ENHANCEMENT OF BRAIN MELANOCORTIN SIGNALING
IN LEAN, ACTIVE RATS

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Appendix 3: List of Abbreviations

Adenylate cyclase activating polypeptide ----------------------------------------------- Adcyap1
Adrenocorticotropic hormone ------------------------------------------------------------- ACTH
Agouti-related protein --------------------------------------------------------------- AgRP
American Medical Association --------------------------------------------------------- AMA
Analysis of covariance -------------------------------------------------------------- ANCOVA
Analysis of variance --------------------------------------------------------------- ANOVA
Area prostema ------------------------------------------------------------------------ AP
Artificial cerebrospinal fluid -------------------------------------------------------- aCSF
Body mass index ---------------------------------------------------------------------- BMI
Brain-derived neurotrophic factor ----------------------------------------------------- BDNF
Carboxypeptidase E --------------------------------------------------------------------- CPE
Central nervous system --------------------------------------------------------------- CNS
Cocaine- and amphetamine-regulated transcript ----------------------------------------- CART
Corticotropin-like-intermediate lobe peptide ------------------------------------------ CLIP
Diacylglycerol ------------------------------------------------------------------------ DAG
Dorsomedial nucleus of the hypothalamus --------------------------------------------- DMN
β-endorphin --------------------------------------------------------------------------- β-EP
Energy expenditure --------------------------------------------------------------------- EE
Fat mass and obesity associated ------------------------------------------------------- FTO
Forkhead box protein O1 --------------------------------------------------------------- FoxO1
Ghrelin receptor ----------------------------------------------------------------------- GHSR
<table>
<thead>
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<th>Term</th>
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<tr>
<td>Paraventricular nucleus</td>
<td>PVN</td>
</tr>
<tr>
<td>Peptidyl α-amidating monooxygenase</td>
<td>PAM</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td>PAG</td>
</tr>
<tr>
<td>Phosphate buffer saline with tween 20</td>
<td>PBST</td>
</tr>
<tr>
<td>Phosphatidylinositol-3 kinase</td>
<td>PI3K</td>
</tr>
<tr>
<td>Prohormone convertases</td>
<td>PC</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>PKC</td>
</tr>
<tr>
<td>Prolylcarboxypeptidase</td>
<td>PRCP</td>
</tr>
<tr>
<td>Proopiomelanocortin</td>
<td>POMC</td>
</tr>
<tr>
<td>Radioimmunoprecipitation buffer</td>
<td>RIPA</td>
</tr>
<tr>
<td>Respiratory exchange ratio</td>
<td>RER</td>
</tr>
<tr>
<td>Restriction fragment length polymorphism</td>
<td>RFLP</td>
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<tr>
<td>Signal transducer and activator of transcription 3</td>
<td>STAT3</td>
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<tr>
<td>Single nucleotide polymorphism</td>
<td>SNP</td>
</tr>
<tr>
<td>Sirtuin</td>
<td>SIRT1</td>
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<tr>
<td>Splicing factor 1</td>
<td>SF1</td>
</tr>
<tr>
<td>Sprague–Dawley</td>
<td>SD</td>
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<tr>
<td>Sympathetic nervous system</td>
<td>SNS</td>
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<tr>
<td>Thyrotropin releasing hormone</td>
<td>TRH</td>
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<tr>
<td>Ventromedial nucleus of the hypothalamus</td>
<td>VMN</td>
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<td>World Health Organization</td>
<td>WHO</td>
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CHAPTER 1: Introduction

1.1 Obesity

The problem of obesity is one that continues to plague society at growing proportions, decreasing the quality of life along with increasing mortality rates (Allison, Fontaine, Manson, Stevens, & VanItallie, 1999; Flegal, Carroll, Ogden, & Johnson, 2002; Heo, Allison, Faith, Zhu, & Fontaine, 2003; Ogden, Carroll, Kit, & Flegal, 2014). Obesity incidence has increased over the years and it is now considered to be a serious health issue all over the world, but particularly in the United States (Fontaine, Redden, Wang, Westfall, & Allison, 2003; Haffner & Taegtmeyer, 2003; Klein, 2004). At the annual meeting in 2013, obesity was declared to be a disease by the American Medical Association (AMA). Obesity is also closely linked to many life-threatening comorbidities; for example, type-2 diabetes can be preceded by metabolic syndrome, which includes cardiovascular diseases and high blood cholesterol (Grundy, 2004; Haslam & James, 2005; Sheehan & Jensen, 2000). As a result, obesity contributes to a large proportion of health-care costs (Østbye, 2013). In the US alone, obesity accounts for almost 21% of all health-care costs, which was about 190.2 billion USD in 2005 (Cawley John, 2012). On average, an obese individual spends $274 more on health care related expenses vs. a non-obese person in a year (Cawley John, 2012).
A western diet and a sedentary lifestyle are important factors that promote obesity. Being a complex condition, there are many contributors to a person’s likelihood of becoming obese, including an individual’s genetic predisposition. What predisposes a person to be resistant or prone to gain weight in an obesogenic environment is still poorly understood, however.

1.2 Physical Activity and its Relevance in the Obesity Epidemic

Sedentary behavior can be defined as sitting or lying down for most parts of the day, which is becoming a common trend in the modern society (Sedentary Behaviour Research, 2012). A good comparison to link lack of physical activity with the increasing prevalence of obesity comes from studies on the Amish community, as their lifestyle has remained almost unchanged over the years. Amish people, both men and women, have an extremely low prevalence of obesity, along with very high daily physical activity levels, when compared to other individuals in North America (Bassett, Schneider, & Huntington, 2004; Tremblay, Esliger, Copeland, Barnes, & Bassett, 2008). This decline in physical activity over time is related to increased prevalence of obesity in today’s society (Church, Earnest, Skinner, & Blair, 2007; Church et al., 2011). Multiple studies have shown that a sedentary lifestyle as well as being physically inactive both increase the risk of metabolic disease as well as cancer (George, Rosenkranz, & Kolt, 2013). Even in people who exercise, sitting for longer periods directly affects insulin sensitivity and can escalate the risk of cardiovascular diseases (Owen, Healy, Matthews, & Dunstan, 2010; Stephens,
The problem has led to the idea that “sitting is the new smoking” and that prolonged inactivity is harmful in itself (George et al., 2013; Owen et al., 2010). More precisely, common sedentary habits in today’s society such as television viewing or sitting for longer durations at work have been linked to a high cardiovascular related mortality rate in males (Warren et al., 2010) and an increased incidence of diabetes and the metabolic syndrome (Hu et al., 2001).

According to the World Health Organization’s (WHO) Global Recommendations on Physical Activity for Health, adults are recommended to engage in a minimum of 150 minutes of moderate-to-vigorous-intensity physical activity per week. In addition to its health benefits and improving the metabolic profile, regular physical activity also significantly improves life expectancy (D. W. Brown et al., 2004b; Moore et al., 2012; Teske, Billington, Kuskowski, & Kotz, 2012). In a randomized control trial in post-menopausal women, overall fitness was found to be proportional to a person’s physical activity and exercise training (Church et al., 2007). The general trend, however, has been in the opposite direction, with daily physical activity in adults, decreasing over the last five decades including occupation-related physical activity (Church et al., 2011; Hamilton, Healy, Dunstan, Zderic, & Owen, 2008).

The tendency to be physically active is not only biologically regulated and a heritable trait (Garland et al., 2011; Novak & Levine, 2007), but also interacts with known genetic determinants of obesity risk including the fat mass and obesity-associated
(FTO) protein (Qi & Cho, 2008). Alpha-ketoglutarate-dependent dioxygenase, commonly known as the FTO protein, is encoded by the FTO gene located on chromosome 16 in humans. Certain variants of the FTO gene have been shown to be strongly associated with obesity in children as well as adults (Frayling et al., 2007). However, physical activity can decrease the likelihood of obesity by up to 30% in adults carrying a variant of this gene (Kilpelainen et al., 2011). Therefore, physical activity can be a highly effective means of regulating body weight, potentially counteracting known risk factors for obesity.

1.3 Energy Expenditure and non-exercise activity thermogenesis (NEAT)

The total energy expended during the day apart from any volitional exercise, sporting activities, sleeping, and eating, can be categorized as non-exercise activity thermogenesis (NEAT) (Levine, 2002; Levine et al., 2005). NEAT, or the activity of daily living, includes small everyday tasks like walking around or fidgeting, cleaning the house, mowing the lawn, shoveling snow, and even cooking or doing laundry. While these activities seem trivial individually, cumulative non-exercise physical activities can contribute greatly towards daily energy expenditure (EE) and affect body weight (D. W. Brown et al., 2004a; Levine, 2002). For most individuals, NEAT, rather than exercise, accounts for the majority of calories expended by activity during a day.

Non-obese individuals have been shown to resist weight gain by increasing their NEAT under increased caloric intake (Levine, Eberhardt, & Jensen, 1999; Levine,
Schleusner, & Jensen, 2000). Compared to their obese and sedentary counterparts, lean people tend to spend more time standing rather than sitting (Levine et al., 1999; Levine et al., 2005). For instance, while the resting metabolic rate between a lean and obese female was found to be similar, obese women expend less energy through activity relative to body weight, which can be accounted for by fewer hours spent per day in sitting and low-activity behaviors, than in standing and high-energy-expending tasks (Johannsen, Welk, Sharp, & Flakoll, 2008). Therefore, NEAT varies considerably between individuals and could explain how some individuals are more physically active and remain lean in an obesogenic environment, while others are more prone to sedentary habits and have higher body weights.

NEAT is an important concept that accounts for a large proportion of total daily EE. There is a need to better understand the physiological and molecular interactions that influence physical activity and EE, having a direct impact on weight gain and obesity propensity. By understanding the regulatory mechanisms of NEAT, we may be able to explain how spontaneous physical activity is possibly controlled by various factors and affects obesity. Overall, NEAT and EE of daily living is an interesting concept to explain the obesogenic trends in today’s population. However, equally important are the neuronal mechanisms that regulate the energy balance pathway. One such circuitry is the brain melanocortin (MC) system.
1.4 Proopiomelanocortin (POMC) processing in the hypothalamus: Regulation of energy balance

Proopiomelanocortin (POMC) is a 31-kDa precursor protein which is synthesized in the arcuate nucleus of the hypothalamus, nucleus of the solitary tract (NTS), the medulla, pituitary, and also in several peripheral tissues (Wardlaw, 2011). The prohormone is processed tissue specifically, yielding many biologically active peptides including α-melanocyte-stimulating hormone (α-MSH), adrenocorticotropic hormone (ACTH), and β-endorphin (β-EP) that are involved in multiple physiological processes throughout the body.

The POMC precursor protein is comprised of an N-terminal sequence that binds to carboxypeptidase E (CPE), which then acts as a sorting signal in the regulated secretory pathway (Cool et al., 1997). During this trafficking process, POMC is proteolytically cleaved into a number of biologically active peptides. The differential expression of prohormone convertases (PCs) in various tissues leads to tissue-specific posttranslational processing of POMC (Castro & Morrison, 1997; Smith & Funder, 1988). Functionally active peptides are produced by endoproteolytic cleavage by the PCs, PC1/3 and PC2 (Benjannet, Rondeau, Day, Chretien, & Seidah, 1991).
Figure 1: Schematic diagram indicating the endoproteolytic cleavage of the Proopiomelanocortin (POMC) molecule including the resulting peptide products.

Adapted from (Wardlaw, 2011), with copyright permission from Elsevier Limited.

(JP = Joining peptide; LPH= Lipotropin; EP = Endorphin; CLIP= Corticotropin-like-intermediate lobe peptide; da-α–MSH = desacetyl α–melanocyte stimulating hormone; PC1/3 and PC2= Prohormone convertases 1/3 and 2; CPE= Carboxypeptidase E; N-AT= N-acetyltransferase; PAM= Peptidyl α–amidating monoxygenase; PRCP = Prolylcarboxypeptidase).
Figure 1 (Wardlaw, 2011) illustrates the POMC processing mechanism. In the anterior pituitary, POMC processing primarily leads to production of ACTH, γ-lipotropin (LPH), and a 16 kDa N-terminal fragment. In the hypothalamus and in the intermediate lobe of the pituitary, POMC is processed to yield α-MSH and corticotropin-like-intermediate lobe peptide (CLIP); β-LPH is processed to γ-LPH and β-EP, and N-terminal is processed to γ3-MSH (Emeson & Eipper, 1986; Pritchard & White, 2007).

POMC is first cleaved by PC1/3 to yield pro-ACTH and β-LPH. Pro-ACTH is then cleaved by PC1/3 to ACTH and the N-terminal. Further processing by PC2 yields ACTH 1–17 and CLIP as well as γ-LPH and β-EP 1–31 (Benjannet et al., 1991). CPE removes the C-terminal basic amino acid residues from ACTH 1–17 to ACTH 1–13 which is then amidated by the enzyme peptidyl α-amidating monooxygenase (PAM) to generate desacetyl α-MSH, an important peptide in central regulation of energy balance and a focus of the work described here. Desacetyl α-MSH is acetylated by an N-acetyltransferase to form α-MSH. Recently, a new processing enzyme, prolylcarboxypeptidase (PRCP), has been identified that is responsible for inactivation of α-MSH by removal of the C-terminal valine (Wallingford et al., 2009).

POMC neurons in the brain play a major role in regulating energy balance,
including the peptides cleaved post-translationally to yield α-MSH and other peptides that interact with melanocortin receptors (MCRs). These neuronal populations have a demonstrated importance in human obesity as well as in animal models of obesity (Bell, Walley, & Froguel, 2005; Pritchard, Turnbull, & White, 2002). Mice having a Pomc gene deletion or ablation of the POMC neuron are obese (Challis et al., 2002; Yaswen, Diehl, Brennan, & Hochgeschwender, 1999), while overexpression of Pomc can attenuate obesity in ob/ob mice (lacking the hormone leptin) and in obese Zucker rats (G. Li, Mobbs, & Scarpase, 2003; Mizuno, Kelley, Pasinetti, Roberts, & Mobbs, 2003). POMC mutations have also been reported in obese humans (Krude et al., 1998). The neuronal transcription factor, nescient helix-loop-helix2 (Nhlh2), which regulates the transcription of PC1/3 and PC2, has also been implicated in human obesity (Fox, Vella, & Good, 2007). Nhlh2 is expressed throughout the hypothalamus, including in POMC neurons.

Other peptides and precursors found alongside POMC in the arcuate and in the hypothalamus also play important roles in mediating energy balance (Harrold, 2004; Wilding, 2002). Agouti-related protein (AgRP) is a natural inverse agonist of MCRs that can modulate the signaling activity of brain MCRs. α-MSH and AgRP are synthesized in distinct neuronal populations within the arcuate nucleus that project into other hypothalamic regions including the paraventricular nucleus (PVN), lateral hypothalamus (LH), and the brainstem. These areas of the hypothalamus and hindbrain are known to be particularly important in regulating energy balance (Cowley et al., 1999; Elmquist, Elias,
While α-MSH inhibits feeding and stimulates EE, AgRP decreases EE and is orexigenic (Murphy et al., 1998). Furthermore, injections of synthetic α-MSH antagonists increase food intake, indicating a constitutive role of α-MSH in appetite suppression (McMinn, Wilkinson, Havel, Woods, & Schwartz, 2000; Murphy et al., 1998). Along with appetite, α-MSH can also affect EE, oxygen consumption, and fuel oxidation, all of which contribute to changes in energy balance (Remmers & Delemarre-van de Waal, 2011). Energy availability modulates this system; POMC expression in the arcuate neurons is suppressed during fasting and stimulated when energy stores are in excess (Elmquist et al., 1999). Additionally, POMC and AgRP neurons can both act as sensors of peripheral energy stores and respond to a variety of dietary, neuronal, and hormonal signals that are known to affect energy balance, such as insulin and leptin (Ibrahim et al., 2003; Levin, Routh, Kang, Sanders, & Dunn-Meynell, 2004). The MC system is known to be the target of numerous mutations and single nucleotide polymorphisms (SNPs) relevant to human obesity, and is therefore a topic of considerable interest for understanding the complex circuitry that regulates energy homeostasis (Zegers, Van Hul, Van Gaal, & Beckers, 2012) and will be expanded upon below.

1.5 Parallel regulation of Pome transcription and peptide processing

Many peripheral signals from circulating hormones such as insulin, ghrelin, and leptin, among others, are sensed in hindbrain and the hypothalamus (Levin et al., 2004).
The transcription of POMC can be regulated in the hypothalamus by these peripheral signals. Levels of peripheral energy stores are sensed by leptin receptors on POMC neurons. (Elias et al., 1999). In addition, mice with selective deletion of leptin receptors on POMC neurons are obese (Balthasar et al., 2004). Though leptin and insulin act through distinct signaling pathways, they also share intracellular signaling pathways with respect to phosphoinositide 3-kinase (PI3K) stimulation in POMC neurons (A. W. Xu et al., 2005). Insulin binds to its receptors on POMC neurons and stimulates phosphatidylinositol-3 kinase (PI3K), leading to phosphorylation of AKT and subsequent phosphorylation and exclusion of forkhead box protein O1 (FoxO1) from the nucleus of POMC neurons (Fukuda et al., 2008). Perinatally, leptin can affect the development of POMC neuronal projections, while insulin also has a stimulatory effect on POMC gene expression (Benoit et al., 2002; Bouret, Draper, & Simerly, 2004). Figure 2 (Wardlaw, 2011) depicts the PC1/3, CPE, PRCP, and POMC processing relative to changes in energy balance and leptin and insulin action. Briefly, via the signal transducer and activator of transcription 3 (STAT3) pathway, leptin stimulates Pome gene transcription and also stimulates the genes Psck1 and Psck2 for the processing enzymes, PC1/3 and PC2. The transcription factor Nhlh2 stimulates Psck1 and Psck2 as well. Simultaneously, FoxO1, which is part of the insulin signaling pathway, inhibits Pome gene transcription and the processing enzyme, CPE; CPE sorts POMC to the regulated secretory pathway and PRCP is responsible for the degradation of α-MSH. Therefore, the ratio of POMC precursor to its processed peptides is constantly regulated by physiological changes in energy balance such as fasting or high-fat diet.
Figure 2: Parallel regulation of proopiomelanocortin (POMC) transcription and peptide processing. Adapted from (Wardlaw, 2011), with copyright permission from Elsevier Limited.

(EP = Endorphin; PC1/3 and PC2= Prohormone convertases 1/3 and 2; CPE= Carboxyptidase E; PRCP = Prolylcarboxyptidase; Nhll2= nescent helix-loop-helix2, MSH= melanocyte stimulating hormone, FoxO1= forkhead box protein O1, STAT3= signal transducer and activator of transcription 3, ACTH= adrenocorticotropic hormone).
The two sets of neurons in the arcuate—AgRP/ neuropeptide Y (NPY) and POMC/cocaine- and amphetamine-regulated transcript (CART) neurons—are impacted by circulating hormones that act as signals of energy surplus or surfeit. The central MC system is important in sensing and interacting with peripheral, neural, and endocrine cues such as insulin, leptin, and ghrelin, which then influence downstream mediators of energy balance (Belgardt & Bruning, 2010; Cone, 2005). Figure 3 summarizes some of the interconnected peripheral and central signals involved in energy balance and the control of energy homeostasis by neurons of the arcuate nucleus. AgRP and NPY stimulate food intake and decrease EE, whereas α-MSH (cleaved from POMC) and CART are neuropeptides that inhibit food intake and increase EE. Insulin and leptin are circulating hormones that increase in response to long-and short-term energy stores in the body, and that inhibit AgRP/NPY neurons and stimulate adjacent POMC/CART neurons. Ghrelin is also a circulating peptide secreted from the stomach that can activate AgRP/NPY neurons, thereby stimulating food intake. Though these are the most studied endocrine signals, there are other important intermediates such as brain-derived neurotrophic factor (BDNF) that converge on hindbrain and hypothalamus to alter energy balance (Rios, 2013).
Figure 3: Summary of some of the inter-related signals involved in energy balance and the control of energy homeostasis by neurons of the arcuate nucleus.

Adapted from (Barsh & Schwartz, 2002), with copyright permission from Nature publishing group.
1.6 Melanocortin Receptors (MCR)

As described above, while POMC expression in the brain is limited to the arcuate nucleus and hindbrain area prostema (AP), it is cleaved post-translationally to yield different bioactive compounds that act on MC receptors. Along with its receptors, the brain MC system plays a key role in energy balance as defects in POMC synthesis or processing, and haploinsufficiency of the MC3 and MC4 receptors have all been reported in human obesity syndromes (Coll, Farooqi, Challis, Yeo, & O'Rahilly, 2004). The biological effects of MCs are mediated by their interactions with MCR. MCRs are members of the rhodopsin family of 7-transmembrane G protein-coupled receptors. MCR signaling activates the enzyme adenylate cyclase, leading to accumulation of cAMP and protein kinase C (PKC) along with diacylglycerol (DAG) (Millington, 2006).

Currently, there are five known members of the MCR system, MCR 1 through 5, each having differing specificities for the MC peptides. The MCs contain the amino acid sequence His-Phe-Arg-Trp, which is required for receptor binding (Millington, 2006). MC1R is predominantly the peripheral α-MSH receptor important in pigmentation, whereas MC2R is the ACTH receptor. Receptor binding studies have shown that β-MSH has higher affinity for MC4R than does α-MSH, with γ-MSH having the lowest affinity. γ-MSH binds to the MC3R with higher affinity than either α- or β-MSH (Getting, 2006). MC5R binds to α-MSH and has been shown to regulate lipolysis and fatty acid oxidation in skeletal muscle (An et al., 2007; Rodrigues, Almeida, & Gouveia, 2012). Among the
three ligands, α-MSH is generally associated with appetite regulation in mammals; β-MSH is not produced in rodents as they lack the proximal di-basic site that is necessary for the proteolytic cleavage event that produces β-MSH (Coll et al., 2004; Y. S. Lee et al., 2006; Tung, Piper, Yeung, O'Rahilly, & Coll, 2006). Summarized in Table 1, the following section discusses these MCR subtypes, their expression, and known functions.
Table 1: Melanocortin receptor (MCR) subtypes and their known functions.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Localization</th>
<th>Function/Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1R</td>
<td>Melanocytes, inflammatory cells (monocytes, neutrophils), testis, ovary</td>
<td>Pigmentation, anti-inflammatory</td>
<td>(Cone et al., 1996; Xia, Wikberg, &amp; Chhajlani, 1995)</td>
</tr>
<tr>
<td>MC2R</td>
<td>Adrenal cortex, skin, testis, white adipose tissue, mononuclear leukocytes</td>
<td>Adrenal steroidogenesis, mediates lipolytic actions of ACTH in mammals</td>
<td>(Boston &amp; Cone, 1996; Slominski, Ermak, &amp; Mihm, 1996; Weber et al., 1995)</td>
</tr>
<tr>
<td>MC3R</td>
<td>Hypothalamus, limbic system, heart, pancreas, testis, stomach, duodenum, placenta, liver, skeletal muscle, adipose tissue, monocytes, macrophages</td>
<td>Energy homeostasis: (processing and release of POMC derived peptides, entrainment to food availability), photoperiod changes, regulation of insulin production, inhibition of pro-inflammatory mediators</td>
<td>(G. M. Sutton et al., 2008) (Cowley et al., 2001; Kumar et al., 2009; Leoni et al., 2008; Nilaweera et al., 2009; G. M. Sutton et al., 2006)</td>
</tr>
<tr>
<td>MC4R</td>
<td>Hypothalamus, cerebral cortex, brainstem</td>
<td>Energy homeostasis: food intake, energy expenditure, glucose homeostasis, insulin secretion and sensitivity, bone mineral content, Erectile activity</td>
<td>(Fan, Boston, Kesterson, Hruby, &amp; Cone, 1997; Huszar et al., 1997; Kumar et al., 2009; G. M. Sutton et al., 2006)</td>
</tr>
<tr>
<td>MC5R</td>
<td>Exocrine glands, skeletal muscle, brain, lymphocytes, adipocytes, liver, heart, eye, adrenals, pancreas, prostate, bone</td>
<td>Exocrine gland secretion, aggression</td>
<td>(Akbulut et al., 2001; Chen et al., 1997; Morgan &amp; Cone, 2006)</td>
</tr>
</tbody>
</table>
1.6.1 MC1R:

MCR1 is known to mediate effects on coat color and skin pigmentation, and is present on melanocytes (Cone et al., 1996). Mutations of the Mc1-r gene have been associated with variations in coat color in animals as well as a red hair and a fair skin phenotype in humans (Valverde, Healy, Jackson, Rees, & Thody, 1995). Some variants are also linked to an increased risk of melanoma and skin cancers (Han, Kraft, Colditz, Wong, & Hunter, 2006). This receptor has also been detected in inflammatory cells and in the periaqueductal gray (PAG) in rodents and humans (Xia et al., 1995). Therefore, this receptor subtype is mainly associated with roles in pigmentation and immunomodulatory and anti-inflammatory effects.

1.6.2 MC2R:

MC2R is expressed in the adrenal cortex, mediating effects of ACTH on glucocorticoid synthesis. Its role in adrenal steroidogenesis is further confirmed by several mutation studies demonstrating a large number of cases of glucocorticoid deficiencies in humans with MC2R mutations (Weber et al., 1995). MC2R is also present in adipocytes in rodents (but not in humans), and may play a role in mediating MC-induced lipolysis (Boston & Cone, 1996).
1.6.3 MC3R and MC4R

These two receptor subtypes are highly expressed in the brain and are specifically associated with areas involved in regulating energy balance.

In the brain, MC3R is mainly localized to the limbic system and the hypothalamus, within the arcuate nucleus on POMC and AgRP neurons, ventromedial hypothalamus (VMN), preoptic nucleus, lateral hypothalamus, and posterior hypothalamic area (Roselli-Rehfuss et al., 1993). MC3R can also be found in the hippocampus, thalamus, amygdala, septum, shell of the nucleus accumbens, and brainstem including the central nucleus of raphe and ventral tegmental area (Lindblom, Schioth, Larsson, Wikberg, & Bergstrom, 1998; Roselli-Rehfuss et al., 1993). Among the peripheral tissues, MC3R is present in the gut, heart, monocytes, and the placenta (Gantz, Konda, et al., 1993).

MC4R, which is the most extensively studied MC receptor present in the brain, is located in the hypothalamus, thalamus, cerebral cortex, brainstem, and the spinal cord; MC4R has not been detected outside of the central nervous system (CNS) thus far (Cowley et al., 1999; Gantz, Miwa, et al., 1993; Mountjoy, Mortrud, Low, Simerly, & Cone, 1994). Within the hypothalamus, the MC4R is extensively present in the PVN and the LH, which are both important in regulating energy balance including food intake, diabetes, hyperphagia, as well as obesity (Fan et al., 1997; Huszar et al., 1997). It is also present at lower concentrations in the dorsomedial hypothalamus (DMN), and VMN (M. Lee & Wardlaw, 2007).
There is also emerging evidence of the role of MC4R in autonomic outflow, where it activates the sympathetic nervous system (SNS) and inhibits the parasympathetic neurons by acting on pre-autonomic areas, and also with receptors on pre- and post-ganglionic neurons (Sohn et al., 2013). For instance, mice deficient in MC4R have normal blood pressure and are protected from hypertension despite their profound obesity, possibly because of the lack of MC4R-stimulated SNS/parasympathetic nervous system (PSNS) function, mediated by the hypothalamic IKK-β and NF-κB mechanisms (Purkayastha, Zhang, & Cai, 2011).

### 1.6.4 MC5R

MC5R is present in numerous peripheral tissues, including fat cells, kidney, lung, mammary glands, ovary, pituitary, testis, uterus, leukocytes, lymph nodes, stomach, spleen, skin, bone marrow, esophagus, spinal cord, and predominantly in exocrine and endocrine glands including prostate, pancreas, adrenals, and thymus, sebaceous glands, as well as in skeletal muscles (Akbulut et al., 2001; Chen et al., 1997; Labbe, Desarnaud, Eggerickx, Vassart, & Parmentier, 1994). The receptor’s most well-defined function is in the regulation of exocrine gland function, and MC5R knockout (KO) mice are known to express an exocrine gland dysfunction (Chen et al., 1997). MC5R has also been reported to modulate immune responses by their expression in lymphocytes (Akbulut et al., 2001). Rodents also contain MC5R in adipocytes. MC5R is the predominant subtype expressed in skeletal muscle and plays a role in fatty acid oxidation via the actions of α-MSH (An et
al., 2007). It is also found in the brain in low amounts (Fathi, Iben, & Parker, 1995; Griffon et al., 1994; Labbe et al., 1994), but any evidence in the current literature to support its physiological role in the brain is absent.

As described above, the receptors MC3R, MC4R, and MC5R are present in the adult mammalian brain, localized in the PVN, LH, DMN, and VMN, among other areas (Pritchard et al., 2002). Since the MC (e.g., α-MSH) projections extend into these areas of the hypothalamus, it is important to know how MC receptors and their ligands exert their actions further downstream in various regions of the brain.

1.7 MCR mutation studies

Several studies have reported hyperphagia and obesity in rodents by the loss or inactivation of MC4R (Butler et al., 2001; Challis et al., 2004; Huszar et al., 1997). Targeted deletion of MC3R also leads to an obese phenotype (Chen et al., 2000). The primary focus of MC actions on the brain to alter energy balance is through MC4R and, to a lesser extent, the MC3R subtype. Mutations in MC3R and MC4R have been associated with decreased EE in mice and rats, partly through a decrease in physical activity (Butler et al., 2001; Chen et al., 2000; Mul, van Boxtel, et al., 2012). Some MC3R and MC4R variants have also been identified to be associated with physical activity in humans (Loos et al., 2005; Yako et al., 2012). Although characterized to be similar to MC3R and MC4R and found in the brain among other tissues, MC5R is less studied, particularly with respect to energy balance (Fathi et al., 1995; Griffon et al.,
1994).

1.7.1 \textit{Mc3r null mice}

The mechanism by which MC3R mediates energy homeostasis is not as well understood as the MC4R, but it is likely related to energy partitioning of fat stores and feeding efficiency. The MC3R-KO mice have an obese phenotype, which is mild relative to the MC4R-KOs. Also, unlike their MC4R counterparts, MC3R KOs are not hyperphagic but still have increased fat and lean body mass (Butler et al., 2000; Chen et al., 2000). In addition, these MC3R-KO mice are generally only mildly hyperinsulinemic and exhibit a higher respiratory quotient and an increased feed efficiency when placed on a high-fat diet (Butler et al., 2000). This indicates that MC3R contributes more to EE than to appetite. Mice lacking both MC3R and MC4R are also significantly more obese compared to mice deficient in either MCR alone (Chen et al., 2000), indicating that both these receptors possibly mediate energy balance independent of each other and that their functions are not completely overlapping.

1.7.2 \textit{Mc4r null mice and rats}

MC4R KO mice were shown to develop a late-onset obese phenotype, which is characterized by hyperphagia, hyperinsulinemia, hyperglycemia, and hyperleptinemia. Mice heterozygous for the mutation exhibit an intermediate phenotype compared to wild type and MC4R-KO mice. Further, it was shown that hyperphagia alone could not
explain the obese phenotype in this case because the KOs weighed more than the wild-type mice when they were pair fed, with equal amounts of food given to both groups (Ste Marie, Miura, Marsh, Yagaloff, & Palmiter, 2000). Increased adiposity and reduced O$_2$ consumption were also seen in MC4R-KO mice. While wild-type mice showed increased diet-induced thermogenesis and physical activity on a high-fat diet, no such response was observed in the MC4R-KO animals (Butler et al., 2001). When MC4R was restored specifically to the PVN, MC4R-KO experienced a decrease in hyperphagia, which led to attenuation of obesity, while the relatively low EE remained unchanged (Balthasar et al., 2005). This implicates different regional concentrations of MC4R in appetite-suppressive vs. EE functions of MC4R. Interestingly, α-MSH analogs also fail to stimulate metabolic rate or suppress feeding in MC4R-KO animals, which suggests that this receptor is primarily responsible for mediating the effects of α-MSH on energy balance (A. S. Chen et al., 2000; Marsh et al., 1999). Young MC4R-deficient mice also show hyperinsulinemia and impaired insulin tolerance before the onset of obesity, which suggests a possible role of the receptor in modulating insulin sensitivity independently of, and not just as a response to, the obese phenotype (Fan et al., 2000).

Recently, a rat model has been developed that lacks functional MC4R. These KO rats have increased body weight, white adipose mass, body length, and hyperphagia. They also exhibit a decrease in melanotan II (MTII)-stimulated grooming behavior and ambulatory activity (Mul, van Boxtel, et al., 2012). Functional loss of the MC4R shows a gene-dosage effect on body weight, with homozygote-KO weighing more than the WT
mice as well as the heterozygotes. Additionally, intracerebroventricular (i.c.v.) administration of MTII induced excessive grooming behavior in wild-type but not in the homozygous KO rats (Mul, Begg, et al., 2012), implicating this receptor in the ability of endogenous MC to exert its effects on behaviors including physical activity.

Pharmacological studies complement the phenotypic findings seen in mutation studies. A common MC3/4 receptor antagonist SHU9119, when injected i.c.v., leads to a decrease in spontaneous physical activity in rats (Adage et al., 2001). This implicates some sort of tonic MC action on spontaneous physical activity. Further, i.c.v. injections of the endogenous MC receptor agonist, α-MSH, have been shown to increase Fos expression in the PVN, which suggests that hypothalamic areas including the PVN likely mediate the downstream effects of MC and its receptors on energy balance (McMinn et al., 2000).

1.7.3 Mc5r null mice

MC5R-KO mice have a suppressed pheromone-induced aggressive behavior. (Caldwell & Lepri, 2002; Morgan & Cone, 2006). There has been limited research focusing on MC5R or its role in energy balance since the structure of this receptor was characterized in rat only in 1994 (Griffon et al., 1994). However, since MC5R is present in the brain, as well as in other tissues, and has a strikingly similar sequence structure to receptors MC3R and MC4R, its possible role in metabolism should not be ignored (Fathi et al., 1995).
In summary, MC is an important peptide system that regulates the energy balance equation by interacting with its receptors MCR3, 4, and 5 that are present in the brain. While it remains poorly addressed in the literature, some of the studies described in this dissertation address the role of MC5R in the brain.

1.8 MCR mutations and human obesity

Mutations and polymorphisms in the MC4R are considered to be the most common monogenic cause of severe obesity in humans (Cauchi et al., 2009; Loos et al., 2008; MacKenzie, 2006). Over the last several years, many human studies have reported MC4R gene mutations in multiple ethnic groups (Farooqi et al., 2000; Hinney et al., 1999; Vaisse et al., 2000; Vaisse, Clement, Guy-Grand, & Froguel, 1998). In a large cohort study of 500 people, 5.8% of the patients with severe early-onset morbid obesity were found to express mutations of the MC4R (Farooqi et al., 2003). As in rodents, obese humans with MC4R mutations also demonstrate hyperphagia, hyperinsulinemia, and an increased linear growth in childhood (Farooqi et al., 2003; Farooqi et al., 2000). These individuals also display increased bone mineral density, fat mass, and lean mass. Although a majority of MC4R mutations were linked to obese patients, some subjects with these variants were found to be non-obese. Therefore, it is possible that such haploinsufficiency mutations promote obesity with variable expressivity. An association between the severity of the functional loss of MC4R and the age of onset of obesity has also been reported, with more severe functional loss of the receptor function being
associated with earlier onset of the obesity (Lubrano-Berthelier et al., 2006). These studies provide compelling evidence for the role of the MC4R in controlling energy balance in the human population. A multitude of population genetic studies have linked many different variants of MC4R to human obesity (Chagnon et al., 1997; Santini et al., 2009; Stutzmann et al., 2007). In addition to monogenetic obesity, which is quite rare, polygenic obesity also leads to an obese phenotype, with increasing effect conferred by each genetic variant being a possible contributor (Hinney & Hebebrand, 2008; Qi & Cho, 2008).

Many MC3R mutations and polymorphisms have been reported in humans, though the data to correlate them with the pathogenesis of human obesity is not as convincing. For instance, one study comparing overweight and non-overweight children identified two polymorphisms in association with high body weight, while the same inactivating polymorphisms had no such correlation in adult females (Feng et al., 2005; W. D. Li et al., 2000). Similarly, a study of 252 morbidly obese adults and 312 controls detected 3 MC3R gene variants with equal frequency in both the study-groups (Schalin-Jantti et al., 2003). Recently, however, a study comparing 290 obese subjects with 215 individuals having normal weight identified three mutations in the MC3R gene in the obese group that also segregated in the members of the families studied (Mencarelli et al., 2008). Such reports implicate MC3R mutations that are dominantly inherited as a cause of obesity. In another study that examined subjects of Italian and French origin, the complete coding region of the MC3R gene was analyzed followed by in vitro functional analysis of the mutations detected (Mencarelli et al., 2011). While the presence of MC3R
variants was not significantly higher in the obese sub-population per se, the mutations that resulted in functional modifications were significantly higher in the obese group. Thus, some of these genetic variants may have a stronger impact on an individual’s tendency to gain weight. Supporting this idea was a report describing 63 severely obese children, of which only 4 were found to have a heterozygous missense mutation; however, all 283 non-obese individuals lacked this mutation (Dubern et al., 2001).

With respect to MC5R mutations, restriction fragment length polymorphism (RFLP) analysis of 124 Quebec families found a linkage or association with obese phenotypes, where parameters like BMI, fat mass, glucose tolerance, resting metabolic rate were examined (Chagnon et al., 1997). Most studies have failed to identify a specific function of these variants; the report identified a similar correlation with MC4R variants, though it was strongest in MC5R polymorphisms.

The role of these MCRs in the brain is yet to be mapped out with respect to their regulatory function of energy balance (Grill, 2006; Grill, Ginsberg, Seeley, & Kaplan, 1998). Also it is unknown whether these receptors work together site specifically to regulate food intake and EE. To better understand the neuronal regulation of MCs, it is critical to find these answers. The studies described in this dissertation in the forthcoming chapters address this question where I have attempted to isolate hypothalamic nuclei that are crucial for the actions of each receptor subtype with respect to energy homeostasis in rats.
1.9 Identification of target proteins within MC system

The brain MC system, consisting of POMC, α-MSH, AgRP, and the MCRs, plays a key role in regulating feeding behavior as well as energy homeostasis in general (Butler, 2006; Fan et al., 1997). A growing number of studies in both rodents and humans that show genetic defects in the synthesis or processing of POMC, or in MCR signaling, clearly implicate the MC system as a critical regulator of energy balance. The role of its receptors in this process, particularly MC4R and MC3R, has been demonstrated by knockout studies as well as a large number of obese patients reported with SNPs affecting these receptors. MC4R mutations are also the most common known monogenic cause of human obesity, with more than 150 reported mutations (Tao, 2010).

Another key component involved in the central MC system are the melanocortin receptor accessory proteins (MRAP), the mechanism in which MCRs, along with other G protein-coupled receptors (GPCRs), signal and traffic to the cell membrane. This could be vital in understanding MCR dysfunction and associated disorders. MRAP, a MC2R accessory protein, is critical for MC2R function. MRAP2 is a homologue, expressed in brain and the adrenal gland, has been shown to interact with all 5 MCRs, including MC3R and MC4R that are associated with obesity (Chan et al., 2009). Mrap2 null mice were recently reported to be obese, with a late-onset of hyperphagia, while there was no significant effect on their EE or respiratory exchange ratio (RER) (Masato Asai, 2013). Additionally, since MRAP can interact with central MC4R and MC3R, a brain-specific
inactivation of this accessory protein also led to an obese phenotype in mice (Masato Asai, 2013). In humans, MRAP2 variants have been identified to be associated with early-onset and severe obesity (Masato Asai, 2013). MRAPs form stable complexes with the receptors and have contrasting effects on MC2 vs. MC5 receptors. While it facilitates surface expression of MC2R, MRAP2 has been shown to prevent MC5R from being expressed in the plasma membrane (Sebag & Hinkle, 2009). Hence, these accessory proteins can potentially influence MC signaling through any of the MCRs, including those in the brain.

In summary, MC pathway integrates with a multitude of signaling intermediates such as leptin, along with other factors such as accessory proteins, which all coexist and play important roles in maintaining energy balance. The leptin-MC pathway is a target of many genetic changes associated with obesity. A more detailed understanding of the regulation of this system can lead to effective new therapies for human obesity including those focused on increasing physical activity.

1.10 Hypothalamic brain regions and energy balance

Several brain regions that process energy balance information have been extensively shown to have overlapping roles, illustrated in Figure 4. Below in this section we describe each one individually.
1.10.1 Arcuate nucleus

The arcuate nucleus is present at the base of the hypothalamus. As described above, there are two main neuronal populations that process energy balance cues in this nucleus identified by their peptide composition, namely POMC/CART cells and NPY/AgRP cells (Dhillo et al., 2002). Different signals involved in regulation of energy homeostasis are integrated by responsive neurons in the arcuate. Lesions in the arcuate nucleus of the hypothalamus result in obesity in mice (Olney, 1969). Changes in diet can alter neuronal activity and gene expression in the arcuate, suggestive of hypothalamic plasticity. Further, upon examining the turnover of hypothalamic neurons in obese mice, it was observed that both a high-fat diet and leptin deficiency led to decreased neurogenesis in the arcuate neurons (McNay, Briancon, Kokoeva, Maratos-Flier, & Flier, 2012). Site-specific depletion of POMC neurons in the arcuate also leads to obesity in mice (Greenman et al., 2013). Thus, these sets of neurons sense peripheral signals, integrate and communicate with each other, then relay that information to other brain regions, including hypothalamic sites. POMC neurons of the hypothalamus that originate in the arcuate nucleus project to other brain regions including the PVN, DMN, and LH (Hakansson, Brown, Ghilardi, Skoda, & Meister, 1998). Some of these regions that are important for the central MC system and energy balance are discussed below.

1.10.2 Paraventricular Nucleus of hypothalamus (PVN or PVH)

The PVN, situated on either side of the 3rd ventricle, has two major subdivisions, magnocellular (large cell) and parvocellular (small cell). Very generally, parvocellular
neuronal subpopulations control the hypothalamic-pituitary-adrenal (HPA) axis, and the hypothalamic-pituitary-thyroid (HPT) axis, whereas the magnocellular neurons integrate the hypothalamic-neurohypophysial system. The HPA axis controls circulating glucocorticoids and is involved in maintenance of energy homeostasis via modulating metabolism and appetite (Castonguay, 1991; Hill, 2012) and both corticotropin- and thyrotropin-releasing hormones in the PVN are central modulators of behaviors that impact energy balance, including physical activity and food intake (R. E. Sutton, Koob, Le Moal, Rivier, & Vale, 1982; Valassi, Scacchi, & Cavagnini, 2008). Both the magnocellular and parvocellular neurons of the PVN express receptors for α-MSH (Ghamari-Langroudi, Srisai, & Cone, 2011). Many of the POMC neurons from the arcuate project to PVN (Bagnol et al., 1999). This nucleus has well-established and heavily documented effects on energy balance. The PVN receives a strong input from POMC and AgRP neurons and regulates a variety of neuroendocrine, behavioral, and autonomic functions (Cowley et al., 1999; Sohn et al., 2013). Lesions to this region result in significant hyperphagia; hence, its normal function is associated with satiety (Mountjoy et al., 1994; Weingarten, Chang, & McDonald, 1985). Restoration of MC4R expression in the PVN in MC4R-KO mice ameliorates the obese phenotype by up to 60% (Balthasar et al., 2005). Site-specific microinjections of MCR ligands into the PVN decrease food intake and increase EE and physical activity (Cowley et al., 1999; Kask & Schioth, 2000; Shukla, Britton, Koch, & Novak, 2012). The importance of this region is also reinforced by the study in which obesity develops in mice and humans with an
altered copy of the Sim1 gene, which is a transcription factor that controls the development of PVN (Holder, Butte, & Zinn, 2000).
Figure 4: Illustrations from a rat brain highlighting the key areas involved in energy balance. Adapted from (Barsh & Schwartz, 2002), with copyright permission from Nature Publishing Group.

a) A sagittal view with olfactory bulb at the anterior end and the caudal hindbrain at the posterior end on the right; b,c) Cross-sections of the brain (indicated by dashed lines in a).

Highlighted regions of interest are: arcuate nucleus (ARC, shown in turquoise), paraventricular nucleus (PVN, shown in green), perifornical area (PFA), lateral hypothalamic area (LHA), ventromedial nucleus (VMN), and the dorsomedial nucleus (DMN). Abbreviations: AM, amygdala; CC, corpus callosum; OC, optic chiasm; SE, septum; TH, thalamus; 3V, third ventricle; fornix (FX).
1.10.3 Lateral hypothalamus (LH) and perifornical nucleus of LH or orexin cell body containing area (PeFLH)

Damage to the lateral hypothalamus inhibits feeding, reducing body weight (Bernardis & Bellinger, 1993). Immunocytochemical studies have shown that both POMC and AgRP fibers project to this nucleus, and have also shown the presence of MC3R and MC4R receptors (Bagnol et al., 1999; Jegou, Boutelet, & Vaudry, 2000; Mountjoy et al., 1994).

Orexin, also called hypocretin, is a neuropeptide that regulates arousal, appetite, and wakefulness (Beck, Kozak, Moar, & Mercer, 2006), in addition to stimulating feeding (Kotz, Teske, Levine, & Wang, 2002). Orexin-expressing cells are located in the lateral, dorsal, and perifornical regions of the LH (Muroya et al., 2004). Orexin neurons also synapse with NPY/AgRP and POMC/CART neurons in the arcuate nucleus (Horvath, Diano, & van den Pol, 1999). Microinjections of orexins into the arcuate, PVN, and LH stimulate feeding (Dube, Kalra, & Kalra, 1999). Additionally, intra PVN microinjections of orexin increase spontaneous physical activity and weight loss in rats (Novak & Levine, 2009).
1.10.4 Dorsomedial Nucleus of the hypothalamus (DMN)

The DMN is also important when considering brain control of metabolism. Lesioning and knife-cut experiments show that the DMN has projections to the PVN, particularly to portions of the PVN involved in autonomic control (Aravich & Sclafani, 1983). Also, these lesions lead to hypophagia and stunted linear growth in rats (Aravich & Sclafani, 1983). In addition to POMC projections leading from the arcuate, also present in the DMN are MC3R, MC4R and MC5R (Bagnol et al., 1999; Jegou et al., 2000). In MC4R-KO mice, the expression of NPY is specifically elevated in the DMN, indicating that this nucleus is likely to be functionally altered when MC signaling is disrupted (Kesterson, Huszar, Lynch, Simerly, & Cone, 1997).

1.10.5 Ventromedial nucleus of the hypothalamus (VMN)

The VMN lies close to the DMN, arcuate nucleus, and third ventricle. It has no known neuroendocrine projections, but has been shown to be directly involved in regulating feeding behavior and body weight (Millington, Tung, Hewson, O'Rahilly, & Dickson, 2001; Satoh et al., 1997). Lesions in the VMN region in rats lead to hyperphagia and weight gain. (Brobeck, Tepperman, & Long, 1943; Grundmann et al., 2005). POMC arcuate projections into this region are sparse, however there is significant expression of MCR 3, 4, and 5 mRNA in the VMN (Bagnol et al., 1999; Jegou et al., 2000; Mountjoy et al., 1994). The common MCR agonist MTII increases neuronal firing in the VMN, and this response is diminished in food-deprived rats (Y. Z. Li & Davidowa, 2004). BDNF is a neurotrophin whose expression is regulated by signals from the MC4R
Compared to other brain regions, the expression of BDNF is most dense in the VMN, and this region also mediates the ability of BDNF to increase EE (Wang, Bomberg, Billington, Levine, & Kotz, 2010).

Other brain regions that might be targets for POMC-mediated effects are supraoptic nucleus (SON), periventricular nucleus, nucleus accumbens, amygdala, and the hindbrain. In this dissertation, I have focused on the hypothalamus, and thus these other regions are outside of the scope of the studies described here.

1.11 Modeling physical activity

Current literature investigating the role of these hypothalamic regions in regulating energy balance focuses much attention on food intake and body weight regulation, and not as much on physical activity. The energy-balance equation is a fine equilibrium between the energy intake and EE; though appetite regulation is important, the impact of daily spontaneous physical activity on obesity should not be ignored. As described in the previous sections, physical activity mediated by the MC system has a direct impact on regulation of body weight. Little is known about how physical activity is potentially regulated by the MC system, particularly by the different receptor subtypes. A study targeting six different regions of the hypothalamus and the hindbrain with site-specific microinjections of the MCR agonist demonstrated that the energy intake and EE regulation of MCR stimulation is distributed across different neuronal populations (Skibicka & Grill, 2009). When these investigators measured food intake, spontaneous
physical activity, heart rate, and core body temperature in these rats, they found an independent and variable response of each parameter to the stimulation of different nuclei. While MC stimulation of the different nuclei did not result in identical behavioral responses, a given nucleus was not exclusively associated with one behavior (e.g., feeding) and in the absence of other responses. Further studies are therefore crucial to understand the regulation of physical activity by the MC system. Studies described in this dissertation focus on this aspect with respect to different MCR subtypes in the brain, namely MC3, 4, and 5 receptors.

1.12 Rat model of obesity and leanness
In 1996, Koch and Britton, now at the University of Michigan, initiated development of rat lines that differ for intrinsic aerobic treadmill running capacity via two-way (divergent) artificial selection, establishing models for leanness vs. obesity: high-capacity runners (HCR) and low capacity runners (LCR), respectively (Koch & Britton, 2001; Koch, Britton, & Wisloff, 2012). HCR rats have consistently higher levels of daily “spontaneous” physical activity relative to the LCR, independent of differences in body weight or lean mass (Gavini et al., 2014). Having an obese phenotype, LCR rats are also prone to metabolic disorders and gain more weight on normal chow as well as high-fat diets (Novak et al., 2010). Some of the other contrasting characteristics of these rats are listed in Table 2.
Since these rats have been shown to significantly differ in daily activity levels and EE, and are genetically intact with a heterogenous background, they make an ideal model to study the role of MC receptors in physical activity.
## Table 2: Comparison between the rat model of obesity and leanness

<table>
<thead>
<tr>
<th></th>
<th><strong>HCR rats</strong></th>
<th><strong>LCR rats</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic Capacity</strong></td>
<td>Bred for high intrinsic treadmill running (aerobic) capacity</td>
<td>Bred for low intrinsic treadmill running (aerobic) capacity</td>
<td>(Koch &amp; Britton, 2001)</td>
</tr>
<tr>
<td><strong>24-hr Physical Activity</strong></td>
<td><strong>HIGH</strong></td>
<td><strong>LOW</strong></td>
<td>(Novak et al., 2010; Novak et al., 2009)</td>
</tr>
<tr>
<td><strong>Economy of activity</strong></td>
<td>Low</td>
<td>High</td>
<td>(Novak et al., 2010)</td>
</tr>
<tr>
<td><strong>Life span</strong></td>
<td>Long-lived</td>
<td>Relatively short life span</td>
<td>(Wisloff et al., 2005)</td>
</tr>
<tr>
<td><strong>Overall body composition</strong></td>
<td>Lean: Low body weight Low body fat</td>
<td>Fat: High body weight High body fat</td>
<td>(Novak et al., 2010; Novak et al., 2009)</td>
</tr>
<tr>
<td><strong>Effect of high fat diet (HFD)</strong></td>
<td>Gain less weight</td>
<td>Gain more weight</td>
<td>(Novak et al., 2010)</td>
</tr>
<tr>
<td><strong>Metabolic profile</strong></td>
<td>Healthy metabolic profile</td>
<td>Prone to metabolic syndrome and cardiovascular disease</td>
<td>(Wisloff et al., 2005)</td>
</tr>
<tr>
<td><strong>Insulin resistance</strong></td>
<td>Sensitive</td>
<td>Resistant</td>
<td>(Noland et al., 2007)</td>
</tr>
<tr>
<td><strong>Anxiety like behavior</strong></td>
<td>Vigilant, anxiotypic coping</td>
<td>Passive coping</td>
<td>(Burghardt et al., 2011; Waters et al., 2010)</td>
</tr>
</tbody>
</table>
Obesity in humans is complex and involves many factors, both genetic composition and environmental (Marti, Martinez-Gonzalez, & Martinez, 2008). Physical activity is one such trait that is inherited, and also varies between individuals (Joosen, Gielen, Vlietinck, & Westerterp, 2005; Levine & Kotz, 2005; Levine et al., 2005). In both rats and humans, aerobic capacity is a key trait that is a predictor of physical activity (Bray, 2000; Koch & Britton, 2001; Novak et al., 2009). Aerobic capacity, even in the absence of exercise, consistently predicts good health and longevity (Kokkinos et al., 2008; Myers et al., 2002), which is also seen in our rat model (Wisloff et al., 2005). While transgenic and KO models can provide useful information in many diseases, obesity is a much more complex syndrome with multifactorial underlying causes (Davies, 2009). The HCR and LCR rats, which are genetically intact but artificially selected for their aerobic capacity starting with a genetically variable population, are untrained for running endurance (Koch & Britton, 2001). This successfully models polygenic obesity and leanness, as seen in the human population (Koch & Britton, 2008). The importance of using this rat model is further highlighted by the fact that subtle differences among neuropeptides and their receptors might not be identifiable using standard rodent models. It has been suggested that the common control or non-obese animal models might essentially have metabolic syndrome since they are housed in a sedentary environment with unrestricted food supply and limited physical challenges (Martin, Ji, Maudsley, & Mattson, 2010). Therefore, the use of HCR, or high-activity rats, is crucial to identify the role of these receptors in a normal, metabolically diverse population. All the studies
described in this dissertation have been based on this rat model in order to focus on different attributes of obesity and regulation of physical activity and EE.

1.13 Specific Aims

Broadly, there are three specific aims addressed in this dissertation. The first aim (Chapter 2) focuses on analyzing the expression of MC receptors in different nuclei of the hypothalamus. The main hypothesis here is that high-activity HCR and low-activity LCR rats display differential expression levels of MC receptors across brain regions. Chapter 3 further describes the composite profile of MC receptor expression in different rat brain areas in a very precise and site-specific manner using laser capture microdissection (LCM), for their prospective regulation of MC circuitry. Next, Chapters 4 and 5 expand on the role of MC receptors in regulating different aspects of the energy balance equation, namely physical activity, EE, and food intake. The primary aim (specific aim 2) of studies described in Chapter 4 was to identify how MCR subtypes in specific brain areas regulate physical activity and EE. I predicted that the elevated response to the agonists and antagonist in high-activity HCR rats would correspond to the specific brain regions identified to have variable MCR expression between the two groups (Chapters 2 and 3). Next, in Chapter 5 (specific aim 3), I tested the hypothesis that the MC neuronal circuitry contributes to satiety signaling via its brain-region-specific receptor expression pattern. I predicted that treatment with these drugs, targeting specific brain regions, would lead to inhibition (agonists) or stimulation (antagonist) of food intake.
Overall, in the studies described in subsequent chapters, the emphasis is on distinguishing between the MCR subtypes individually, for their differential expression in the brain, as well as their specific functional role in regulating physical activity and/or food intake. Together, these studies will help in filling current gaps in our understanding of the MC circuitry as it closely integrates with other complex pathways to regulate the metabolic profile.
CHAPTER 2: Expression of Brain Melanocortin Receptors (MCRs) in
HCR-LCR rats

The brain MC system is one of numerous overlapping systems regulating energy
balance (Zemel & Shi, 2000). The prohormone POMC, the precursor peptide central to
the MC system, is cleaved into several bioactive peptides, one of which is α-MSH. These
peptides act as ligands for MC receptor subtypes MCR1-5; three of these are found in the
adult mammalian brain and mediate a multitude of physiological and behavioral effects
(Tao, 2010). Mutations and deficiencies in POMC and MC receptors lead to obesity and
there is evidence linking this system to physical activity in humans as well as animal
models of obesity (Farooqi et al., 2003). Thus, brain MC circuitry is relevant for a
naturally occurring obese phenotype.

While reports link the MC system including the MCRs to different aspects of
energy balance, most studies focus on appetite rather than physical activity. Moreover,
since the receptors are present across different regions of the brain in addition to many
other organs, determining the role of each receptor in the context of multiple brain
regions adds complexity to any study. Here I use the rat model of obesity and leanness;
these rats show consistent differences in physical activity and EE (Gavini et al., 2014;
Koch & Britton, 2001; Novak et al., 2010). Initially bred for their intrinsic aerobic
capacity, we see significant differences in the baseline physical activity of HCR-LCR rats, making them a good model system to investigate differences in the brain that may lead to such activity differences. To test the hypothesis that brain MC modulates physical activity and EE, I first compared brain MCR profiles in these rats that consistently show high vs. low levels of ‘spontaneous’ daily physical activity. Focusing on individual nuclei of the hypothalamus, here I examine the baseline expression of POMC and MCRs that are present in the brain (MC3R, MC4R, MC5R), along with other peptides that are closely integrated with this pathway. The hypothalamic regions examined here were the arcuate nucleus, PVN, and PeFLH, where orexin-containing cell bodies are present. As described above, these nuclei have all been shown to be relevant in the energy balance pathway.

2.1 STUDY 1: Relative quantitative mRNA expression in specific brain regions.
I tested the hypothesis that high-activity HCR and low-activity LCR rats display differential expression levels of MC receptors in different brain regions. To quantify receptor mRNA expression, quantitative RT-PCR was performed on brain micropunches from the PVN and PeFLH. POMC mRNA was also quantitated in the arcuate nucleus, where it is synthesized.

2.1.1 Methods:
HCR-LCR rats:
Eight each of HCR and LCR male rats (generation 21) were obtained from the University of Michigan. Each rat was individually housed on a 12:12 light: dark cycle with lights on at 0700 EST. Rats received *ad libitum* water and rodent chow (Lab Diet 5001; Lab Diet, Richmond, Indiana, USA) for 28 days. All procedures and handling were in accordance and approval of Kent State University’s Institutional Animal Care and Use committee (IACUC).

**Brain micropunches**

Brains were extracted after a rapid decapitation and placed in ice-cold saline for 20 min. Each brain was then sectioned using a Hatton apparatus. With optic chiasm as the baseline, a 100µm slice was taken by making cuts at −0.1 mm and −0.2 mm; a second, 200µm slice was then cut. The slices were placed onto slides and frozen on dry ice. From the first slice, PVN was isolated using a 2 mm micropuncher, and PeFLH with a 1 mm puncher bilaterally. Two more punches of the PeFLH were taken on the second (200 µm) slice. All punches were flash frozen with liquid nitrogen, and stored at -80° C for further processing.

**Gene expression**

Tissue from the punches was homogenized for RNA isolation to be measured using quantitative real-time PCR (Q-PCR). The samples were purified and total RNA was extracted using an RNA purification kit (Ribopure; Ambion Life Technologies, Grand Island, New York, USA). The RNA concentration and purity was measured using
NANODROP (ND-1000; Nanodrop Technologies, Wilmington, Delaware, USA) with A260/280 ratio ranging from 1.8 – 2.1. Only samples with optimum RNA integrity numbers were used for further processing. Purified total RNA was reverse transcribed using the Applied Biosystems (ABI) Reverse Transcription reagents kit (Carlsbad, California, USA), using random hexamers with thermal cycling at 25°C for 10 minutes, 48°C for 30 minutes, 95°C for 5 minutes. Next, 20-100 ng of cDNA was used for quantifying the expression of the genes of interest using Taqman probes (Applied Biosystems). The starting concentration of cDNA was kept the same within the nuclei examined. All samples were run in triplicate on the StratageneMx3005P Real-Time PCR System (Agilent, Carlsbad, California, USA), with annealing temperature of 60°C, for 40 cycles. The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used as control for all assays and the relative expression was calculated using the comparative Ct method (ΔΔCt) method.

Analyses and statistics:

The HCR mean ΔΔCt values were used to define 100%, and each animal’s data were calculated as a percentage of the mean. Mean and variance values were calculated and unpaired two-tailed t-tests were used for analyses. Differences with \( p<0.05 \) were considered to be significant.
2.1.2 Results

**Lean rats have greater site-specific mRNA expression of MC receptors**

First, I found no significant difference in the mRNA expression of the precursor POMC, or the processing enzymes PC1 as well as PC2, in the arcuate nucleus (Figure 5). Next, we examined the basal mRNA expression of MC receptors MC3R, MC4R, and MC5R in two brain hypothalamic regions: the PVN and the PeFLH. Q-PCR data are shown in Table 3 (Shukla et al., 2012). Upon comparing HCR with LCR rats, the MC3R mRNA expression level was significantly higher in HCR in the PVN (p = 0.008; Figure 6) (Shukla et al., 2012). No significant difference was observed in the PeFLH region for this receptor subtype. Both MC4R and MC5R mRNA, however, were significantly higher in HCR in the PeFLH only (p = 0.01 and 0.02 respectively; Figure 6), but not in the PVN. mRNA expression of MC precursor polypeptide POMC and the processing enzymes PC1 and PC2 in the arcuate nucleus were not found to be significantly different between LCR and the lean HCR (Table 3). I also measured mRNA expression for the other peptides such as BDNF, thyrotropin-releasing hormone (TRH), ghrelin receptor (GHSR), and NHLH2, which are all closely linked in energy balance regulation. Of the other peptides examined (Table 3), only the expression of BDNF in the PeFLH was found to differ significantly between groups (HCR>LCR).

MC5R has the widest peripheral distribution among the three brain-associated MCR subtypes. Outside of the CNS, on comparing the mRNA expression of MC5R in the
skeletal muscle samples (gastrocnemius) from HCR-LCR rats, no difference was found between the groups (Figure 7).
There was no significant difference in the mRNA expression levels of the precursor Proopiomelanocortin (POMC) or the processing enzymes pro-hormone convertase 1 (PC1) and pro-hormone convertase 2 (PC2) between the low-capacity runner (LCR), and high-capacity runner (HCR) rats.

Figure 5: Micropunches: Quantitative PCR - Arcuate nucleus
Figure 6: Micropunches: Quantitative PCR in target brain regions. Taken from Fig 1A (Shukla et al., 2012). Compared to low-capacity runner (LCR), high-capacity runner (HCR) rats have greater expression of MC3R in the paraventricular nucleus (PVN); MC4R and MC5R are higher in the perifornical lateral hypothalamus (PeFLH); *$p<0.05$, NS: non significant.
Table 3: Quantitative mRNA measurement in hypothalamic nuclei obtained from micropunches. Taken from Table 1 (Shukla et al., 2012)

<table>
<thead>
<tr>
<th>Brain Region Micropunch</th>
<th>Q-PCR Probe</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arcuate nucleus (ARC)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>POMC</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>GHSR</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>NHLH2</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td><strong>Perifornical lateral hypothalamus (PeFLH)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC3R</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>MC4R</td>
<td>0.02*</td>
<td></td>
</tr>
<tr>
<td>MC5R</td>
<td>0.02*</td>
<td></td>
</tr>
<tr>
<td>BDNF</td>
<td>0.03*</td>
<td></td>
</tr>
<tr>
<td>TRH</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>GHSR</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td><strong>Paraventricular nucleus (PVN)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC3R</td>
<td>&lt;0.01*</td>
<td></td>
</tr>
<tr>
<td>MC4R</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>MC5R</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>BDNF</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>TRH</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>GHSR</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

Melanocortin 3, 4, and 5 receptor (MC3R, MC4R, MC5R), proopiomelanocortin (POMC), prohormone convertases (PC1, PC2), brain derived neurotropic factor (BDNF), thyrotropin-releasing hormone (TRH), ghrelin receptors (GHSR), and nescient helix-loop-helix 2 (NHLH2) in target brain regions: arcuate nucleus, paraventricular nucleus (PVN), and perifornical lateral hypothalamic area (PeFLH); *p <0.05 (HCR>LCR).
Figure 7: Quantitative PCR in the gastrocnemius muscle tissue: Melanocortin 5 Receptor (MC5R).

No significant difference was observed in MC5R expression between low-capacity runner (LCR) and high-capacity runner (HCR) rats.
2.2  Study 2: Protein expression of MCRs in paraventricular nucleus (PVN) and the perifornical region of the hypothalamus (PeFLH)

Since it is not always feasible to obtain good quality RNA as well as protein from the same tissue samples while working with relatively small brain punches (individual hypothalamic nuclei), I conducted a separate study to determine the protein concentrations in these regions. The goal of these experiments is to verify that the differential gene expression, as noted above, corresponds to differences in protein expression as well.

2.2.1  Methods

Rats and brain micropunches

10 of each HCR and LCR male rats (generation 27) were used for this study. Rats were euthanized, and brains were quickly removed and frozen in isopentane cooled in dry ice and liquid nitrogen, after which they were stored at -80°C until further processing.

Brains were sectioned at 100 µm on a cryostat; sections were placed onto slides and frozen on dry ice. Tissue sites containing the PVN and PeFLH were then micropunched; the PVN was isolated using a 2 mm micropuncher, and PeFLH with a 1 mm puncher bilaterally (Novak et al., 2010). All punches were flash frozen with liquid nitrogen and stored at -80°C for further processing.
Western blotting

The punch samples were placed in microcentrifuge tubes and frozen on dry ice. Punches were sonicated in 35 µl of ice-cold radioimmunoprecipitation buffer (RIPA; Thermo Scientific) supplemented with protease inhibitor cocktail (Roche Diagnostics) followed by 30 min incubation on ice. The total homogenates were then centrifuged and the supernatant (total lysate) was transferred to new clear tubes for analysis. Total protein concentration was determined by the Bradford method. Equal quantities (5-10 µg) of total lysate were resolved by SDS-PAGE, then used for Western blot analysis. MC3R, MC4R, and MC5R protein levels were examined using actin as a loading control. Equal quantities of supernatant and sample buffer (150mM tris-HCl pH 6.8, Trizma-base for pH, 6% SDS, 30% glycerol, 0.03% pyronin-Y, DTT) were mixed and tubes were heated at 90°C for 3 minutes. Samples containing equal quantity of protein were loaded onto a gradient gel (4-15%) (Bio Rad) and electrophoresed using SDS running buffer (0.384M glycine, 0.05M Trizma bas, 0.1% SDS) at constant voltage (150V) for 30 minutes. The gel was blotted on to a PVDF membrane using semi-dry blotting apparatus and transfer buffer (49.6mM Trizma base, 384mM glycine, 17.5% methanol, 0.01% SDS) at constant current (400mAmp). The blot was incubated overnight in a blocking solution of 5% Blotto in 1XPBST (Phosphate buffered saline; 84 mM sodium hydrogen phosphate, 16mM sodium dihydrogen phosphate, 100mM sodium chloride, Tween20), then rinsed using 1X PBST. Primary antibodies used for MC3R, MC4R, and MC5R (Santa Cruz Biotechnology sc6878, 6879, and sc7644, respectively) were diluted in blocking solution.
at a ratio of 1:2000 and incubated overnight at 4°C. After three washes in PBST, the membrane was exposed to a 1:5000 dilution of a donkey anti-goat IgG-HRP secondary antibody (Santa Cruz Biotechnology sc2020) and incubated for 1 hour at room temperature. After washing, the blots were developed using a chemiluminiscence detector using an Amersham kit (GE Healthcare, UK; 1ml solution A, 1ml solution B). The expression levels relative to β-actin were plotted as a percent of the reference value (with HCR as 100%).

2.2.2 Results

On comparing HCR vs. LCR in the PeFLH, protein expression of MC4R and MC5R were significantly higher in HCR, while the MC3R was higher in the PVN region of HCR. No differences were seen in MC3R protein levels (Figure 10) in the PeFLH (Figure 8), or in MC4R and MC5R in the PVN (Figure 9), consistent with predictions based on the mRNA profile.
Figure 8: Protein expression of melanocortin receptors (MCRs) in the perifornical region of the hypothalamus (PeFLH).

Compared to low-capacity runners (LCR), high-capacity runners (HCR) have higher expression of MC4R and MC5R proteins in the PeFLH. MC3R was not significantly different in this region; *p<0.05
Figure 9: Protein expression of melanocortin receptors (MCRs) in the paraventricular nucleus (PVN).

Compared to low-capacity runners (LCR), high-capacity runners (HCR) have higher expression of MC3R in the PVN. MC4R and MC4R were not significantly different in this region; *$p<0.05$
Figure 10: Western blot images showing the expression of melanocortin receptors 3, 4, and 5 (MC3R, MC4R, MC5R) along with actin as the loading control in PVN and the PeFLH.
2.3 Discussion:

These studies revealed enhanced gene expression levels of MCRs in the hypothalamic nuclei examined. Since this variable expression was region- and subtype-specific, it suggests that these changes may not be simply secondary to a generalized systemic increase in α-MSH release. Further, the gene expression was reflected in the receptor protein levels as well, strengthening the assertion that these differences in receptor expression have the potential to exert behavioral effects. To summarize, highly specific, differential regional expression of brain MCRs affected different aspects of energy balance, particularly physical activity. These results implicate brain MC receptors in the heightened EE and activity seen in association with high endurance capacity.

Although POMC expression in the brain is mainly limited to the arcuate nucleus and area prostrema, it is cleaved post-translationally to yield different bioactive compounds that act on MCRs in numerous brain regions to alter several components of energy balance (Cauchi et al., 2009; Chen et al., 2000; MacKenzie, 2006; Mountjoy et al., 1994). Elevated POMC results in upregulation of α-MSH and decreased MCRs (Pritchard et al., 2002; Zemel & Shi, 2000), suggesting the possibility that the heightened MCR observed in the high-activity HCR might be secondary to the altered overall MC ‘tone’ in these rats. However, these data do not support this interpretation. First, it is not likely that differences in activity levels in the rats originate with altered arcuate MC as I found no significant difference in precursor POMC mRNA expression (Figure 5), and
only a trend in the processing enzyme PC1 (not significantly higher in HCR, p=0.06, Table 3). Second, higher global MC release would be expected to alter MCRs similarly irrespective of subtype or brain region, whereas these studies revealed a region-specific and subtype-specific expression pattern. Altogether, these data suggest that the profile of MCR expression may be intrinsic to the high-activity, high-endurance phenotype.

These are the first studies to focus on MCR subtypes and their specific expression patterns in different brain regions of the high- and low-activity phenotypes. While the expression of MC3R and MC4R in PVN compared to PeFLH suggests a key role of receptor subtype as well as the region examined, it also implies that high-activity HCR rats do not have an overall increased MC tone. Instead, the MC pathway is possibly being regulated in a more precise manner as it moves downstream from the arcuate to the projecting neurons of the hypothalamus. These data are particularly interesting with respect to MC5R. The current literature does not recognize the presence of this receptor subtype in measurable quantities in the brain. Though a few isolated reports have documented MC5R to be present in the brain in very low amounts (Griffon et al., 1994), most of these studies have been in the whole brain in contrast to the nucleus-specific identification in my studies. It is possible that central MC5R is much lower compared to the predominant and the more studied receptors MC3R and MC4R, and may not be detectable in mRNA or protein measurements when considering bigger tissue portions. Additionally, I estimated relative gene expression in HCR PeFLH to be more than 1000 times greater than in LCR, as seen in Figure 6; depending on the animal model studied
and the tissue examined, such a large difference could explain why a different model or brain region may or may not have sufficient quantities of MC5R present to be detectable. Interestingly, one organism that has been reported to have high levels of MC5R in the brain is goldfish; its role has not been investigated, however (Cerda-Reverter, Ling, Schioth, & Peter, 2003).

In subsequent studies, I have expanded upon these results by measuring receptor mRNA in a more region-specific and precise manner. One downside of using micropunch samples is inability to isolate brain regions without getting surrounding cells as well. Since I have focused on different hypothalamic nuclei, which are in relatively close proximity to one another, it was preferable to isolate the regions of interest in a more precise and site-specific manner using Laser Capture Microdissection (LCM), described in Chapter 3.
CHAPTER 3: Laser Capture Microdissection (LCM): Precise Measurement of
Brain MCR Expression in HCR LCR rats.

As a technique, LCM allows the precise procurement of enriched cell populations from a heterogeneous tissue or cell culture while under direct microscopic visualization. Histologically enriched cell populations or regions can be isolated by harvesting cells of interest directly, or by ablating unwanted cells. The basic steps of LCM are (a) visualization of cells via light microscopy, (b) transfer of laser energy (infrared (IR) or ultraviolet) to a thermolabile polymer with the formation of a polymer-cell composite, and (c) removal of cells of interest from the heterogeneous tissue section (Golubeva, Salcedo, Mueller, Liotta, & Espina, 2013). For my study I used the IR laser-capture method, which uses laser energy (810nm) to capture cells of interest without any contamination from surrounding cells.

Figure 11 further outlines the steps from a typical IR capture. Briefly, the system uses a stationary near-infrared laser mounted in the optical axis of the microscope to melt a thermolabile polymer film. The cap, which is placed on the specimen slide via a robotic arm, acts as an optic for focusing the laser in the same plane as the tissue section. Next, the polymer melts only in the vicinity of the laser pulse, forming a polymer-cell composite. Finally, microdissection occurs when the polymer is removed from the tissue.
surface and the embedded cells of interest are picked up from the slide, on to the cap. The exact cellular morphology, as well as the RNA of the procured cells, remain intact and can be processed for further downstream analysis.

Here, I used the relatively new and more precise technique of LCM to map the expression of MCRs and other key precursors and peptides in the hypothalamus in a precise as well as site-specific manner. I expanded on the previous results and set out to outline a composite map of MC receptor expression in different rat brain areas for their prospective regulation of MC circuitry, and hence the physical activity differences between high- and low-activity rats. In addition to the two nuclei examined earlier, we now also examined the VMN and the DMN in these rats. I expected to find higher relative mRNA expressions of MC3R, MC4R, and MC5R in high-activity rats compared to low-activity obesity-prone rats in some or all of the brain regions dissected using LCM.
Figure 11: Schematic representation of infrared capture of tissue of interest from laser capture microdissection (LCM).
3.1 Methods:

Harvesting of brains from the rats

Adult HCR and LCR rats, generation 30 (12 females in each group) were obtained from University of Michigan and individually housed for 28 days. While I used males in the previous study with micropunches (Chapter 2), female rats were used here because the physical activity studies described in Chapters 4 and 5 utilized female rats, and I predicted that these differences would occur irrespective of sex. Moreover, we often see a sex bias in animal studies, particularly in behavioral neuroscience, which is a serious disadvantage when pursuing a research problem (Beery & Zucker, 2011; Zucker & Beery, 2010). After being acclimated to our facility, rats were euthanized by rapid decapitation, brains quickly removed and frozen in isopentane cooled in dry ice and liquid nitrogen, after which they were stored at -80°C until further processing.

Preparation of Tissue Sections for Arcturus Infrared Capture Mode

Brains were sectioned into 12 µm thick sections on a cryostat and mounted onto SuperfrostPlus slides. Sections were stained using a quick protocol to allow visual identification of target areas, namely: arcuate, PVN, PeFLH, VMN, and DMN. As illustrated above, these regions were chosen because of their documented presence of MC receptors in these areas and the actions of MC on metabolism. Figures 12-15 are illustrations of a coronal section of a rat brain [adapted from “The rat brain in stereotaxic coordinates” (Watson)], indicating the positions of the nuclei examined.
Once on the slide, first the sections were fixed in a 75% EtOH solution for 30 seconds, rinsed in water to remove excess EtOH from the slide, and then immersed in Hemotoxylin for 90 seconds. The slides were then taken through an alcohol dehydration series of 75%, 95%, and 100% EtOH for 30 seconds each, followed by immersion in xylenes for 5 minutes. These slides were then used with the LCM machine (Arcturus XT™). Target areas were identified and captured into CapSure® HS LCM Caps (Molecular Devices). Typically, captured tissues from 6-12 sections were pooled on to one cap for each nucleus for every sample. While it is possible that during capturing from the slide, a few cells are transferred from regions outside the target site, utmost care was taken to confirm each capture by examination of both pre and post-capture images of the tissue. I estimated that any extra or undesired cells would comprise less than 1% of the total captured material. Figure 16 and Figure 19 show hematoxylin-stained brain sections with visible nuclei of interest before and after capture with LCM.

**RNA purification and Q-PCR:**

The samples were purified using an RNA purification kit (Picopure from Molecular Devices) and only samples with optimum RNA integrity numbers were used for further processing. As previously described, I then used quantitative real-time PCR to determine the relative expression of target genes using Taqman probes from Agilent. GAPDH was used as the control gene and relative expression was calculated using comparative Ct method (ΔΔCt) method.
**Data analysis and results:**

HCR mean ΔΔCt values were used to define 100%, and each animal’s data were calculated as a percentage of this mean. I also calculated mean and variance and analyzed data using unpaired 2-tailed t-tests. Values with p<0.05 were considered significant for these results.
Figure 12: Illustration of a coronal section of a rat brain. Highlighted are the paraventricular nucleus (PVN) and the arcuate nucleus. Adapted from *The rat brain in stereotaxic coordinates* (Watson).
Figure 13: Illustration of a coronal section of a rat brain. Highlighted is the VMN region (also called ventral medial nucleus of the hypothalamus, VMH). Adapted from The rat brain in stereotaxic coordinates (Watson).
Figure 14: Illustration of a coronal section of a rat brain. Highlighted is the perifornical region of the lateral hypothalamus (PeFLH), orexin cell body containing area of the hypothalamus. Adapted from *The rat brain in stereotaxic coordinates* (Watson).
Figure 15: Illustration of a coronal section of a rat brain. Highlighted is the
dorsomedial nucleus (DMN), a region of interest in the hypothalamus. Adapted from
The rat brain in stereotaxic coordinates (Watson).
Figure 16: Hematoxylin stained brain section with visible paraventricular nucleus (PVN) region before (A) and after (B) laser capture microdissection (LCM).
3.2 Results:

Results are represented in Figures 17, 18, 20, 21 and Table 4. In the PVN, HCR rats had significantly higher MC3R mRNA expression \((p=0.02)\), whereas in the PeFLH, MC4R \((p=0.04)\) and MC5R \((p=0.01)\) were significantly higher in HCR compared to LCR rats. No significant differences in MCRs were detected in the VMN and the DMN region.
Figure 17: Laser capture microdissection: Quantitative PCR in the perifornical region of lateral hypothalamus (PeFLH) region.

Compared to low-capacity runner (LCR), high-capacity runner (HCR) rats have greater expression of MC4R and MC5R in the perifornical lateral hypothalamus (PeFLH). No significant difference was observed in MC3R expression * different from HCR, p < 0.05.
Figure 18: Laser capture microdissection: Quantitative PCR in the paraventricular nucleus (PVN).

Compared to low-capacity runner (LCR), high-capacity runner (HCR) rats have greater expression of MC3R in the paraventricular nucleus (PVN), no significant difference was observed in MC4R and MC5R expression. * different from HCR, p < 0.05.
Figure 19: Hematoxylin-stained brain section with visible ventromedial nucleus (VMN) and arcuate regions after laser capture microdissection (LCM).

(A) Shows the section after an arcuate capture by LCM.

(B) Shows the section after an arcuate and VMN capture from the same section.
Figure 20: Laser capture microdissection: Quantitative PCR in the ventromedial nucleus (VMN). No significant difference was observed in MCRs between low-capacity runner (LCR) and high-capacity runner (HCR) rats.
Figure 21: Laser capture microdissection: Quantitative PCR in the dorsomedial nucleus (DMN).

No significant difference was observed in MCRs between low-capacity runner (LCR) and high-capacity runner (HCR) rats.
Table 4: Quantitative mRNA measurement in hypothalamic nuclei obtained from laser capture microdissection (LCM); *p<0.05 (HCR>LCR).

<table>
<thead>
<tr>
<th>Brain Region LCM</th>
<th>Q-PCR Probe</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arcuate nucleus</td>
<td>PC1</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>PC2</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>POMC</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>AgRP</td>
<td>0.08</td>
</tr>
<tr>
<td>Perifornical lateral hypothalamus (PeFLH)</td>
<td>MC3R</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>0.04*</td>
</tr>
<tr>
<td></td>
<td>MC5R</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>BDNF</td>
<td>0.04*</td>
</tr>
<tr>
<td>Paraventricular nucleus (PVN)</td>
<td>MC3R</td>
<td>0.02*</td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>MC5R</td>
<td>0.99</td>
</tr>
<tr>
<td>Ventromedial nucleus (VMN)</td>
<td>MC3R</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>MC5R</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Sirt1</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>SF1</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>ADCYAP1</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>BDNF</td>
<td>0.06</td>
</tr>
<tr>
<td>Dorsomedial nucleus (DMN)</td>
<td>MC3R</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>MC5R</td>
<td>Low levels/undetectable</td>
</tr>
</tbody>
</table>

Melanocortin 3, 4, and 5 receptor (MC3R, MC4R, MC5R), proopiomelanocortin (POMC), prohormone convertases (PC1, PC2), agouti-related peptide (AgRP), brain derived neurotropic factor (BDNF), sirtuin (SIRT1), adenylate cyclase activating polypeptide (Adcyap1), and splicing factor 1 (SF1) in target brain regions: arcuate nucleus, paraventricular nucleus (PVN), perifornical lateral hypothalamic area (PeFLH), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN).
3.3 Discussion:

I found site- as well as receptor subtype-specific differences in MCR expression in two of the different hypothalamic brain regions examined (PVN and PeFLH). These results implicate brain MC receptors in the heightened EE and activity seen in association with high endurance capacity. This effect is probably not just a consequence of variations or a generalized systemic increase of α-MSH secretion because we found no difference in POMC and no significant or only minimal changes in the processing enzymes PC1 and PC2 (Table 4). Moreover, the changes in receptor expression identified were highly site-specific as well as subtype-specific.

With the results from this study, I further establish that the differences in MC receptor expression between high-activity HCR and low-activity LCR that are subtype- and region-specific, confirming the micropunches data, replicating the basic effect. Further, the previous study using micropunches was done in male HCR-LCR rats, while females were used in the LCM study, showing that this variable MCR expression is consistent across sex. This identifies brain regions of interest that may mediate the actions of MC on receptors and their relevance in mediating physical activity.

Thus far I have shown that the mRNA levels of POMC in the arcuate did not differ between high-activity and low-activity rats. This suggests that the regulation is likely to be further downstream in the processing of its peptides and their interaction with its receptors. AgRP is an inverse antagonist of the MCRs and is also known to alter food
intake (Stutz, Morrison, & Argyropoulos, 2005). On examining AgRP mRNA levels in the arcuate nucleus, I did not detect a difference between HCR and LCR (Table 3 and 4). A differential expression of MC receptors could also be due to the action of its promoters, their post-translational modifications, or mediation by other peripheral signals/mediators. For example, the transcriptional factor Nhlh2 regulates the hypothalamic expression of MC4R mRNA (Wankhade & Good, 2011). Though I did not find any difference in the Nhlh2 mRNA expression in the arcuate, it is only one of the many possible mediators, along with the MRAPs in target cells (Cooray & Clark, 2011), that may modulate the actions of this system in a region- or cell-specific manner. Further studies will be required to shed light on these variations and inter-individual differences in activity and its associated EE that could be accounted for by the MCRs. For example, we could investigate various epigenetic markers that may alter promoter regulation that is region-specific.

MC is known to interact with several other neuropeptide systems, including orexin (Novak et al., 2010; Novak et al., 2009; Novak & Levine, 2009). Therefore, I examined other potential downstream neuropeptide mediators including BDNF and TRH, as well as GHSR, all of which are important in these hypothalamic circuits (Wang et al., 2010). In the regions examined, BDNF showed a significant difference, being significantly higher in the PeFLH of the high-activity HCR (Tables 3 and 4). BDNF has been described previously as an important component of the hypothalamic pathway that regulates body weight and energy homeostasis (Wang et al., 2010). My results are
consistent with the idea that BDNF may be a critical component in the melanocortinergic brain system regulating spontaneous physical activity and that this system may differ between lean and obesity-prone individuals.

Here, I have focused on hypothalamic circuits, although forebrain reward systems and hindbrain MC are also known to affect energy balance and may potentially differ between high-activity and low activity individuals. Thus, there is a need to better understand the physiological and molecular interactions that might influence physical activity and EE and have a direct impact on weight gain and obesity propensity.

Thus far I have identified that high-activity lean rats have more MCRs than the obese, low-activity rats, but only in some specific regions of the brain. Now, I will focus on the functional role of these differences in behavioral or energy balance regulation. In the latter half of the studies described in this dissertation, the focus is on understanding how differential expression of MCRs in the brain may account for behavioral differences in our rat model. In subsequent studies described, I will further be able to illustrate a role of MC receptors in physical activity and the lean phenotype showcasing these receptor-mediated effects individually by using specific receptor agonists and antagonists. In this dissertation, I expect to provide sufficient evidence to support the hypothesis that region-specific roles of MC receptors mediate regulation of physical activity, EE, and possibly food intake.
CHAPTER 4: Effects of MCR agonists/antagonists treatment on physical activity and Energy expenditure in HCR-LCR rats

To follow up on the molecular findings including the gene expression profile and differential expression of MCRs in the hypothalamus in our rat model, I focused on investigating a direct, functional link between MCRs and spontaneous activity in the lean phenotype. Here, to further elucidate the role of MCRs in physical activity, I used the pharmacological approach of targeting these receptors in the identified nuclei, by using specific agonists and antagonists, and then assessing the change (if any) in the rats’ spontaneous physical activity.

The primary aim of these studies was to identify how MCR subtypes in specific brain areas regulate physical activity and EE. This contributes to the long-term goal of understanding the mechanisms underlying low and high physical activity as they relate to obesity. Since, with these HCR-LCR rats, we see distinct phenotypes differing in physical activity according to their propensity to be lean or obese, I set out to further demonstrate how the regional MC receptor expression within the hypothalamus contributes to individual differences in physical activity and EE.

4.1 Methods

HCR-LCR rats
Adult female HCR-LCR rats were obtained from the University of Michigan and were individually housed on a 12:12-hr light-dark cycle with lights on at 0700 Eastern Standard Time. Rats received ad libitum water and rodent chow (Lab Diet 5001; Lab Diet, Richmond, Indiana, USA) for 28 days.

Three different sets of HCR-LCR rats were used for studies described subsequently in Chapters 4 and 5 (from selection generations 27 and 31), with n =10-12/group. Within a study, all the HCR and LCR rats used had overlapping ranges of body weight. The females selected for each study were generally representative of the phenotypes of that generation, which was verified by comparing the aerobic capacity treadmill phenotyping analysis (including body weight and best time, distance and speed, and work performed) conducted for each rat at 11 week of age at the breeding facility in Michigan.

I used females throughout my behavioral studies mainly because HCR and LCR males have very little overlap in body weights during adulthood, whereas we see relatively minor variations in body weight in adult female rats over time. This is important when conducting any body weight or EE study to factor out differences due to body weight because body weight and composition are the primary contributors to EE, and inaccurate comparisons of lean vs. obese phenotypes have led to spurious conclusions regarding the contribution of EE to energy balance (Butler & Kozak, 2010; Goran, 2005; Tschop et al., 2012; White, Blackburn, & Seymour, 2009). Although there
are possible sex differences in metabolism, and subtle variations in daily physical activity may exist that are estrous cycle-related (data not shown), the phenotypic differences in activity we observe are robust and seen irrespective of sex (Novak et al., 2010). Further it has been shown that any variance related to the estrous cycle has minimal effect on EE (Giles et al., 2010).

All animal procedures followed were approved by the Institutional Animal Care and Use Committee of Kent State University and were in accordance with the Guide for the Care and Use of Laboratory Animals.

**Implanting guide cannulae to different hypothalamic nuclei**

After the rats acclimated to our facility, stereotaxic surgeries were performed to chronically implant guide cannulae aimed at PVN or PeFLH (Novak et al., 2010; Novak, Zhang, & Levine, 2006). While it was possible to target other hypothalamic areas of interest to look for a possible correlation, for the purpose of clarity, I only focused on these 2 areas for my behavioral studies. Also, these were the 2 nuclei that showed a differential expression of receptors in HCR-LCR rats (Chapters 2 and 3). Inhaled isoflurane was used for surgical anesthesia and care was taken to minimize the suffering of animals at each stage of the experiment. Briefly, rats were anesthetized and placed in the stereotaxic apparatus. The following coordinates were used for PVN: anterior-posterior, -1.92 mm; medial-lateral, +0.5; dorsal-ventral, −7 to -7.3; injection needle, 1mm projection (Figure 22). For the PeFLH, the stereotaxic coordinates were anterior-
posterior, -2.6 to -2.7 mm; medial-lateral, +1.7; dorsal-ventral, -7.3; injection needle, 1mm projection (Figure 23). The guide cannula was affixed to the skull using a sterile Michel wound clip and dental cement.

**Body composition measurements**

Following surgery, rats were allowed to recover for a minimum of 7 days, after which a body composition measurement was taken using magnetic resonance spectroscopy with an EchoMRI-700 (Echo Medical Systems, Houston, Texas, USA) to determine fat and lean mass (Nixon et al., 2010). The instrument measures total water as well as fat and lean mass, from which we calculate fat-free mass (total mass minus fat mass; includes bone, skin, and fur). With this, the fat and lean mass (in g) of each rat can be determined, which was later used for EE calculations.
Figure 22: Illustration of a sagittal section of hypothalamus from -1.9 posterior to Bregma, indicating the target site for paraventricular nucleus (PVN) cannulations.

Adapted from *The rat brain in stereotaxic coordinates* (Watson).
Figure 23: Illustration of a sagittal section of hypothalamus from -2.6 to -2.7 posterior to Bregma, indicating the target site of the perifornical region of the lateral hypothalamus (PeFLH) cannulations. Adapted from *The rat brain in stereotaxic coordinates* (Watson).
Measurement of physical activity and energy expenditure (EE)

The rats were then placed in our calorimetry room, which is an isolation chamber, where they were allowed to acclimate in their testing cage (7.5 X12 X 9 inches) for a minimum of 48 hours before the start of any experiment to avoid any novelty-induced changes in calorimetric parameters. The temperature of the room was set at thermoneutral (about 25.9 °C) for rats (N.-M. P. L. a. J. W. Brown, 2008; Overton, 2010) and the light cycle was the same as the main housing room. All rats also had food and water available ad lib during the acclimation period. Rats were divided in groups of four, since our calorimetry setup allows monitoring of four chambers during a single run. In each run, I used 2 HCR and 2 LCR rats at a time to randomize the experiment to avoid any potential bias. The assignment of each rat to a chamber was also random except making sure no single chamber/HCR (or LCR) combination is overrepresented. I performed small-animal indirect calorimetry using a 4-chamber Oxymax FAST system (Columbus Instruments, Columbus, OH) after treating the rats with site-specific microinjections. Rats were microinjected with either vehicle, artificial cerebrospinal fluid (aCSF) or the drug (MCR agonist or antagonist) over 30 seconds. The day before their first injection, each rat was also lightly restrained and was given a sham injection to get them used to the handling and injection protocol to avoid any anxiety-related activity behavior on the first day.

On the day of microinjection, rats were weighed, microinjected, and placed back in the testing cages in the chamber, which was then sealed. The rats had ad libitum food and water present during all studies with the antagonist treatment since this took place
overnight; drug was microinjected just before lights-off time and data were collected over a 12-hour period until the following morning. However, for the agonist treatment, 3-4 h measurements were taken post-injection (during the daytime) and any food was removed from the cages while they still had access to water. This was done to factor out any variations in thermic effect of food or in spontaneous physical activity and movements due to feeding episodes as a result of changes in appetite and satiety behavior due to treatment. MCRs have a known role in appetite and satiety in some areas of the hypothalamus, such as the PeFLH. For this set of experiments (Chapter 4), I exclusively focused on physical activity and any associated EE as a function of MCR activation. A separate set of studies was performed to look at food intake alone (described in Chapter 5).

The calorimeter was calibrated using primary gas standards. Air was pumped into the chamber at 1.5-2.5 liters/min (depending on the weight of the rat), and chamber air was sampled at 0.4 liters/min. Measurement of gas exchange took place every 30 seconds, except for a room-air reference and settle period of 3.5-min after each 60-sample interval. Physical activity data were collected, using infrared beam-break counts, every 10 s in the x- and z-axes; the initial 20 minutes of data was excluded from the analysis to account for handling-induced activity and to allow the air exchange to settle. EE (kcal/h) and physical activity (counts/min) were measured for a total of 3 h. Rats were also weighed at the end of the experiment.
Brain harvesting and site checks:

After the completion of all data collection, placement of the cannula and the potential spread of microinjected compounds were determined at the end of the study by histologically examining the anatomic placement of an injection of India ink. Briefly, after rats were euthanized with FatalPlus, 200 nl of dye (India Ink) was injected into the guide cannula to allow easy identification of the injection site. The brains were removed and fixed in formalin and placed in 10% phosphate buffered formalin with 30% sucrose. Brains were sectioned at 50 µm using a cryostat and mounted onto slides (Fisher Superfrost Plus); slides were dehydrated, stained using cresyl violet, and the injection sites were determined using a microscope. Only rats whose dye injection site corresponded to the stereotaxic coordinates (within 250 µm) were used for data analysis.

Data analysis and Interpretation.

For each rat, and for the 2 hypothalamic nuclei examined (PVN and PeFLH), I obtained:

1. Energy expenditure (EE; kcal/ hr)
2. Physical activity (counts/min)

EE data (kcal/h) were averaged, and physical activity data were expressed as mean beam breaks per minute. Data associated with each target region (PVN and PeFLH) was analyzed using a 2x4 analysis of variance (ANOVA), with dose as the within-subjects independent variable and high- and low-activity rat (HCR-LCR) as the between-subjects independent variable. The precise p value for the individual studies are summarized in Table 5. Physical activity and EE were analyzed separately. Some of the
other factors also measured by an Oxymax calorimeter are VO$_2$, VCO$_2$, RER, but they were either not significant or not the focus of this study (data not shown).

I predicted that the elevated response to the agonists and antagonist in high-activity HCR rats would correspond to the specific brain regions identified to have variable MCR expression between the two groups (Chapters 2 and 3). In other words, I predicted that since I identified high MCR in brain regions (PVN or PeFLH) in HCR, these rats would show more of a physical activity response to an agonist stimulation or suppression by an antagonist. This would support the hypothesis that baseline receptor expression in these brain areas corresponds to the region-specific increase in sensitivity that we may see from receptor activation/suppression, and hence suggest that brain region-specific effect of MCRs can potentially underlie individual differences in daily physical activity.

4.2 STUDY 1: Non-specific MCR agonist melanotan II (MTII), and PVN

Here, I hypothesized that brain MC signaling, mediated by its receptors, can partially account for individual differences in physical activity. First, to determine the dose range to use in HCR-LCRs, the same experimental procedure was followed in six Sprague-Dawley (SD) rats targeting PVN or PeFLH. As a result, I was able to identify the optimal dose range and also proceed with fewer total microinjections by eliminating one dose in the higher range for further experiments. Using SD rats, I was also able to demonstrate that intra-PVN microinjections of MTII increased physical activity (Figure
Rats were microinjected with either a vehicle (aCSF) or the MCR 3, 4, 5 (non-specific) agonist MTII (Phoenix Pharmaceuticals, Burlingame, California, USA). Three different doses of MTII (5, 10, and 20 pmol in 200 nl aCSF) were used to establish a dose-response curve, with each rat receiving each dose in a random order, separated by at least 48 h between subsequent injections. I also used the latin square formula to determine the order and dose of each rat, in such a way that each rat received every dose in a random order, through its subsequent runs.

A 4x2 mixed ANOVA was used, with dose of MTII as the within-subjects independent variable, and high-activity HCR vs. low-activity LCR rats as the between-subjects independent variable. I analyzed physical activity and EE as an effect of MTII treatment in separate analyses, comparing each rat’s activity with its vehicle value in a repeated measures analysis to account for individual differences in activity. I also used analysis of covariance (ANCOVA), with body weight or lean mass as the covariate, to analyze EE data due to the large effect of body weight and composition on EE. This helps to statistically factor out the influence of body weight and lean mass on EE.

Compared to the vehicle, physical activity increased with the MTII treatment in SD rats in a dose-dependent manner (Figure 24). The activity peaked at the dose of 10pm/200 nl, which was thus selected as the optimum dose for future studies. Based on
these results, I also predicted that if high physical activity is regulated by increased MC signaling in high-activity rats (HCR), a similar treatment with MTII would induce a greater increase in short-term, daytime NEAT in high-activity rats compared to low-activity rats.

**MCR agonists in specific brain nuclei increase short-term physical activity and energy expenditure (EE)**

A short-term increase in home-cage physical activity was evident after PVN microinjections of MTII in SD rats (Figure 24). Therefore, I next focused on targeting the PVN region when examining MTII-induced physical activity in HCR vs. LCR rats, and found it to be significantly greater in HCRs (Figure 25). 3 h activity increased after an MTII injection (significantly greater than vehicle at doses 5 and 10 pm/200nl) in HCR. The spontaneous activity in LCRs only increased marginally, with an effect not significantly higher than vehicle at any of the doses (Figure 25). These data demonstrate that the HCR were more responsive to the MTII-induced enhancement of activity. EE analysis revealed significant interactions; follow-up analyses of EE at the 5pmol/200nl dose showed significantly greater MTII-induced EE for HCR than LCR (Figure 26). Therefore, HCR rats have greater sensitivity and an increased response to MTII compared to the low-activity LCR rats.
Site-specific microinjection of MC3/4 receptor agonist melanotan II (MTII) in the PVN increases physical activity in rats.

Figure 24: Paraventricular nucleus (PVN) and melanotan II (MTII): Physical activity in Sprague-Dawley (SD) rats

Intra-PVN microinjections of melanocortin receptor agonist MTII induced physical activity in male SD rats. *Significantly greater than vehicle, $p<0.05$. veh, vehicle (aCSF) (Shukla et al., 2012)
Figure 25: Melanotan II (MTII) and physical activity in high-capacity runner (HCR) and low-capacity runner (LCR) rats.

In the paraventricular nucleus (PVN), MTII-induced activity was significantly greater in HCR compared with LCR rats. *Significantly greater than vehicle, $p<0.05$. (Shukla et al., 2012).
Figure 26: Melanotan II in the paraventricular nucleus (PVN): Energy expenditure (EE)

Compared to vehicle, there was a significant increase in EE in high-capacity runner (HCR) rats after MTII (5 pm/200nl) but not in low-capacity runner (LCR) rats. *MTII induced a significant increase in EE compared to vehicle in HCR only; p< 0.05 agonist.
One of the LCR rats died during the study of an unrelated cause and I used simulation-based statistical software (NORM) to estimate the missing activity value on the basis of multiple imputations for one of the doses (NORM, 1999); however, the imputed data point was not included in post-hoc analyses. I used a 4X2 mixed ANOVA, with a dose of MTII as the within-subjects independent variable and HCR vs. LCR as the between-subjects independent variable; the dependent variables (physical activity and EE) were analyzed separately. I compared each rat’s activity with its vehicle value to account for individual differences in activity.

Following up on these data, next I targeted each MCR subtype more specifically in the 2 nuclei, the PVN and PeFLH.

4.3 Study 2: MCR subtype-specific agonist and antagonist treatments in PeFLH and PVN

To further establish that physical activity differences seen as a result of MCR activation are region specific and may directly correspond to brain regions identified in the molecular studies, I used receptor subtype-specific agonists and antagonists to investigate the role of each MCR receptor (MC3R, MC4R, and MC5R) individually. Stereotaxic surgeries, microinjections, and analyses were done the same way as described above. I focused mainly on the PeFLH since it showed differential expression of MC4R and MC5R (but not MC3R) between HCR and LCR rats. I also targeted the PVN for some of the compounds.
I hypothesized that MC receptors mediate region-specific activation of neuronal circuitry that in turn modulates physical activity. It is important to note that the protein sequence for all three MC receptors found in the brain is quite similar, agonists/antagonists that have a good selectivity for each one are critical in such studies. Depending on what the role of a particular receptor is in a region, we can get conflicting results using an antagonist like SHU9119, for instance, which is not as specific. The commonly used antagonist SHU9119, is antagonistic to MC3R and MC4R but is also an agonist to MC5R. By using specific drugs targeting only one particular receptor subtype at a time, I predicted a similar correlation (as seen above with MTII and PVN) with physical activity in these target areas.

If high physical activity is due to an increased MC signaling in high-activity rats, we would predict that HCR rats would have greater sensitivity and/or responsiveness to MC receptor activation in their physical activity and EE, and that this heightened physical activity is mediated by site-specific activation of MC receptors. While using high-activity HCR and low-activity LCR rats to test the effect of different receptor agonists/antagonists, one would expect to see a greater increase in physical activity in HCRs than in LCRs. This would support the hypothesis that baseline receptor expression in brain areas corresponds to that region’s ability to alter physical activity and EE in response to receptor activation, and hence suggests that brain region-specific effect of MC receptors can potentially underlie individual differences in daily physical activity.
**MC5R and PeFLH**

Since MC5R is the receptor that has been least studied in the brain, the main focus was to investigate its role in the activity-EE mechanism. I was able to obtain highly specific peptides, not yet commercially available, from collaborators to test the hypothesis that site-specific microinjections of MC5R agonists will induce a greater increase in short-term, daytime NEAT in high-activity rats compared to low-activity rats. Similarly, with an MC5R antagonist, I predicted a greater suppression of activity compared to LCR rats. These peptides are selective for MC5R vs. MC3R (about 2000 fold) and MC4R (about 200-fold) (Bednarek et al., 2007; Grieco et al., 2008). This would also argue for a higher relative nighttime MC “tone” in HCR, and that endogenous daytime MC5R receptor activity in this region sustains their higher activity levels in HCR.

**Experiment (i):** MC5R agonist (obtained from MedImmune, Compound 7 (Bednarek et al., 2007).

**Experiment (ii):** MC5R antagonist (obtained in collaboration with Dr. Victor J. Hruby) (Grieco et al., 2008).

Since these peptides have only been characterized chemically and not yet tested before in vivo, SD rats were used once again to determine the optimum dose. The sequence of the peptide is similar to MTII and some of the other MCR compounds available, so I predicted the dose range to be similar to other compounds that have been used in the past.
Each rat was microinjected with either a vehicle (aCSF) or MC5R agonist (Ac-Nle-cyclo(Asp-Oic-D-4,4'-Bip-Pip-Trp-Lys)-amide). Three different doses of the agonist (5, 10, and 20 pmol in 200 nl aCSF) were used to establish a dose-response curve (Figure 27). A second MC5R agonist (compound 6) (Bednarek et al., 2007) was also tested in SD rats, but no significant effect on activity was found at any of these doses (data not shown). I therefore decided to proceed only with the first agonist (compound 7) (Bednarek et al., 2007) for these studies.

Next, using the 10pm/200 nl of MC5R agonist determined above, increases in 3-hr physical activity and EE were found that were significant at this dose compared to vehicle in HCR but not in LCR rats (Figure 28 and Figure 29). Physical activity and associated EE in the HCR rats was significantly suppressed with an MC5R antagonist treatment (Figure 30 and Figure 31). Since rats are nocturnal, in order to detect suppression, this experiment was performed during the dark phase of the cycle and the compounds were injected just before the time of lights-off. The MC5R antagonist suppressed HCR physical activity to the level of LCR, but no significant change was seen in the activity of LCR (Figure 30). This supports the hypothesis that MCR 5 enhances physical activity in rats via the PeFLH brain region. These results implicate brain MC5R in the elevated physical activity associated with high endurance capacity.
Figure 27: MC5R agonist and Sprague-Dawley (SD) rats: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

MC5R agonist-induced an increase in 3-hr physical activity in SD rats, with the dose of 10pm/200nl significant vs. vehicle; *p< 0.05, compared to vehicle.
Figure 28: MC5R agonist: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

Site-specific microinjections of a MC5R agonist (Ac-Nle-cyclo(Asp-Oic-D-4,4’-Bip-Pip-Trp-Lys)-amide) induced a greater increase in short-term daytime activity in lean high-capacity runner (HCR) rats compared to low-capacity runner (LCR) rats; *p< 0.05; agonist induced significant increase in physical activity compared to vehicle in high capacity runner (HCR) only.
Figure 29: MC5R agonist: Energy expenditure (EE) in perifornical region of lateral hypothalamus (PeFLH)

Site-specific microinjections of a MC5R agonist (Ac-Nle-cyclo(Asp-Oic-D-4’,Bip-Pip-Trp-Lys)-amide) induced a greater increase in EE in lean, high-capacity runner (HCR) compared to low-capacity runner (LCR) rats. *p< 0.05; agonist induced significant increase in EE compared to vehicle in HCR only.
Figure 30: MC5R antagonist: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

High-capacity runner (HCR) rats showed a greater MC5R-antagonist (Tyr-Val-Nle-Gly-His-DNal(2’)-Arg-Dtrp-Asp-Arg-Phe-Gly-NH2)-induced suppression of physical activity compared to low-capacity runners (LCRs). $p<0.05$; *significant suppression of night-time physical activity in HCR by MC5R antagonist compared to vehicle. ** Significant difference in night-time activity between HCR vs. LCR with vehicle treatment.
Figure 31: MC5R antagonist: Energy expenditure (EE) in perifornical region of lateral hypothalamus (PeFLH)

High-capacity runner (HCR) rats showed a greater MC5R-antagonist-induced suppression of EE compared to low-capacity runner (LCR) rats; \( p<0.05 \); *significant suppression of night-time EE in HCR by MC5R antagonist compared to vehicle.

**Significant difference in night-time activity between HCR vs. LCR with vehicle treatment.
PeFLH and MC4R

Targeting the MC4R using the agonist (Cyclo(βAla-His-D·Phe-Arg-Trp·Glu)NH2 (Phoenix Pharmaceuticals), both groups showed a significant increase in short-term activity, however HCR rats once again showed an enhanced response compared to LCR; activity increased about 3-fold compared to vehicle treatment (Figure 32). Similarly, EE also increased significantly more in the HCR vs. LCR rats (Figure 33). This clearly indicates that the HCR group showed a stronger response, driven by their internal receptor expression in the PeFLH.

Using a highly specific MC4R antagonist (HS014 from Tocris biosciences), the 12-hr nighttime physical activity decreased significantly only in HCR rats, compared to vehicle (aCSF) treatment (Figure 34). No difference in nighttime activity was detected in the LCR. The HS014-induced suppression of activity seen in the HCR group was also significant at the 3-hr time point (data not shown). With EE, there was a significant difference between the groups in activity levels after the vehicle treatment during the night, which can be attributed to the intrinsic high-activity phenotype of HCR. Further, the antagonist treatment significantly reduced the EE in HCR but not in LCR rats (Figure 35).
Figure 32: MC4R agonist: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

Intra PeFLH treatment with the MC4R agonist (Cyclo(βAla-His-D-Phe-Arg-Trp·Glu)NH2) induced a greater increase in physical activity compared to vehicle in high-capacity runner (HCR) vs. low-capacity runner (LCR) rats, indicated by** (3 fold vs. 2 fold). *significant increase in activity after agonist treatment vs. vehicle; $p<0.05$
Figure 33: MC4R agonist: Energy expenditure (EE) in perifornical region of lateral hypothalamus (PeFLH)

Intra PeFLH treatment with the MC4R agonist induced a greater increase in EE, compared to vehicle in high-capacity runner (HCR) vs. low-capacity runner (LCR) rats;

*Significant increase compared to vehicle; $p<0.05$
Figure 34: MC4R antagonist: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

The MC4R antagonist (HS014) suppressed 12-hr nighttime physical activity in high-capacity runner (HCR) rats compared to vehicle treatment; (no significant change in low-capacity runner (LCR) group). *Significantly different from vehicle p<0.05
Figure 35: MC4R antagonist: Energy expenditure (EE) in perifornical region of lateral hypothalamus (PeFLH)

High-capacity runner (HCR) rats showed a greater MC4R-antagonist-induced suppression of EE compared to low-capacity runner (LCR). *Significant suppression of night-time EE by MC5R antagonist in HCR compared to vehicle. **Significant difference in night-time activity between HCR vs. LCR with vehicle treatment $p<0.05$. 
PeFLH and MC3R

With the MC3R agonist (D-Trp8-γ-MSH from Tocris Biosciences), both physical activity and EE increased in HCR as well as LCR in a similar fashion (Figure 36 and Figure 37). This is particularly interesting because we did not detect a variable expression of MC3R in PeFLH (Chapters 2 and 3). This supports the hypothesis that the MCR-driven activity and EE is regulated by the brain in a region and receptor subtype-specific manner.
Figure 36: MC3R agonist: Physical activity in perifornical region of lateral hypothalamus (PeFLH)

The MC3R agonist (D-Trp8-γ-MSH) increased short-term physical activity, but no difference was detected between the high-capacity runner (HCR) and low-capacity runner (LCR) rats; *p<0.05 (treatment vs. vehicle).
Figure 37: MC3R agonist: Energy expenditure (EE) in perifornical region of lateral hypothalamus (PeFLH)

The MC3R agonist (D-Trp8-γ-MSH) increased short-term EE, but no difference was detected between the high-capacity runner (HCR) and low-capacity runner (LCR); *p<0.05 (treatment vs. vehicle).
**PeFLH and MTII**

Next, the ability of the non-specific agonist MTII to induce physical activity after microinjection into the PeFLH was investigated. Being an agonist to the 3, 4, and also 5 subtypes, it cannot differentiate between the three. On measuring a dose-response curve in SD rats, the activity and EE increased only slightly, though not significantly, at any of the doses compared to vehicle (Figure 38 and Figure 40). On comparing HCR with LCR rats, MTII in the PeFLH significantly increased physical activity, but this effect was not different between groups (Figure 39). While these data show that MC4R and MC5R are differentially expressed between HCR and LCR, and targeting the MC4R and MC5R specifically also increases physical activity to a greater extent in HCR, the results with MTII roughly mirror the effect of MC3R in PeFLH (rather than the effects of MC4R and MC5R in this region). This demonstrates the importance of targeting one receptor subtype at a time; a compound like MTII that does not distinguish between the MCR subtype activated did not reveal a variable effect between the rat groups even though specific agonists for two out of the 3 receptors subtypes in this region (MC4R and MC5R, but not MC3R) were more effective in the PeFLH in stimulating physical activity of HCR compared to LCR.
Figure 38: Perifornical region of the lateral hypothalamus (PeFLH) and melanotan II (MTII): Physical activity in Sprague–Dawley (SD) rats

A non-significant trend towards an increase in physical activity with increasing doses of MTII in the PeFLH of SD rats.
Figure 39: Perifornical region of the lateral hypothalamus (PeFLH) and melanotan II (MTII): Physical activity in high-capacity runner (HCR) and low-capacity runner (LCR) rats

The non-specific agonist MTII in the PeFLH increased short-term physical activity, but no difference was detected between the HCR and LCR rats; *p<0.05 (treatment vs. vehicle).
Figure 40: Perifornical region of the lateral hypothalamus (PeFLH) and melanotan II (MTII): Energy expenditure (EE) in high-capacity runner (HCR) and low-capacity runner (LCR) rats

There was no significant change in the EE after MTII compared to vehicle, with microinjections in the PeFLH in HCR or LCR.
PVN

While the main focus of these studies was MC5R, especially its actions in the PeFLH nucleus to demonstrate novel MCR function, I also completed most of the parallel studies in the PVN. The earlier chapters described how the PVN region shows higher expression levels of MC3R in HCR vs. in LCR, with no difference in MC4R and MC5R (as observed in figures 41-45). In view of that, I predicted that there would be no difference in the MC4R-or MC5R-mediated effects on physical activity between the two rat groups.
Figure 41: MC5R agonist: Physical activity in paraventricular nucleus (PVN)

MC5R agonist (Ac-Nle-cyclo (Asp-Oic-D-4,4’-Bip-Pip-Trp-Lys)-amide) increased spontaneous physical activity in both groups; high-capacity runners (HCR) and low-capacity runners (LCR) were not significantly different from each other. *Significant increase in activity compared to vehicle; no difference between HCR and LCR (treatment vs. vehicle).
Figure 42: MC5R agonist in the paraventricular nucleus (PVN): Energy expenditure (EE)

EE increased significantly in both the groups of rats similarly; high-capacity runners (HCR) and low-capacity runners (LCR) were not significant from each other.

*Significantly different from vehicle; p<0.05.
The MC5R antagonist did not significantly suppress nighttime activity in either high-capacity runner (HCR) or low-capacity runners (LCR). **Significant difference between HCR-LCR, both with vehicle and treatment; \( p < 0.05 \). No significant effect of antagonist treatment was seen.
Figure 44: MC5R antagonist: Energy expenditure (EE) in paraventricular nucleus (PVN)

No significant effect on nighttime EE in either of the group with the MC5R antagonist.

**Significant difference between high-capacity runner (HCR) and low-capacity runner (LCR), both with vehicle and antagonist treatment; $p<0.05$.**
MC5R treatment (neither agonist nor antagonist) of the PVN did not significantly alter physical activity levels in our rat model (Figures 41-45). The overall spontaneous physical activity was higher in HCR vs. LCR after both the vehicle and antagonist treatments during the nighttime (Figure 43 and Figure 44). This reflects the intrinsic high activity phenotype of HCR rats. Since experiments with the antagonists were performed over the 12-hr dark cycle, this allowed sufficient time for such activity differences to emerge, compared to shorter 3-hr periods (such as in agonist experiments) where average overall HCR activity counts are rarely significantly elevated. Also, because rats are nocturnal, most of the activity seen in these rats is during the night.
Figure 45: MC4R agonist: Physical activity in paraventricular nucleus (PVN)

MC4R agonist (Cyclo(βAla-His-D·Phe-Arg-Trp·Glu)NH2) increased spontaneous activity in both the groups; high-capacity runners (HCR) vs. low-capacity runners (LCR) not significant; *p<0.05, treatment vs. vehicle.
Table 5: Summary of statistical analysis and corresponding p values (ANOVA) obtained from the analyses of physical activity (PA) and energy expenditure (EE) differences as an effect of agonist/antagonist treatment in the paraventricular nucleus (PVN) and the perifornical region of the hypothalamus (PeFLH).

<table>
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<tr>
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<tr>
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<td>MTII</td>
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</tr>
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</table>
4.4 Discussion

Physical activity differences seen as a result of specific agonist and antagonist treatments were region specific and directly correspond to brain regions identified earlier to have a differential expression in MCR subtypes (Chapters 2 and 3). Additionally, these studies were able to identify the PVN and PeFLH as having the ability to increase activity in response to activation of MC receptors. These findings might also show how a normal distribution of different receptor profiles in a population may cause one individual to be more or less prone to be sedentary and obese, compared to another individual. Different brain MCR profiles may be reflected in different individuals’ daily physical activity and EE. HCR and LCR rats that were used in these studies have been shown to significantly differ in daily activity levels and EE, and represent the upper and lower percentages of a normal distribution of population (Koch et al., 2012). Therefore, these findings support the hypothesis that physical activity differences between individual rats or humans could potentially stem from different MCR profiles in specific brain regions. These are the first studies to investigate the role of activity and EE focusing on each MCR subtype individually in a region-specific manner. Being similar in structure, the functional role of individual MCR subtype is often misrepresented by the use of the common mixed MCR agonist MTII. Given the importance of this system in human energy balance, and the potential to target these receptors pharmacologically to treat obesity, there is a need to further partition the representation and functional role of MCRs in different brain regions.
The pharmacological approach to target the receptors is a good way to look at short-term effects of the brain MC system on behavioral changes. However, GPCRs are often associated with a complex phenotype since they have a variety of physiological roles. A more effective way to investigate the role of MCRs in a high-activity phenotype over longer periods would be to use viral-vector-mediated gene transfer or direct injections of siRNA. These genetic tools enhancing or suppressing the receptor of interest can possibly target a specific brain area with the utmost specificity and selectivity (Garza, Kim, Liu, Zhang, & Lu, 2008; Liu, Garza, Li, & Lu, 2013) and more effectively test hypotheses regarding the roles a specific receptor normally plays in a given behavior.

Next, I dissect the brain circuitry between physical activity and appetite in association with MCs (Chapter 5). The ultimate goal is to differentiate between region- and receptor subtype-specific effects on different aspects of energy balance. Accordingly, I aim to identify the role of MC receptor on physical activity compared to food intake.

**Possible damage to site during multiple injections:**

When doing multiple microinjections through a cannula implant, it is possible that repeated injections can damage the site and affect the data. To avoid this problem, utmost care was taken to be gentle while injecting throughout the study. Also, by using different sets of rats for various studies, I minimized the total number of injections per rat to a maximum of 8-10. Further, our preliminary studies (not all data shown here) to determine the optimal dose of each compound used, also cut down the total number of
injections required for each study. This was done by completing a dose-response curve, where the dose of 10pm/200 nl was identified to be most effective in eliciting physical activity changes.
CHAPTER 5: Melanocortin Receptors (MCRs) and Food Intake

The neuroendocrine control of energy balance and food intake is a complex process that involves multiple integrated pathways. Being a satiety signal, MC is one such system that regulates food intake. While wild-type mice exhibit an anorectic response with i.c.v. injections of MTII, mice with double-KO of MC3R and MC4R show no MTII effect on food intake (Rowland, Schaub, Robertson, Andreasen, & Haskell-Luevano, 2010), implicating these receptor subtypes MTII-induced food intake. Also, MC3R or MC4R KO alone has been shown to have a partial anorectic response, underlining the importance of each of these receptors in appetite control. i.c.v (3rd ventricle) injections of the commonly used mixed MC agonist MTII have been shown to decrease food intake, while the antagonist SHU9119 increases appetite in rats (Murphy et al., 1998).

Therefore, to the aim of these studies was to measure the influence of specific MCR agonists and antagonists on food intake and body weight in high-activity (HCR) and low-activity (LCR) rats. Using specific MCR agonists and antagonists in our rat model, I investigated whether the activation of MC receptors in hypothalamic nuclei partly affects appetite and satiety in these rats, and if HCR rats may be more sensitive to this MC-mediated satiety signaling (as they are with physical activity). I hypothesized
that MC neuronal circuitry contributes to satiety signaling via its brain-region-specific receptor expression pattern. I predicted that treatment with these drugs, targeting specific brain regions, would lead to inhibition (agonists) or stimulation (antagonist) of food intake. Furthermore, I predicted that site-specific microinjections of MCR agonist would induce a greater anorectic effect in HCR compared to LCR rats. Conversely, I predicted that specific receptor antagonists will lead to a stimulation of food intake, and this effect will be greater in HCR rats compared to LCR rats. These studies would highlight the role of MC receptors in specific brain areas, as well as their role in food intake.

Because MTII administration in the PVN also has an effect of decreasing food intake (Rowland et al., 2010; Skibicka & Grill, 2009), and given that HCR rats are significantly more responsive to other functions of these receptors (physical activity and EE), I expected this effect to be greater in high-activity rats compared to low-activity rats. For the feeding studies, I used some of the compounds described above, once again mainly focusing on receptor 5 with an MC5R agonist and MC5R antagonist, but additionally using an MC4R agonist and antagonist. If the MC receptors examined here are involved in food intake, then I predict that site-specific activation with receptor-subtype specific agonists would result in decreased food intake. I also predicted that this effect would be receptor- and target region-specific. Since the effect of the agonist occurs in the short term over only a few hours, a change in food intake as an effect of dose is not necessarily apparent. So for all food intake studies, only the higher dose of 20pm/200 nl was used to optimize the ability to detect an effect on food intake.
5.1 **Experimental Design:**

To evaluate the role of MCs in spontaneous feeding, I used MC receptor agonists and antagonists, microinjected aimed at specific hypothalamic regions (PVN or PeFLH). Though these were independent microinjection studies, the same set of HCR and LCR rats was used (10-12 rats/group in each study) from the studies described in Chapter 4. For treatment with the agonists, each rat was deprived of food overnight (18 h) before being microinjected with the drug or a vehicle (aCSF), in order to detect a decrease in food intake. For the antagonist, each rat was treated in the absence of food deprivation. If the antagonist increased food intake, this would implicate endogenous MC stimulation of receptors in that particular nucleus in modulation of food intake. Again, for the agonist as well as the antagonist, I used a single dose (20pm/200nl), which was higher than the one I used in activity/EE studies, as described previously.

After the microinjection, rats were placed back in their home cages along with weighed amounts of food pellets. Food intake was then determined at specific time intervals: 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, and 48 hours after injection. Body weight measurements were also taken at each time point. The experiment was repeated after a period of 7 days so that each rat was injected with vehicle as well as the drug (order counterbalanced), in addition to allowing sufficient washout time for any residual drug effects.
Data analysis:

Food intake of treated rats was compared between agonist or antagonist vs. vehicle (aCSF) treatment, using a 2X2 mixed (split-plot) ANOVA, with HCR-LCR rats as the between-subjects independent variable, drug/vehicle as the within-subjects independent variable, and food intake (g) or body weight as dependent variables in separate analyses.

5.2 Results

In the experiment, I once again used the SD rats to test the ability of the MC5R agonist to suppress food intake. Again, since the behavioral functions of these MCR peptides are yet to be characterized and this is the first food intake study using this compound, the appropriate doses have not yet been characterized in vivo. Here, I first used the same dose (10pm/200nl) that was used in my activity/EE experiments, to determine if a treatment that affects physical activity and EE (Chapter 4) has an effect on food intake as well. No differences in the food intake pattern or body weight were observed at any time point during 48 hours with an MC5R agonist, compared to vehicle (Figure 46 and Figure 47). Thus, I decided to use the higher dose of 20 pm/200 nl to proceed with the experiments in the HCR-LCR model.
Figure 46: MC5R agonist in Sprague-Dawley (SD) rats: Cumulative food intake

In SD rats, no significant differences were seen in cumulative food intake during 48 hours after microinjections of the MC5R agonist compared to vehicle treatment, after an overnight fast.
Figure 47: MC5R agonist in Sprague-Dawley (SD) rats: Body weight

In SD rats, no differences were seen in body weight during 48 hours after microinjections of the MC5R agonist compared to vehicle treatment, after an overnight fast.
In the HCR-LCR experiment with the MC5R agonist, once again no significant differences in the food intake or body weight pattern were evident up to 48 hours after treatment, compared to the vehicle (aCSF) (Figure 48 and Figure 49). The LCR rat groups weighed slightly more than the HCR to start with, but no significant difference was seen in the change in body weight (in g) over time in either HCR or LCR rats. Similarly, I did not detect any correlation with food intake or body weight as a function of MC5R antagonist in the PeFLH (Figure 50 and Figure 51).

In a separate experiment, the MC5R agonist and antagonist were injected into the PVN and no significant effects of treatment were observed (data not shown). Another compound tested was an MC4R agonist in the PVN and PeFLH and no significant induction or suppression of food intake was observed.
Figure 48: MC5R agonist in high-capacity runner (HCR) and low-capacity runner (LCR) rats: Cumulative food intake

Upon comparing HCR with LCR rats, no differences were seen in cumulative food intake over 48 hours after microinjecting with MC5R agonist compared to vehicle treatment (after an overnight fast).
Figure 49: MC5R agonist in high-capacity runner (HCR) and low-capacity runner (LCR) rats: Body weight

In both HCR and LCR rats, no significant differences were seen in body weights with MC5R agonist, compared to vehicle treatment.
Figure 50: MC5R antagonist in high-capacity runner (HCR) and low-capacity runner (LCR) rats: Cumulative food intake.

No significant differences were seen in cumulative food intake over 48 hours in response to microinjections of the MC5R antagonist, compared to vehicle treatment, in either HCR or LCR rats.
Figure 51: MC5R antagonist in high-capacity runner (HCR) and low-capacity runner (LCR) rats: Body weight

No significant differences were seen in body weight over 48 hours in response to microinjections of the MC5R antagonist, compared to vehicle treatment, in either HCR and LCR rats.
5.3 Discussion

While the MC system impacts appetite through a complex pathway involving multiple receptors and peptides, MC5R agonists and antagonists in the PeFLH were not found to alter food intake or body weight. In contrast to our prediction, we found that agonists did not decrease the food intake over a 48-hour period in either HCR or LCR. In addition, the antagonists did not increase the cumulative food intake over 48 hours at any given time point. These results, though contrary to prediction, are interesting because they implicate MC5R more in physical activity than in appetite. Though MC is usually associated with appetite more than physical activity, there is an intriguing possibility that MC5R predominantly functions in the regulation of physical activity.

The role of brain MC in modulating food intake may be harder to interpret than its role in EE. There are many other appetite and satiety signals coming from multiple organs such as the stomach, pancreas, and adipose tissue, to name a few, which then need to be integrated in the brain. Altogether, as in most systems, each component contributes to the signal to influence feeding behavior, and while MCs may still be relevant, further studies may be required to better understand the system.

This study demonstrates that, first, MC5R in the PeFLH was not found to alter food intake or body weight at a dose (the same or higher) that significantly affected physical activity in the same rats and in the same regions. While earlier reports have shown MTII and SHU9119 to alter food intake (Murphy et al., 1998), such a non-specific
agonist or antagonist cannot reflect the role of each MCR subtype individually. It is possible that with the MTII, MC3R may be driving the actions of MTII, and MC4R and MC5R are not relevant to the same extent, as seen from the activity/EE data in the PeFLH. While the literature on the role of MC5R in the brain is extremely limited, hypothalamic down-regulation of MC5R in sea bass has been shown to have no effect on its food intake (Sanchez, Rubio, & Cerda-Reverter, 2009).

When evaluating MC4R, as with MC5R, no significant effect on food intake was observed in our rat model. These results contrast with a number of studies that associate MC4R with appetite and satiety (Balthasar et al., 2005; Benoit et al., 2000; Noble, Billington, Kotz, & Wang, 2011). The apparent discrepancy of these results could be because HCR-LCR is a very different model compared to regular SD rats that have been used for most studies focusing on the response of appetite to MC agonists. Given the complexity of the appetite and satiety signaling mechanism, it is likely that other factors may differ in these rats, which may contribute to its phenotype. Similarly, it is also possible that I did not use the optimal dose range (particularly with the MC5R peptides which haven’t been characterized in vivo) to effect a short-term change in feeding behavior in these rats.

In addition, the role of BDNF in this pathway cannot be ignored. The MC4R also activates neurons containing BDNF, which then exerts its actions through the TrkB receptors in the hypothalamus and has been shown to decrease food intake and increase
EE in mice, demonstrating that BDNF plays a role in regulating food balance, possibly mediating the function of MC4R (Nakagawa et al., 2000; B. Xu et al., 2003). This may be particularly relevant in our experimental model for two reasons. First, I saw a differential expression of BDNF mRNA between HCR- and LCR in the PeFLH (Table 3), which could interact and alter the role of MC4R appetite regulation. Second, BDNF is also known to interact with estrogen receptors (Scharfman & MacLusky, 2006; Solum & Handa, 2002; Zhu, Liu, Senthil Kumar, Zhang, & Shi, 2013), which could be a factor in these studies, since I used females for these experiments. Many previous food intake studies have used male mice and some reports even fail to mention the sex of the species used (Balthasar et al., 2005; Beery & Zucker, 2011; Zucker & Beery, 2010). The possible confounds of a sex bias in such studies have recently been noted. While it was an advantage to use the female model to characterize the EE phenotype, it may be important to do a comparative study in males to better understand the role of MCRs in appetite and satiety.

Rarely are these two dependent variables—food intake and physical activity—studied together. However, the signals regulating food intake and EE overlap almost universally (Skibicka & Grill, 2009), so if we are to discern the regulation of one, it’s not ideal to ignore the other. Previously, we have used this rat model of leanness vs. metabolic syndrome to highlight these effects (appetite and physical activity) and how they vary during high-fat feeding. The current study focuses on differentiating between region- and receptor subtype-specific effects on these aspects of energy balance. Through
these results, we were able to suggest a possible role of MC receptors, specifically MC4R and MC5R, in the PeFLH on physical activity, and not on appetite.
CHAPTER 6: General Discussion

The energy expended through spontaneous physical activity may confer resistance to obesity and its deleterious health consequences, particularly in an obesogenic environment (Church et al., 2007; Hamilton, Hamilton, & Zderic, 2007; Levine et al., 1999; Levine & Kotz, 2005; Woolf et al., 2008). Apart from EE, the simple act of being sedentary for long periods of time increases risks for diabetes, cardiovascular disease, metabolic syndrome, and mortality (Dunstan et al., 2012; George et al., 2013; Moore et al., 2012; van der Ploeg, Chey, Korda, Banks, & Bauman, 2012). Though numerous physiological factors can affect physical activity and NEAT, mechanisms underlying individual differences in activity levels associated with obesity propensity are not clearly understood (Kotz, Teske, & Billington, 2008; Novak & Levine, 2007; Teske, Billington, & Kotz, 2008). Since intrinsic aerobic capacity is a critical predictor of high spontaneous activity in both rats and humans (Novak et al., 2009), and low activity is not just secondary to a high body weight (Gavini et al., 2014), high physical activity and intrinsic aerobic capacity may be mechanistically related and could be characteristic features of the lean phenotype (Novak & Levine, 2009). Here, I investigated one of the potential mechanisms that modulate daily activity in high- and low-capacity rats.

The brain MC system, including POMC, α-MSH, AgRP, and MCRs, plus the various metabolic cues it transduces and systems it impacts, is known to play an important role in satiety and obesity propensity. This has been corroborated by genetic
studies in humans showing a number of point mutations and polymorphisms related to human obesity (Cauchi et al., 2009; Chen et al., 2000; MacKenzie, 2006; Mountjoy et al., 1994). However, how these genetic differences may interact with altered energy status or energetic challenges remains poorly understood. Here, I have shown for the first time that regional differences in brain MC responses are associated with individual differences in physical activity, and these are both linked to obesity propensity or resistance to obesity (Cauchi et al., 2009; Chen et al., 2000; MacKenzie, 2006; Mountjoy & Wild, 1998). These findings may provide insight into how individuals differ in their brain MC responses, which could in turn make them more or less physically active. Specifically, these data implicate MC3R in the PVN, and MC4R and MC5R in the PeFLH, as potential mechanisms underlying the elevated levels of daily physical activity we consistently see in HCR (Gavini et al., 2014; Novak et al., 2010; Shukla et al., 2012).

MC5R has the widest peripheral distribution among the three brain-associated MCR subtypes. Outside of the CNS, on comparing the mRNA expression of MC5R in the skeletal muscle samples (gastrocnemius) from HCR-LCR rats, no difference was found between the groups (Figure 7). While this class of GPCRs has a wide variety of physiological roles in the body, how only some tissues or cellular populations get altered (by secondary signals) remains to be understood.

Many maternal programming and developmental functions impact physical
activity, appetite, and underlying neural systems (Chang, Gaysinskaya, Karatayev, & Leibowitz, 2008). For example, maternal nutrition in sheep has been shown to affect fetal hypothalamic glucocorticoid receptors and also the appetite-regulating neuropeptides, including POMC and neuropeptide Y, which it regulates (Stevens et al., 2010). Our rats are housed in similar conditions to each other, including access to food. However, secondary factors (including known and unknown genetic/epigenetic differences between the two rat lines) could be a source of signals that drive promoter driven effects and other post-transcriptional modifications. Peripheral signals including ghrelin, leptin, opioid peptides, and NPY through their interactions with their respective receptors and peptides could also be altering MCR development and differentiation as they are integrated in the hypothalamus. It is conceivable that these maternal programming factors, such as diet or stress exposure, may impact the brain MC system and MC receptors to promote weight gain and obesity in offspring, potentially through modulation of physical activity levels. In fact, under-nutrition during gestation negatively impacts physical activity levels of the offspring (Bellinger, Sculley, & Langley-Evans, 2006). It is not known whether these or similar maternal programming treatments operate through or influence the development of the brain MC system or its receptors.

Numerous MCR mutations have been identified in obese humans in various ethnic groups. This includes MC4R, the most common monogenic cause of human obesity, MC3R, and also MC5R mutations (Cauchi et al., 2009; Chagnon et al., 1997; Farooqi et al., 2000; Feng et al., 2005). Indeed, loss of function mutations of MC4R in
humans can result in obesity that is even resistant to bariatric surgery (Aslan et al., 2011). While we see similar correlation between MCR and obesity or obesity risk in animal models, how these genetic differences in MCRs affect brain functioning and behavior—food intake and EE—in HCR-LCR rats (and other models of obesity) remains to be discerned. My data implicate lower levels of daily physical activity in the weight gain seen in these individuals, though the relevance of physical activity to the obesity seen with mutations in the brain MC system has yet to be demonstrated.

These data demonstrate that high-activity rats show enhanced expression of MC4R and MC5R, but not MC3R, in PeFLH. Conversely, the PVN of high-activity rats shows higher expression of MC3R, but not of MC4R or MC5R. Next, I found that intra-PVN microinjections of MTII increase physical activity and EE of activity (NEAT), and this increase in activity was greater in lean HCR compared to obesity-prone LCR rats. This elevated EE after intra-PVN MTII can be attributed to activity EE, but the contribution of intra-PVN MC to brown adipose tissue thermogenesis cannot be ruled out (Song, Jackson, Harris, Richard, & Bartness, 2005). These results illustrate possible mechanisms through which individual differences in brain MC contribute to changes in energy balance—specifically EE—that predispose an individual to be lean or obese. It is possible that MC peptide activate downstream effectors—including orexin in the PeFLH, CRH or TRH in the PVN—that may then impact motor systems such as dopamine in the basal ganglia to increase physical activity (Teske, Billington, & Kotz, 2014).
The same pattern of MCR expression was evident in two different sets of HCR-LCR rats from different generations, one set of males and females each, and also using different isolation techniques (micropunches vs. LCM), underlining the consistency of this effect. In these studies, an interesting finding has emerged regarding the profile of MC receptor expression, which reflects the behavioral phenotype. One possible mechanism to explain this pattern in receptor expression would be epigenetic regulation of expression. There are instances where one neurotransmitter (or peptide) can drive changes in the promoter region (histone modifications such as acetylations), in a tissue-specific manner (Gozen, Balkan, Yildirim, Koylu, & Pogun, 2013). A number of theoretical models exist that explain how the nervous system utilizes the kinetics of epigenetic changes to direct neurogenesis or changes in neuronal cell populations (Tan, Zong, & Xie, 2013). This may also affect epigenetic modifications leading to differential activation of brain regions, ultimately affecting behaviors including physical activity (Kumsta, Hummel, Chen, & Heinrichs, 2013). These epigenetic effects may act in concert with the underlying genetic differences between HCR and LCR rats.

Increased POMC results in upregulation of α-MSH and decreased MC receptor expression (Pritchard et al., 2002; Zemel & Shi, 2000), thus it is possible that the heightened MCR seen in the high-activity rats might be secondary to a higher overall MC “tone”. Our data do not support this interpretation, however. First, I found no significant difference in POMC mRNA expression and only a trend in the processing enzyme PC1 (not significantly higher in HCR, $p=0.06$, Table 1) (Shukla et al., 2012). Second,
contrary to our findings, globally elevated MC release would be expected to alter MC receptors similarly regardless of subtype or brain region (that is, cause a down-regulation of all receptors in all regions). Altogether, these data suggest that a specific MC receptor expression profile may be intrinsic to the high-activity, high-endurance phenotype.

Though others have demonstrated the relevance of MC3R and MC4R in the regulation of energy balance, satiety, and human obesity, the potential importance of brain MC5R is yet to be considered (Bromberg-Martin & Hikosaka, 2009; Butler et al., 2000; Mountjoy & Wild, 1998). Pronounced differences were identified specifically in the MC5R in the PeFLH in two different studies (Figure 6 and Figure 17) where the HCR levels were almost 1000 times higher than LCR. Further, HCR respond to MC5R manipulations by increasing (agonists) or decreasing (antagonists) their physical activity and associated EE, while the LCR rats did not show an effect. Experiments with the antagonist injections in Chapter 4 showed that it is possible that the intrinsic high activity phenotype of the HCR could be suppressed (becoming LCR-like) by simply blocking the MC5R in the PeFLH, see Figure 30. The findings described in this dissertation strongly suggest that region-specific differences in MC5R, specifically within the PeFLH, could contribute to individual differences in physical activity. These results are a good example of the utility of examining the lean phenotype. It has been suggested that standard “non-obese” animal models might essentially have metabolic syndrome due to housing in a sedentary environment with unrestricted food supply and limited physical challenges (Martin et al., 2010). It is conceivable that MC5R is low or undetectable in the PeFLH of
standard rodent models, as it is in the obesity-prone, low-activity LCR (Figure 6).

Several lines of evidence highlight the close association between high intrinsic aerobic capacity and high daily physical activity. In both rats and humans, for example, those with high running endurance are also found to be more physically active as measured using an objective (i.e., non-questionnaire-based) method (Novak et al., 2010). The present studies suggest that differences in the brain MC system in HCR-LCR rats, with regional distribution of MC receptors in particular, could underlie individual differences in the tendency to be more or less active. Thus, the mechanistic link between aerobic capacity and physical activity may partly lie with MC receptors. Here, we found that highly specific, differential regional activation of brain MC receptors could affect different aspects of energy balance, particularly physical activity (Song et al., 2005). Though these studies focused on hypothalamic circuits, forebrain reward systems and hindbrain MC are also known to affect energy balance, and may potentially differ between high- and low-activity individuals as well (Bromberg-Martin & Hikosaka, 2009). For instance, hindbrain regions or forebrain reward-mediating areas might be interesting for the investigation of potential receptor-mediated physical activity differences.
Table 6: Summary of hypothalamic nuclei examined for gene expression and physical activity differences as an effect of agonist/antagonist treatment in the paraventricular nucleus (PVN) and the perifornical region of the hypothalamus (PeFLH) (Chapters 2, 3, and 4). Green and red signify concordant vs. discordant results, respectively.

<table>
<thead>
<tr>
<th>Region</th>
<th>Receptor</th>
<th>Gene expression (HCR vs. LCR)</th>
<th>Drug/compound used for microinjections</th>
<th>Differential activation/suppression in HCR vs. LCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PeFLH</td>
<td>MC3R</td>
<td>No difference</td>
<td>Agonist: D-Trp8-γ-MSH (Tocris biosciences)</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td>MC4R</td>
<td>HCR &gt; LCR</td>
<td>Agonist: (Cyclo(βAla-His-D-Phe-Arg-Trp·Glu)NH2 Phoenix pharmaceuticals)</td>
<td>HCR &gt; LCR</td>
</tr>
<tr>
<td></td>
<td>MC5R</td>
<td>HCR &gt; LCR</td>
<td>Agonist: (Ac-Nle-cyclo(Asp-Oic-D-4,4'-Bip-Pip-Trp-Lys)-amide) (Dr. Bednarek from MedImmune)</td>
<td>HCR &gt; LCR</td>
</tr>
<tr>
<td></td>
<td>MC3/4/5R</td>
<td>Variable expression</td>
<td>MTII: non-specific MCR agonist (Phoenix Pharmaceuticals)</td>
<td>HCR &gt; LCR</td>
</tr>
<tr>
<td>PVN</td>
<td>MC3R</td>
<td>HCR&gt;LCR</td>
<td>Agonist: (Cyclo(βAla-His-D-Phe-Arg-Trp·Glu)NH2 Phoenix pharmaceuticals)</td>
<td>No difference</td>
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<tr>
<td></td>
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<td>No difference</td>
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</tr>
</tbody>
</table>
6.1 Future perspectives

To summarize, here I examined brain differences in a physiologically and genetically intact model of the lean phenotype, and was able to identify promising targets in the brain MC system that may contribute to the variability in energy balance via physical activity and EE. Further, I show that MC receptors possibly play a role in some (but not all) aspects of energy balance.

In addition to the directions described in earlier chapters, namely the use of viral vectors to target brain region of interest more specifically, extending the food intake studies to male HCR-LCR rats, as well as focusing on other parts of the brain including hindbrain, this work could also be expanded upon by examining other neuroendocrine intermediates and their functional relevance in regulating physical activity and the associated EE. For example, BDNF expression in the PeFLH differed between HCR and LCR; this peptide is known to be an important mediator for some MC effects on energy balance (Noble et al., 2011).

The studies described here have possible implications for how we consider metabolism and intrinsic physical activity when attempting to prevent or treat obesity. Targeting of pathways that may enhance the daily activity levels in an individual may take advantage of already-existing mechanisms, which are endogenously employed, to a greater extent in naturally lean people (as in HCR rats).
These findings also have a great potential for developing pharmaceutical targets to address obesity. While the MC4R is already a popular target being researched and developed as a therapeutic approach to treat obesity, most of these drugs have been found unsafe for use in humans. There are at least three important challenges that arise in the search for effective MC4R anti-obesity drugs. First, the search is for agonists, which must bind and then activate the receptor unlike antagonists, where only receptor binding is required. Second, as the MC4R is a member of subgroup of GPCRs and a family of five receptors with diverse physiological functions, this adds the complication of subtype selectivity (Tao, 2010). Lastly, MC4R is widely expressed within the central nervous system and has other physiological roles that can also be affected by these drugs.

Over the years, multiple drug discovery attempts for anti-obesity MC4R agonists have been curbed by emerging evidence linking MC4R activation with therapeutically adverse effects such as hypertension, erectile dysfunction, and inflammation (Corander, Fenech, & Coll, 2009; Greenfield et al., 2009; King et al., 2007; Maier & Hoyer, 2010; Sayk et al., 2010). It remains to be seen whether these concerns can be overcome. If sites for MC4R energy homeostatic control are anatomically distinct from those affecting cardiovascular functions, differential modulation of MC4R activation could also be attempted through targeting of accessory proteins such as MRAPs, mahogunin, and β-defensins if expression of these proteins were site-specific (Chan et al., 2009; Kaelin et al., 2008; Perez-Oliva, Olivares, Jimenez-Cervantes, & Garcia-Borron, 2009; Walker &
Gunn, 2010). Alternatively, the different sites could be mined for signaling molecules downstream of MC4R (and/or MC3R and MC5R) by transcriptome analysis (Kaelin et al., 2008) and targeted if expressed in a site-specific manner. My findings, which clearly show a pattern of differential expression of MC3R and MC5R (in addition to MC4R) in the brain, may thus address some setbacks in MC drug-discovery so far. While it is possible that MC3R and MC5R do not have therapeutic potential in obesity-prone individuals who have only MC4R mutations, they may be important for others suffering from obesity. More work is needed to develop better drugs, considering the complete MC system and its complex expression in multiple tissues. The findings from these studies may lead to an alternative approach, which has been ignored thus far, in order to develop treatment strategies.
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