), and when the DR A/J strain is exposed to a 35% HFD for 14 weeks, these

mice gain less weight and also have increased *Pomc* mRNA levels compared to the BL6 strain (354). This idea is also supported by studies investigating the effects of melanocortin signaling on substrate preference. It has been shown that models in which melanocortin activity is reduced, such as Pomc null, A^y and MC4-R KO mice, that these animals have increased preference for fat, while MTII decreases preference for fat (26, 300-304). Furthermore, published work in the rhesus monkey showed that POMC peptide levels in cerebrospinal fluid (CSF) were inversely related to body mass index (BMI), supporting the idea that POMC and POMC-derived peptides could be protective against weight gain (358).

Both Agrp mRNA and peptide levels were suppressed with chronic HFD feeding; these effects on mRNA are consistent with what has been previously reported in the literature (339, 340, 359), however the effects on AgRP peptide are new. This suppression could be due hormones such as insulin or leptin or lipids which have recently been shown modulate melanocortin activity (360). The effects of the HFD on AgRP peptide levels had not been previously explored, however one study in rats on a 13% kcal fat high-palatable energy rich diet (vs. 9% kcal from fat in control diet) found that C-terminal AgRP in the hypothalamus was elevated both 2 and 8 weeks after initiation of the diet (106). This is clearly a very different paradigm than what was studied in this experiment as the % kcal from fat difference between the palatable diet and control group was only 4% while the difference in % kcal from fat in our studies is 50% (10% in control vs. 60% in HFD). Thus, this is the first report of AgRP peptide levels after HFD initiation. Of note, an earlier timeframe (3 days) did not modulate AgRP peptide levels.

Another observation from Experiment 1, independent of HFD feeding, is that POMC peptide levels are approximately 4-fold higher in the MBH compared to the AH, while α-MSH and β-EP peptide levels are relatively equivalent between sections. This is an interesting finding as it appears that the POMC-processed peptides are found in equal distribution in areas of synthesis and utilization (as dense MC4-R populations are observed in the AH), while the POMC precursor peptide is found predominantly in the area of Pomc synthesis.

These preliminary studies indicate that both acute and chronic HFD exposure modulate melanocortin gene and peptide expression. We show that for the first time, the HFD increases POMC precursor peptide levels after both acute and chronic HFD administration. In the chronic study increased POMC peptide levels were observed even without an increase in *Pomc* mRNA suggesting that changes in peptide processing or release could contribute to this effect. However, this study is limited by the fact that only content was measured and future studies should investigate HFD-induced changes in POMC and POMC-derived peptide release to further understand this effect. Chronic HFD exposure also suppressed *Agrp* mRNA levels, consistent with previous reports, however another novel finding of this study is that chronic HFD exposure can also suppress AgRP peptide levels. Data from the chronic study is also supportive of the hypothesis that increased melanocortin activity may offer some protection against HFD-induced weight gain. Firstly, DR mice tended to have increased POMC precursor peptide levels compared to control diet fed mice, while this was not as evident in DIO mice that had higher leptin levels. Additionally, β-EP levels were significantly lower in the AH of DR mice compared to DIO mice and the α-MSH/β-EP ratios were higher in the MBH of DR mice. *Prcp* mRNA levels were also suppressed in the MBH of DR mice, which is consistent with the α-

MSH/β-EP ratio. However, these are only preliminary data and future studies are required to further understand how the melanocortin system may mediate HFD feeding. A major limitation of the enzyme measurements in these studies is the fact that *Pcsk1*, *Pcsk2* and *Cpe* mRNA hypothalamic enzyme measurements may not directly reflect POMC processing as these enzymes are also responsible for cleaving other pro-peptides in the hypothalamus (78, 83, 91-93). Furthermore, measurements of these enzymes may not reflect protein activity. Future studies which focus on obtaining protein levels of these enzymes in specific hypothalamic nuclei relevant to POMC processing, such as the arcuate and PVN should be pursued to further understand how the HFD may affect POMC processing. Another limitation of this study is in regard to the stratification of mice in the chronic HFD experiment. Our stratification was based on a natural divide in body weight between the HFD mice and consequently our DR mice were still significantly different with respect to body weight, adiposity, and other parameters versus control mice. Future studies should use larger groups of animals with more stringent criteria in which the DR group is more similar to the control diet fed mice. This may help amplify the effect of some of the trends observed in these studies. Despite these limitations, these data provide a greater insight into the effects of the HFD on melanocortin gene and peptide expression and support further study to understand how the melanocortin system responds to HFD feeding as well as how it may protect from the effects of HFD feeding.

TABLE 5.1: *Effects of HFD feeding on melanocortin gene and peptide expression*

Exp 1a (MBH)	**Control diet**	**3 day HFD**	
POMC/AgRP	0.28±0.01	0.32±0.02	
POMC/α-MSH	2.2±0.1	2.2±0.1	
POMC/β-EP	1.2±0.05	1.2±0.07	
AgRP/α-MSH	8.1±0.6	7.0±0.4	
Exp 2	**Control diet**	**7 day HFD**	
POMC/α-MSH	1.6±0.1	1.6±0.1	
POMC/β-EP	1.1±0.1	1.4±0.1	
Exp 3 (MBH and AH)	**Control diet**	**8 week HFD**	
MBH			
POMC/α-MSH	1.44±0.07	1.54±0.12	
POMC/β-EP	0.79±0.05	0.93±0.05	
AgRP/α-MSH	7.16±0.48	5.50±0.35*	
	Control diet	**8 week HFD-DIO**	**8 week HFD-DR**
MBH			
POMC precursor (fmol/mg protein)	225.2±11.0	255.8±18.1	265±.7±13.5
α-MSH (fmol/mg protein)	159.1±10.5	181.6±22.6	178.9±10.4
β-EP (fmol/mg protein)	295.1±22.1	297.7±28.4	273.3±17.5
POMC/α-MSH	1.44±0.07	1.55±0.20	1.52±0.13
POMC/β-EP	0.79±0.05	0.89±0.07	0.99±0.06*
AgRP/α-MSH	7.16±0.48	5.16±0.56*	5.94±0.35
α-MSH/β-EP	0.55±.03	0.61±.04	0.66±.04†
Pcsk1 mRNA (AU)	1.00±.08	0.92±.04	0.84±.07
Pcsk2 mRNA (AU)	1.00±.06	0.95±.06	0.88±0.05
Cpe mRNA (AU)	1.00±.05	0.96±.06	0.83±.02*
Prcp mRNA (AU)	1.00±.09	0.90±.05	0.80±.06*
AH			
α-MSH (fmol/mg protein)	222.4±13.2	258.3±16.5	222.9±15.9
β-EP (fmol/mg protein)	408.2±15.9	455.2±22.9	394.7±18.8#

All measurements reported at sacrifice in *ad lib* fed mice. Values are mean ± SE. *p<.05, †p=.05 vs. Control diet #p<.05 vs. DIO

TABLE 5.2: *Body composition data in mice switched to a chronic HFD*

	Control diet	**8 week HFD**	
Body Weight Gain (g)	2.0±0.3	12.3±1.1**	
Fat Mass at Sacrifice (g)	2.7±0.5	11.6±1.1**	
Fat Mass Gain (g)	0.2±0.5g	8.7±1.0**	
Lean Mass at Sacrifice (g)	21.7±0.3	23.1±0.5	
Lean Mass Gain (g)	1.7±0.6	3.6±0.4*	
	Control diet	**8 week HFD-DIO**	**8 week HFD-DR**
Body Weight (g)	29.0±0.4	43.4±0.9**	34.0±0.9**, ††
Body Weight Gain (g)	2.0±0.3	15.3±1.2**	8.6±0.7**,††
Fat Mass at Sacrifice (g)	2.7±0.5	14.5±1.0**	7.8±0.9**,††
Fat Mass Gain (g)	0.2±0.5	11.3±0.9**	5.4±0.8**,††
Leptin (ng/ml)	5.0±1.4	59.8±3.8**	27.5±5.3 **,††

All measurements reported at sacrifice in *ad lib* fed mice. Values are mean ± SE. **p<.001 *p<.05, vs. Control diet; †p <.05, ††p<.001 vs. DIO

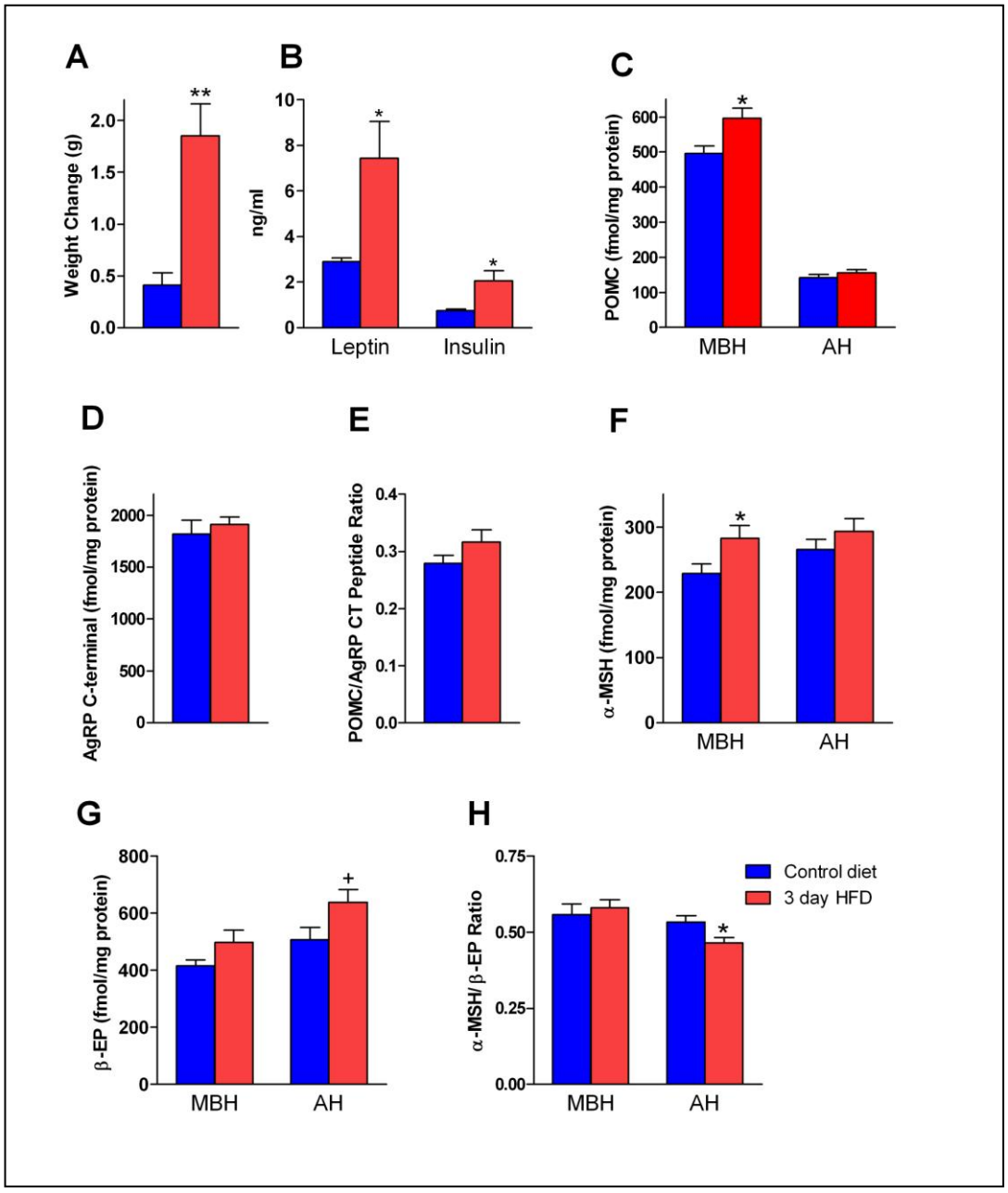
A
Weight Change (g)
2.0
1.5
1.0
0.5
0.0
**
B
ng/ml
10
8
6
4
2
0
*
*
Leptin
Insulin
C
POMC (fmol/mg protein)
600
500
400
300
200
100
0
*
MBH
AH
D
AgRP C-terminal (fmol/mg protein)
2000
1500
1000
500
0
E
POMC/AgRP CT Peptide Ratio
0.4
0.3
0.2
0.1
0.0
F
α-MSH (fmol/mg protein)
300
200
100
0
*
MBH
AH
G
β-EP (fmol/mg protein)
800
600
400
200
0
+
MBH
AH
H
α-MSH/ β-EP Ratio
0.75
0.50
0.25
0.00
*
MBH
AH
Control diet
3 day HFD

Figure 5.1: Effects of 3 days of high fat feeding on hypothalamic melanocortin peptide expression. (A,B) Body weight gain, leptin and insulin levels were all significantly elevated after 3 days on the HFD. **(C)** POMC precursor peptide was significantly elevated in the MBH, but not the AH of 3 day HFD-fed mice. (D) AgRP C-terminal peptide levels were not different between groups. (E) The POMC/AgRP C-terminal peptide ratio was not different between groups. **(F)** α-MSH was significantly elevated in the MBH of 3 day HFD-fed mice. **(G)** β-EP was elevated in the AH of 3 day HFD-fed mice. **(H)** The α-MSH/β-EP ratio was significantly suppressed in the AH of the 3 day HFD-fed mice. *p<.05, +p=.05 vs control diet

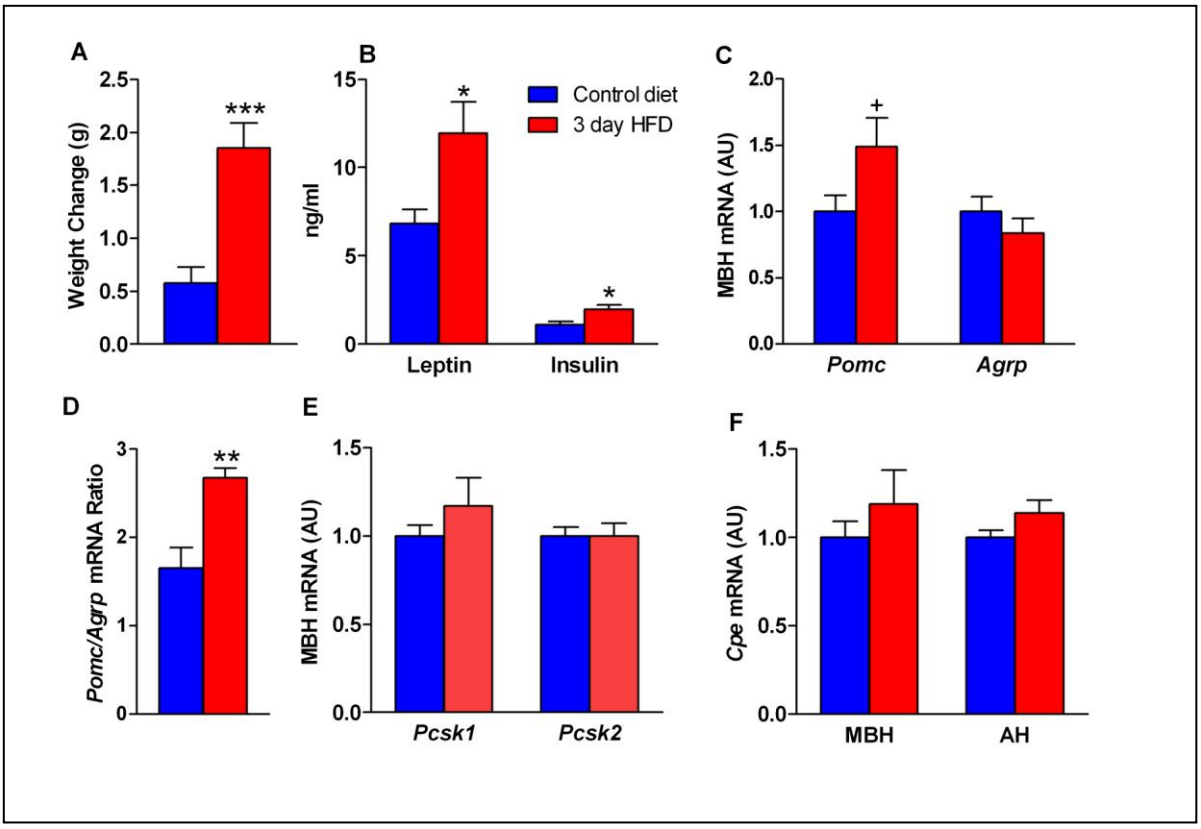

Figure 5.2: Effects of 3 days of high fat feeding on hypothalamic melanocortin gene expression. (A-B) Weight change, leptin and insulin levels were all significantly higher in 3 day HFD-fed mice. **(C)** *Pomc* mRNA tended to be elevated after 3 days on the HFD, without any change in *Agrp* mRNA. **(D)** The *Pomc/Agrp* ratio was significantly elevated after 3 days on the HFD. **(E)** *Pcsk1* and *Pcsk2* mRNA levels in the MBH were not different. **(F)** *Cpe* mRNA in the MBH and AH were not different between groups.

+p=.07, *p<.05, **p<.01, ***p<.001 vs control diet

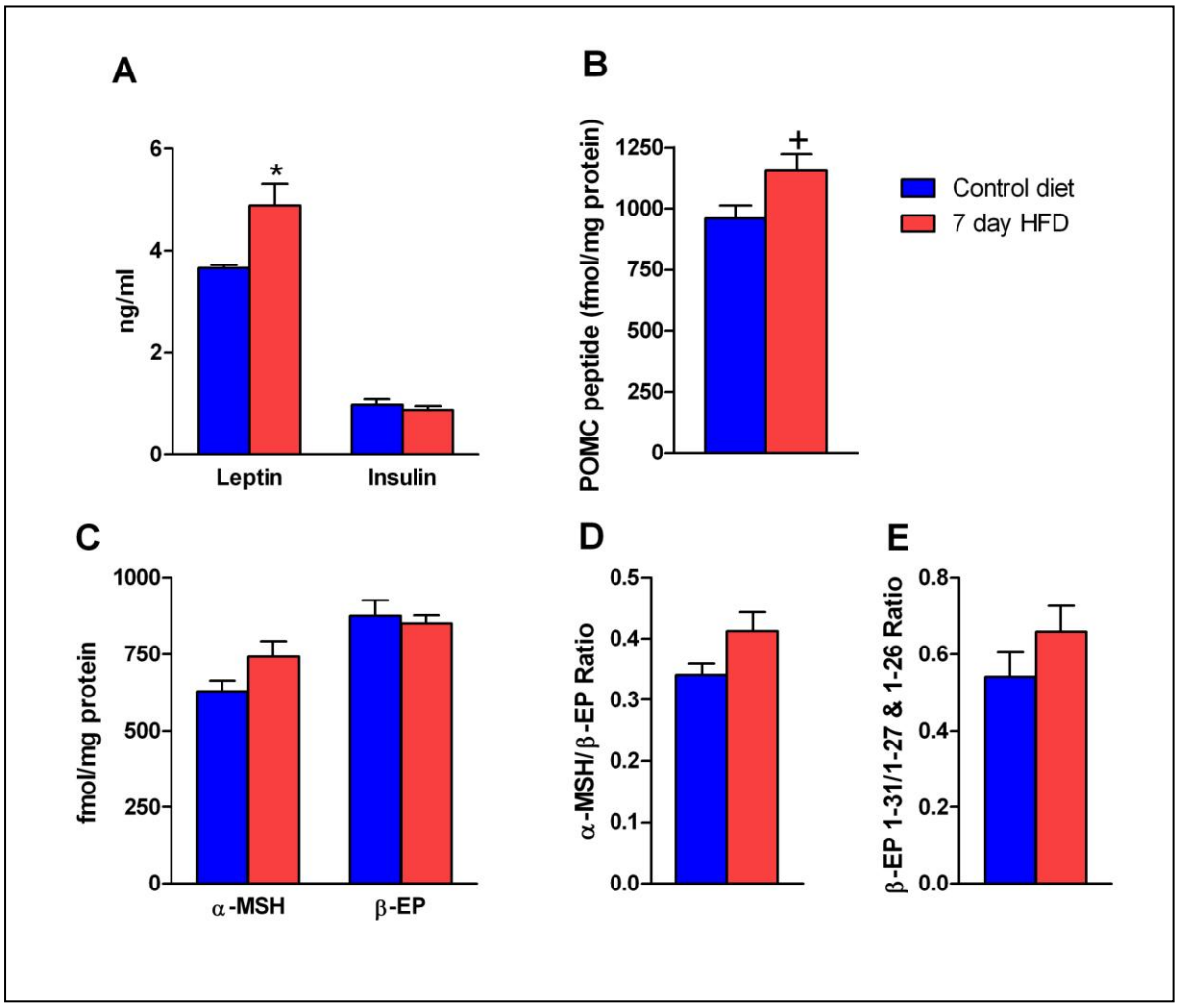

Figure 5.3: Effects of 7 days of high fat feeding on hypothalamic melanocortin peptide expression. **(A)** Leptin, but not insulin levels were higher in 7 day HFD-fed mice. **(B)** POMC precursor peptide levels were elevated in 7 day HFD-treated mice. **(C)** No change in α-MSH or β-EP was detected. **(D)** The α-MSH/β-EP ratio tended to be elevated by the 7 day HFD (p=.08). **(E)** β-EP processing was similar between groups. *p<.05, +p=.05 vs control diet

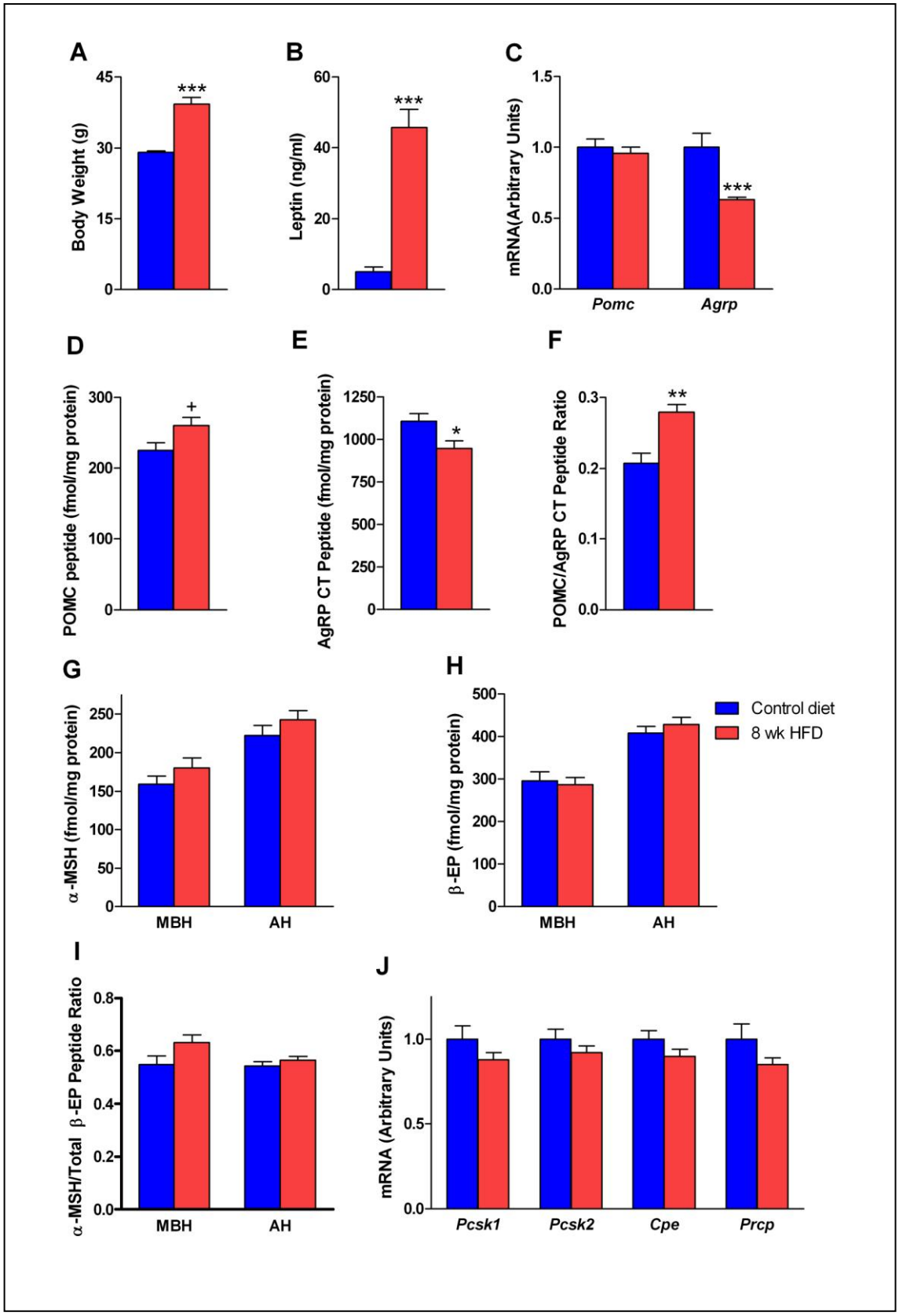
A
Body Weight (g)
45
30
15
0
***
B
Leptin (ng/ml)
60
40
20
0
***
C
mRNA(Arbitrary Units)
1.5
1.0
0.5
0.0
***
Pomc
Agrp
D
POMC peptide (fmol/mg protein)
300
200
100
0
+
E
AgRP CT Peptide (fmol/mg protein)
1250
1000
750
500
250
0
*
F
POMC/AgRP CT Peptide Ratio
0.3
0.2
0.1
0.0
**
G
α-MSH (fmol/mg protein)
250
200
150
100
50
0
MBH
AH
H
β-EP (fmol/mg protein)
500
400
300
200
100
0
MBH
AH
Control diet
8 wk HFD
I
α-MSH/Total β-EP Peptide Ratio
0.8
0.6
0.4
0.2
0.0
MBH
AH
J
mRNA (Arbitrary Units)
1.0
0.5
0.0
Pcsk1
Pcsk2
Cpe
Prcp

Figure 5.4: Effects of 8 weeks of high fat feeding on hypothalamic melanocortin gene and peptide expression. (A-B) Body weight and leptin levels were significantly higher in mice on the HFD for 8 weeks. **(C)** *Pomc* mRNA was unchanged but *Agrp* mRNA levels were significantly suppressed by the HFD. **(D-F)** In the MBH, POMC precursor peptide levels were higher, AgRP peptide levels were lower and the POMC/AgRP peptide ratio was significantly elevated by the HFD. **(G-I)** α-MSH and β-EP peptide were unchanged in either the MBH or AH, but the α-MSH/β-EP ratio tended (p=.08) to be elevated in the MBH, with no change in the AH. **(J)** POMC-processing enzymes *Pcsk1* (p=.13), *Pcsk2* (p=.23), *Cpe* (p=.14) and *Prcp* (p=.08) mRNA all tended to be lower in the HFD group.

+p=.05, *p<.05, **p<.001, ***p<.0001 vs control diet

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

The hypothalamic melanocortin system plays an important role in the regulation of energy balance and metabolism in both animals and humans. Humans with mutations in POMC peptide production or processing as well as MC4-R mutations yield profound effects on obesity (5, 225). Furthermore, MC-4R mutations are the most common monogenic form of severe obesity, with studies citing between 2-6% prevalence in the obese population (226-228). Genetic manipulation of the melanocortin system in animal models further supports the human data as mice with POMC or MC4-R deletion possess obese phenotypes, as do mice with deletion or manipulation in POMC processing enzymes (3, 5). The goal of this thesis was to further study unexplored aspects of melanocortin regulation of energy balance including POMC-derived peptide interaction, the effect of the HFD on POMC processing, melanocortin regulation of prolactin and the effects of an AgRP inhibitor on metabolic parameters in rodents.

One aim of this thesis was to further understand how POMC processing affects energy balance. The effect of β-EP on energy balance is somewhat perplexing as a single icv injection acutely stimulates food intake while selective deletion of β-EP produces a mildly obese phenotype (59, 60, 62, 64). **Chapter 2** focused on the effects of β-EP on food intake and energy balance, both alone and in interaction with α-MSH. In our study, β-EP 1-31 acutely stimulated food intake during the light cycle, consistent with previous reports (59, 60, 62), as well as during the dark cycle. When β-EP 1-31 was infused chronically, no stimulatory effects on food intake or weight gain were detected, and in fact chronic high dose administration decreased food intake after 3 days of infusion. This reduction in food intake may be due to suppressed *Agrp* mRNA levels observed in these rats, a novel finding that persisted despite a fall in leptin levels which would

normally be associated with increased *Agrp* mRNA (290, 361). However, there are opioid receptors on AgRP neurons and a recent study showed that opioids inhibit AgRP neuronal activity which would be consistent with these findings (260, 276). Furthermore, the chronic β-EP data corroborates with the obese phenotype of the β-EP KO mouse (64). In this chapter, it was also observed that a subthreshold dose of β-EP 1-31, which did not stimulate food intake alone, could prevent the suppressive effects of α-MSH on food intake and body weight gain in both an acute fasting and refeeding model and chronic infusion model. However, the antagonism of β-EP on α-MSH did not persist after 3 days in the chronic infusion model. This effect is likely due to opioid tolerance and downregulation which is well-documented to occur with β-EP treatment (272, 273). Rats in this experiment were sacrificed after 7 days of treatment, when the α-MSH and α-MSH+β-EP groups' weights were equally lower than that of the saline group. However, future studies in which the same experiment is repeated in rats sacrificed after 2-3 days, when β-EP is still actively antagonizing α-MSH's effects, could help elucidate if β-EP not only antagonizes α-MSH's effects on food intake and body weight but other aspects of energy balance such as EE, glucose metabolism, and peripheral lipid metabolism. In this study we also observed that levels of *Prcp* mRNA, an enzyme which inactivates α-MSH (99), were decreased in two separate studies of β-EP infusion; this could be another potential mechanism by which mice with chronic β-EP treatment reduce their food intake as decreased *Prcp* could potentially yield increased α-MSH levels. Future studies could directly investigate this effect by treating rodents with either opioids or icv β-EP 1-31 and measuring PRCP levels. Alternatively, the effects of icv β-EP in PRCP null mice, whose lean phenotype has been described, could be evaluated (99). Another key finding of this study is that β-EP 1-31 and 1-27 differentially

regulate feeding and weight gain. Although β-EP 1-31 increased food intake during the light cycle and blocked α-MSH-induced suppression in food intake and body weight gain in an acute fasting and refeeding model, β-EP 1-27 failed to produce these effects. This is the first report of β-EP 1-27 on feeding in a mammalian model and further supports that C-terminal processing of β-EP contributes to the regulation of energy balance.

POMC is processed to α-MSH, which binds to the stimulatory G-protein coupled MC4-R, and β-EP, which binds to the inhibitory G-protein coupled MOR (255, 362). It appears that the mechanism by which these peptides may oppose one another is via functional antagonism at their respective receptors as activation of these receptors yields opposing levels of cAMP. As β-EP 1-27 binds to the MOR but does not activate signal transduction as β-EP 1-31 does, this is likely the mechanism by which β-EP 1-27's effects on feeding are different than β-EP 1-31. In fact, the opposing effects of α-MSH and β-EP 1-31 as well as β-EP 1-31 and 1-27 have been shown previously in models relevant to reproduction and analgesia (65, 216, 262, 363-365). At present it is thought that α-MSH and β-EP are sorted to the same secretory granules and are released together. However, this has not been studied carefully. There is evidence that pro-TRH products can be sorted to different secretory vesicles in the hypothalamus and there is also novel subcellular localization of β-LPH and β-EP, but not ACTH or α-MSH, in the peroxisomes in the pituitary (286, 287). Future studies could use electron microscopy to determine if α-MSH and β-EP can be localized to different secretory vesicles to further understand how these products are sorted and secreted. It is also evident that α-MSH and β-EP can be further processed and inactivated and that this process may be regulated. For example, α-MSH can be deactivated by PRCP and β-EP 1-31 can be C-terminally cleaved to opioids with reduced activity. Future

studies should understand how this process is regulated and how the balance between α-MSH and β-EP is maintained.

Although not included as a chapter in this thesis, the author contributed to a publication which showed that mice with selective deletion of FOXO1 in POMC neurons had a lean phenotype owing to increased *Cpe* levels, which were responsible for the anorexigenic profile of increased α-MSH and increased C-terminal processing of β-EP (171). Contributing to this project afforded this author the opportunity to study a model in which endogenous β-EP processing contributed to a lean phenotype. Preliminary data from **Chapter 5** also investigated how altered melanocortin gene and peptide expression could contribute to a resulting metabolic effect. Mice introduced to a 60% HFD were found to have increased POMC peptide levels after both acute (3 and 7 days) and chronic (8 week) HFD exposure. After 3 days, the increase in POMC peptide was accompanied by an increase in *Pomc* mRNA as well as an increase in α-MSH and β-EP levels. However, the increase in POMC peptide observed at 8 weeks was without any change in *Pomc* mRNA or α-MSH and β-EP, suggesting that altered sorting or release could be involved. POMC is targeted to the secretory pathway by a number of sorting signals including *Cpe*, and its absence has been shown to misroute POMC to the constitutive pathway, an action that could potentially increase degradation and contribute to changes in peptide levels (97). As POMC peptide, but not α-MSH and β-EP levels, were increased possibly suggests that there is increased release of these processed peptides but not increased release of POMC. This has been shown with naltrexone, which increases POMC levels with an associated decrease in α-MSH and β-EP levels; this decrease in processed peptides has been shown to be due to increased release (174, 356). In this chapter we also showed for the first time that chronic HFD treatment decreases

AgRP peptide levels. This is accompanied by a decrease in AgRP mRNA levels. It is thus likely that AgRP turnover and peptide release are decreased by the HFD. It has been previously reported that both leptin and insulin decrease AgRP peptide release (109), and it would be worthwhile to determine if the HFD would yield the same effects on AgRP release as the HFD is shown to increase levels of these hormones. Future studies should also investigate the effects of the HFD on the release of POMC and POMC-processed peptides, as well as the effects of nutrients pertinent to the HFD on the release these peptides.

Chapter 2 demonstrated that POMC-derived peptides interact to regulate energy balance, however this concept of α-MSH and β-EP interacting to regulate physiological functions was inspired by a study in ovariectomized rhesus monkeys in which acute icv administration of α-MSH was able to block β-EP-induced stimulation of prolactin release (216). Although a number of studies have shown that acute icv injections of α-MSH decrease prolactin release, while icv AgRP and β-EP increase prolactin release, the effects of chronic modulation of the melanocortin system on prolactin is unknown (211, 214-216). **Chapter 4** investigates blood and pituitary prolactin content in genetic models of altered melanocortin activity including mice overexpressing an N-terminal POMC transgene (Tg-MSH) that includes α- and γ_3-MSH (but not ACTH or β-EP) which were previously reported to possess a lean phenotype (50, 51) and mice with selective AgRP deletion, which were reported to possess a metabolic phenotype similar to WT counterparts (127). We observed that prolactin levels were lower in Tg-MSH and AgRP KO male mice under both stressed and unstressed conditions. Furthermore, prolactin content in the pituitary was significantly lower in male AgRP KO mice. Previous studies have shown that the acute effects of α-MSH and β-EP on prolactin secretion are mediated via dopamine (211, 213,

219, 223), which is well established to chronically inhibit prolactin synthesis and release (218). *Tyrosine hydroxylase* mRNA expression, the rate-limiting step in dopamine synthesis, was measured in both Tg-MSH and AgRP KO mice, but was not significantly different in either group compared to WT mice. Hypothalamic dopamine and DOPAC content were also measured in male AgRP KO and WT mice, however no differences in content were detected either. To investigate a more dynamic measurement, a dopamine type 2 receptor (D2R) antagonist was administered to AgRP KO and WT mice with the hypothesis that the fold-increase in prolactin levels would be greater in the AgRP KO mice. However, D2R antagonism increased prolactin levels to the same degree in both AgRP KO and WT mice. These data by no means precludes dopamine's role in mediating the effects on prolactin release observed in MSH-Tg and AgRP KO mice. It is possible that the chronically lower pituitary prolactin content in these mice affected their response to metoclopramide. Furthermore, these measurements investigated dopamine content, not turnover, which could be different between groups. Future studies could examine dopamine turnover to further investigate if dopamine mediates the effects observed on prolactin release in these genetic models.

Another interesting observation from this study is that while prolactin levels were reduced in male AgRP KO mice, female AgRP KO mice had blood and pituitary levels of prolactin similar to WT mice. However, as prolactin is critical to reproduction and fertility, it is possible that these females can compensate; indeed developmental compensation has been reported with AgRP KO mice (127, 307). Reproduction was not a major endpoint in this study, however AgRP KO and WT mice appeared to reproduce with similar frequency and litter sizes. It has recently been shown that ablation of the AgRP neuron in *ob/ob* mice can restore metabolic

derangements and fertility in both male and female mice (366). However, more importantly, AgRP peptide deletion in leptin receptor deficient *db/db* female mice dramatically improves fertility and these effects are independent of metabolic parameters as Agrp deletion did not reduce body weight or adiposity in these mice (although it did improve glucose homeostasis) (312). Interestingly, in this study Agrp deletion did not restore fertility in male *db/db* mice which suggests that there could be sexually dimorphic effects of the melanocortin system on reproduction. We also observed sexually dimorphic effects on prolactin release in our study as prolactin levels were significantly lower in male, but not female mice. However, we did not assess prolactin levels in females during pregnancy or lactation, when this hormone serves an important role (307). Studies investigating prolactin levels during this period would help determine if this genotype affects prolactin levels in females.

Despite the well described role prolactin has been shown to play in reproduction, prolactin is also associated with energy balance. Both central and peripheral injection of prolactin have been shown to increase food intake (331-333) while prolactin receptor-deficient mice have a slightly leaner phenotype (334). Additionally, prolactin has been shown to induce leptin resistance and stimulate AgRP (330, 367). Evidence suggests that prolactin levels are increased in human obesity and it is hypothesized the elevations in prolactin may result from decreased inhibitory tone from hypothalamic dopaminergic neurons (336, 368). It is therefore possible that increased prolactin levels could serve as a marker for decreased dopaminergic activity and thus reduced melanocortin activity and suggests that circulating prolactin levels could serve as a biomarker of central melanocortinergic and dopaminergic tone. However, this idea requires further study in both animal and human models.

The phenotype of AgRP KO mice is reported as relatively mild and this is purported to be due to developmental compensation (127). In light of these findings, we sought to further investigate the role of the AgRP peptide in the regulation of energy balance using a novel inhibitor of AgRP developed by TransTech Pharma Inc, (**Chapter 3**) which circumvents the developmental compensation issues evident in AgRP KO mice. The AgRP inhibitor was shown in *in vitro* studies by TransTech Pharma Inc, to prevent AgRP from antagonizing α-MSH-induced increases in cAMP activity. We utilized this compound in numerous *in vivo* studies as a tool to study the role of the AgRP peptide as well as investigate the potential of a new therapeutic intervention for obesity. The effects of TTP2515 were first investigated in icv cannulated rats receiving either icv AgRP or saline and oral TTP2515 or water. TTP2515 attenuated AgRP-induced increases in food intake and body weight gain either after a single AgRP injection or after continuous AgRP infusion. TTP2515 also reversed AgRP-induced increases in adiposity and suppression in total and free T4 levels. Importantly, these effects were not observed in rats receiving icv saline and TTP2515-treatment, which suggested that these effects were due to AgRP antagonism. Subsequent studies investigating endogenous AgRP antagonism in DIO and leptin-deficient mice showed that TTP2515 suppressed food intake, weight gain, and adiposity and decreased RQ scores consistent with increased lipid utilization. However, a transient reduction in VO2, EE and activity, inconsistent with AgRP's purported effects, was also observed (128). The effects on food intake, body weight and adiposity were also largely observed in DIO AgRP KO mice, although the effects, particularly on adiposity, appeared to be less pronounced in AgRP KO mice compared to WT mice. Furthermore the effects on VO2, EE and activity were also observed in AgRP KO mice showing that these effects are not due to AgRP antagonism. Although it is

difficult to determine if the dramatic effects observed in leptin-deficient *ob/ob* are due to AgRP antagonism as the appropriate control would be leptin-receptor deficient AgRP KO mice, the same 50 mg/kg dose which produces such profound effects in *ob/ob* mice does not affect food intake, body weight or adiposity in WT mice. Furthermore, *ob/ob* mice are reported to have immense elevations in *Agrp* mRNA which we assume is contributing to the dramatic phenotype observed with TTP2515 treatment (120). Furthermore, given the results of a recent paper showing that deletion of the Agrp peptide in *db/db* mice restores fertility in females (312), it would be interesting to determine if TTP2515 could also accomplish this.

TTP2515 also potently inhibited food intake when mice were acutely switched from a chow diet to a HFD and simultaneously started treatment with the drug; this is drastically different than the hyperphagic effect typically observed in WT mice switched to HFD (297). TTP2515-treated mice recover from this initial anorectic response and resume daily food intake comparable to WT mice, however they maintained a reduction in cumulative food intake and body weight for the duration of this study. This is quite remarkable as TTP2515-treated mice maintained on a chow diet with the same or higher doses of the drug are unaffected. When this experiment was repeated in AgRP KO mice, these mice also potently reduced their food intake after exposure to a HFD, and this was to the same degree as WT mice. This demonstrates that the potent effects on HFD-feeding, although consistent with AgRP's reported effects on increasing fat preference (300, 301), are not AgRP-mediated. In this chapter it was also reported that TTP2515 administered for 4 or more days increased total T4 and free T4 levels and was able to reverse fasting-induced suppression in T4 levels after chronic administration. However, 36 h of treatment was not able to reverse the suppression in T4 levels after a fast and TTP2515 did not

affect T3 levels in any of the paradigms studied. Furthermore, the increase in T4 levels was equally observed in AgRP KO mice. Thus, although the effects on T4 are consistent with AgRP's inhibitory effect on the thyroid axis, it appears that the effects on T4 are not AgRP mediated and does call into question if the effects observed on T4 are due to interference with binding proteins in the assay. As AgRP levels are elevated by fasting (289, 290), the effects of TTP2515 in AgRP KO and WT mice were also examined in a fasting and refeeding model. Specific effects of TTP2515 on refeeding in WT, but not AgRP KO mice, were observed when a moderate (25 mg/kg) dose was used, however higher doses (50 mg/kg) attenuated refeeding in both WT and AgRP KO mice, although effects on weight gain were selectively attenuated in WT mice at this higher dose. This suggests that the more moderate dose yields specific effects on AgRP antagonism but higher doses may result in off-target effects on refeeding.

The data from these experiments demonstrate that TTP2515 exerts numerous metabolic effects in rodents consistent with AgRP inhibition. The ability of the AgRP inhibitor to prevent AgRP's effects is supported by *in vitro* work in which TTP2515 prevented AgRP, but not SHU9119, induced suppression in MSH-induced increases in cAMP activity and *in vivo* work in which TTP2515 was only effective in icv cannulated rats receiving exogenous AgRP. However, studies in the AgRP KO mouse model demonstrate that some of these effects are not AgRP-mediated. TTP2515 was most effective in obese models including DIO and *ob/ob* mice, as well as during initiation of a HFD and during refeeding after a fast. Despite these remarkable effects, reductions in food intake and weight gain were not observed in lean mice on a low fat diet. These results have direct implications for human obesity. The data suggest that TTP2515 would be useful in individuals after weight loss as a means to prevent weight regain, when a fall in

leptin levels may contribute to increases in AgRP. This compound could also be used before initiation of a high-fat meal. It would be interesting to test if humans are also more responsive to this drug under this paradigm. Although we did not directly test substrate preference using a food choice/ reference test, we would hypothesize that mice administered TTP2515 and the choice of a low-fat or high-fat diet would choose the low-fat diet. This effect is somewhat reminiscent of what is reported after gastric bypass surgery, in which humans and rodents are reported to prefer low fat versus high fat foods (369-371). Although this could provide a potentially novel use for this compound, human feeding is infinitely more complex and it is unknown if the physiological effects of the drug would overcome environmental cues to high-fat feeding. The effectiveness of TTP2515 warrants further exploration to determine the contribution of additional mechanisms of action. Although a likely candidate is *Agouti* as it possesses 25% sequence homology to AgRP, it is only present in the skin and fur of mice where it serves a paracrine function (120). A more likely candidate is one that strongly influences preference for fat intake and may even be localized to the gut. Although an AgRP inhibitor provides a potential advantage over melanocortin agonists as AgRP projects to more limited brain regions than the MC4-R, investigation of this drug's effects on hypertension and cardiac function should also be pursued as these side effects have been reported with melanocortin agonists (292, 293, 296, 372).

Conclusions

Although great strides have been made in furthering the understanding of the regulation of energy balance and metabolism, the obesity epidemic continues to rise with increased numbers

of individuals qualifying as overweight or obese. The hypothalamic melanocortin system is only one component which contributes to obesity, however its robust effects in both humans and animals support its critical role in the pathogenesis of this disease. This thesis furthered the understanding of a number of different aspects of melanocortin regulation of energy balance including how POMC-derived peptides interact to regulate energy balance and how environmental factors such as the HFD can modulate processing of the POMC peptide. This thesis also showed that chronic melanocortin stimulation can decrease prolactin levels and investigated the effects of a potential therapeutic agent for obesity that prevents AgRP's actions. Further understanding of how specific aspects of the melanocortin system can contribute to obesity will help uncover potential therapeutic targets to combat the obesity epidemic.

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