

EFFECTS OF A HIGH FAT DIET ON POMC, AGRP AND MC4R GENE  
EXPRESSION IN THE ARCUATE NUCLEUS AND PARAVENTRICULAR  
NUCLEUS OF THE HYPOTHALAMUS

by

KARI ELIZABETH BOLT

(Under the direction of Silvia Giraud)

ABSTRACT

This study investigated the levels of mRNA of POMC, AgRP and MC4R in the arcuate nucleus and paraventricular nucleus of the hypothalamus in thirty Sprague Dawley rats fed a low fat diet or a high fat diet over the course of 12 weeks. The HF group had higher levels of POMC ( $p=0.013$ ) and AgRP ( $p=0.001$ ) in the ARC after 7 weeks of feeding. The HF group did have higher levels of MC4R ( $p=0.038$ ) in the PVN after 12 weeks of feeding. POMC and AgRP mRNA expression in the ARC seemed to increase and decrease in a similar pattern during high fat feeding, while the varying levels of MC4R mRNA expression in the PVN seems to be inversely related to AgRP and POMC expression in the ARC. This may indicate a link in regulation or a breakdown energy balance that supports a state positive energy balance.

INDEX WORDS: Melanocortin System, POMC mRNA, AgRP mRNA, MC4R mRNA, High fat diet, Diet-induced obesity, Energy balance, Sprague Dawley, Gene expression

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## CHAPTER 1

### INTRODUCTION

The United States continues to see an increase in overweight and obese individuals each year [20]. This prevalence of overweight and obesity is not limited to the United States, but is also being detected world wide in both developed and less developed countries [54]. The prevalence of obesity is a major health concern due to its associations with several diseases including, but not limited to, type 2 diabetes mellitus, osteoarthritis, hypertension, dyslipidemia, coronary heart disease, stroke, heart failure and numerous types of cancer [39].

Obesity results from an imbalance between energy intake and output and is characterized by an increase in the number and size of adipocytes [36]. Energy intake is simply the amount of food absorbed, while output is the energy expended through resting metabolic rate, the thermic effect of food and physical activity [74]. Research has uncovered all types of central and peripheral regulation sites, hormones, peptides, receptors and genes involved in the regulation of energy intake and output. One of the regulation systems recognized to play a predominant role in energy homeostasis is the melanocortin (MC) system [17,64,73]. The parts of the MC system known to be involved in energy balance include the melanocortin receptors 3 and 4 (MC3R and MC4R), the principle agonist of these receptors, alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH), and the receptor antagonist, agouti-related protein (AgRP).  $\alpha$ -MSH is derived from the post-translational of pro-opiomelanocortin (POMC) prohormone.

Diets high in fat are known to lead to excessive intake because of the ability of fat to increase palatability and energy density of food consumed. The implications of a high fat diet on the development of obesity in humans and animals are a subject of much research focus [4,8,77]. Rodent models that become obese through a diet high in fat (DIO) have shown to have changes in hypothalamic AgRP, POMC and MC4R mRNA expression [9,48,62,69].

To better understand how the MC operates during the development of obesity, the main objective of this study was to further assess long-term changes in hypothalamic expression of AgRP, POMC and MC4R mRNA in the arcuate nucleus and paraventricular nucleus of the hypothalamus in thirty Sprague Dawley rats fed a low fat diet or a high fat diet over the course of 12 weeks.

## CHAPTER 2

### LITERATURE REVIEW

#### Obesity

Approximately 65% of the United States population can currently be classified as overweight or obese, and this percentage is predicted to increase in the following years [20]. Unfortunately this high prevalence of body weight gain is not limited to the United States, but is also being detected world wide in both developed and less developed countries [54]. The prevalence of obesity is a major health concern due to its associations with several diseases including, but not limited to, type 2 diabetes mellitus, osteoarthritis, hypertension, dyslipidemia, coronary heart disease, stroke, heart failure and numerous types of cancer [39].

Obesity results from an imbalance between energy intake and output and is characterized by an increase in the number and size of adipocytes [36]. The interaction between environment and genotype contribute to this imbalance and resulting weight gain [7]. Since there have been no major changes in gene pool, environmental changes are labeled as the root cause of the obesity epidemic [26,55]. Some lifestyle and environmental components associated with the development of obesity include food, drugs, viruses, toxins and low physical activity [55]. Out of these components listed, more and more information is emerging on the utilization of food, or nutrients, in the body and how dietary fat may influence an increase in adiposity stores which then can lead to obesity [36].

The predominant energy-yielding nutrients are alcohol, protein, carbohydrate and fat. Body adiposity stores are influenced by the way these ingested nutrients are utilized once they are absorbed into the body. The body will immediately use the nutrients it needs upon consumption and any nutrient that is consumed in excess will be either stored or used [5,19,67]. There is limited storage capacity for excess protein and carbohydrate, no storage for alcohol and unlimited storage capacity for fat in the adipose tissue. Once protein and carbohydrate storage pools are full, the body then prefers to oxidize excess protein and carbohydrate at the expense of fat oxidation [19]. In addition to the metabolic priority of protein and carbohydrate oxidation over fat oxidation, the energy cost of absorbing, processing and storing fat is lower in comparison to protein and carbohydrate. Thus, fat has a great metabolic potential to increase adiposity in the body no matter which nutrient in the diet is consumed in excess [3]. Despite the fact that any nutrient consumed in excess will lead to increased adiposity, diets high in fat are known to lead to excessive intake because of the ability of fat to increase palatability and energy density of food consumed [3,5]. The implications of a high fat diet on the development of obesity in humans and animals are a subject of much research focus [4,8,77] and will be discussed in more detail later in this review. Unfortunately, even with all the information known about the utilization of nutrients after absorption, the pathology of obesity is proving to be far more complex than just an over-consumption of nutrients.

### Energy Balance

The entire energy balance network must be elucidated to fully understand the etiology of obesity. As stated earlier, obesity results from an imbalance between energy intake and output, which promotes energy storage. Energy intake is simply the amount of

food absorbed, while output is the energy expended through resting metabolic rate, the thermic effect of food, thermoregulation and physical activity [74]. Research has uncovered all types of central and peripheral regulation sites, hormones, peptides, receptors and genes involved in energy balance. These individual components all work together to form complex central and peripheral control systems [63,76]. This elaborate network of systems is designed to continually correct for positive and negative energy balance. Positive energy balance, which leads to obesity, happens when energy intake exceeds energy expenditure. Negative energy balance is just the opposite and results in weight loss [76].

Maintaining equilibrium within the body is highly dependent upon the anabolic and catabolic pathways between the periphery and the brain. Anabolic pathways will stimulate food intake, decrease energy expenditure and promote fat storage, while catabolic pathways will reduce food intake, increase energy expenditure and a decrease fat mass. When these feedback mechanisms are in place, energy balance is maintained and body adiposity is stable [74,76].

Hormones secreted by peripheral tissues and organs are major determinants of metabolic status [63]. Their production conveys information from the body to regulatory sites in the brain [63,76]. The arcuate nucleus of the hypothalamus (ARC) and the nucleus of the tractus solitarius (NTS) of the brainstem are two of the main neural regulatory sites that receive short-term and long-term hormonal information from the body [18]. Short-term regulation is involved in triggering hunger and food intake or producing satiety and meal termination. Long-term regulation is more involved with the regulation of weight or adiposity.

### Circulating Hormones

There are also several hormones produced in the gastrointestinal tract that aid in short-term control, also known as meal-to-meal regulation. These hormones are involved with several neurotransmitter systems to help trigger hunger and food intake or produce satiety and meal termination [40,52]. Ghrelin is a hormone primarily produced in the stomach and is known to play a role in the initiation of food intake [30,40]. The concentration of this hormone usually peaks before a meal or during times of negative energy balance. It is thought to initiate food intake primarily through activating neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons located in the arcuate nucleus of the hypothalamus (ARC), which are involved in promoting food intake [30,65].

Peptide YY (PYY) and cholecystokinin (CCK) are gut hormones that work in opposition to ghrelin [40,52]. They are released following a meal to reduce food intake. PYY is secreted by the L-cells in the mucosa of the small intestine, large intestine and rectum in proportion to the amount of energy ingested and CCK is produced by the I-cells in the mucosa and is found in high concentrations in various parts of the brain where it acts as a satiety signal [40,52].

Insulin is known predominantly for its regulation of blood glucose. It is released from beta cells of the pancreas during meals as a response to an increase in glucose levels in the blood and remains elevated about long as blood glucose is elevated. It aids in removing glucose from the blood so that the glucose can be stored as either glycogen and/or triglyceride in the body [75]. In addition to this function, insulin is also a key peripheral regulator of food intake and body adiposity [6,45]. It has been found to enter

the brain through a saturable transport process [27,77] and bind to insulin receptors located in brain regions known to be involved in the energy balance regulation, for example the arcuate nucleus (ARC) of the hypothalamus [6,45]. In short-term energy balance, an increase of insulin in the blood or administration of insulin into the brain reduces food intake and increases energy expenditure [1,45,57]. Insulin is also known to be an adiposity signal given that fasting plasma levels of insulin are directly correlated with the degree of body adiposity [56,57]. When adipose tissue is reduced, less insulin is secreted. Conversely, when adipose stores are increased, the body has higher basal insulin levels and more insulin is secreted in response to a meal [6,45]. Increased fat mass or body adiposity is believed to contribute the development of insulin resistance. This is thought to be one of the main causes of type 2 diabetes mellitus in obese individuals [6,53].

Leptin is another key regulator of food intake and body adiposity. It is secreted from adipocytes in proportion to body adiposity with low levels indicating depletion in body fat stores and higher levels indicating an increase in fat tissue [45,75]. Leptin binds to receptors found in the brain and peripheral tissues [50]. Once leptin crosses the blood-brain barrier, it then has the ability to exert its actions by binding to receptors found on two different populations of hypothalamic neurons. Through binding to the leptin receptors on these neurons, leptin has shown to reduce food intake and increase energy expenditure [6,45]. Unfortunately, leptin does not have the same effect on food intake and energy expenditure once an individual becomes obese and develops leptin resistance [63]. It is thought that leptin resistance occurs in obese individuals from either a defect in leptin transport across the blood brain barrier and/or possibly a decreased sensitivity of

receptors in the hypothalamus [50]. Levin et al suggested that a reduction in hypothalamic but not brain stem leptin signaling might contribute to the development of DIO when dietary fat and caloric density are increased [43].

### Neural Regulation

The neuropeptide Y (NPY) system and the melanocortin (MC) system are two central regulatory pathways found in the hypothalamus that are influenced by food intake and by changes in leptin and insulin levels [9]. These systems stem from two distinct populations of neurons found predominantly in the ARC. One population of neurons co-express neuropeptide Y (NPY) and agouti-related peptide (AgRP), and the other neurons co-express pro-opiomelanocortin (POMC) and cocaine amphetamine-related transcript (CART). These systems work in opposition even though they are co-localized in the ARC. The NPY system is a catabolic system that works to stimulate feeding and energy storage, while the MC system is involved in utilizing stored energy and suppressing food intake [45].

### Melanocortin System

The melanocortin (MC) system is recognized to play a predominant role in energy homeostasis [17,64,73]. It is also involved in many diverse metabolic processes in addition to energy regulation. In fact this system was first identified from examining skin pigmentation in frogs. Other than pigmentation and energy balance the melanocortin system is also involved in exocrine secretion, inflammation, sexual function, immunomodulation, temperature control, cardiovascular regulation and many other functions [46].

The MC system is composed of melanocortin peptides, receptors, endogenous antagonists and mediators. The four melanocortin peptides  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte-stimulating hormone ( $\alpha$ -,  $\beta$ -,  $\gamma$ -MSH) and adrenocorticotrophic hormone (ACTH) are collectively known as the melanocortins since they share the core amino acid sequence His-Phe-Arg-Trp [21,28]. They are derived from post-translational processing of the pro-opiomelanocortin (POMC) pre-prohormone [21,46], whose cell bodies are found in the ARC and the NTS with neurons projecting into several areas of the brain, such as the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) [21,46]. The five melanocortin receptors (MCR), named according to the order in which they were discovered (MC1R- MC5R). Four of the receptors are found in the brain in addition to several peripheral tissues leaving MC2R residing only in the periphery. The receptors belong to the G-protein coupled receptor super-family [21]. The endogenous melanocortin proteins of the MC system include two antagonists, agouti and agouti-related protein (AgRP), and two mediators, mahogany and syndecan-3 [21,78]. The parts of the MC system known to be involved in energy balance include the melanocortin receptors 3 and 4 (MC3R and MC4R); the principle agonist of these receptors, alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH); the receptor antagonist, agouti-related protein (AgRP); and the two AgRP mediators, mahogany and syndecan-3 [21,64,78].

### *Melanocortin 3 and 4 Receptors*

The third melanocortin receptor was first isolated in the early 90's by Gantz et al [22]. It is expressed in several different peripheral tissues and many areas of the brain in humans and rodents [24,60]. MC3R mRNA is present in more than thirty different sites in the rat brain, including the septum, hippocampus, thalamus, amygdala and brainstem.

MC3R is activated by all four melanocortins derived from POMC mRNA [34,60], and is selectively expressed on the POMC and AgRP neurons in the ARC [34,60].

Confirmation of its role in energy metabolism, particularly feed efficiency, has been demonstrated in studies with MC3R knockout mice. The MC3R deficient mice exhibit a 50-60% increase in adipose mass, a 50% reduction in physical activity and an unusual increase in respiratory quotient suggesting a reduced ratio of fat/carbohydrate oxidation [11]. MC3R null mice have also been shown to have a reduction in lean body mass, higher feed efficiency, and in some cases develop mild hyperleptinemia [13].

Unlike the MC3R, the MC4R is expressed primarily within the central nervous system and it is known to have a pivotal role in regulating food intake and energy expenditure [64]. The MC4R is the most abundant and widely distributed melanocortin receptor in the brain. It is expressed in virtually every region of the central nervous system (CNS) [38], including the cortex, thalamus, hypothalamus, brain stem and spinal cord [23,38,49]. In the rat brain, one of the regions with the highest densities of the MC4R mRNA expression can be found in the hypothalamus, specifically in the paraventricular nucleus (PVN) [38], arcuate nucleus (ARC) and lateral hypothalamic area (LHA) [49]. The transmembrane regions of the rat and human MC4R have few differences in the amino acid sequences. In fact the human MC4R has approximately 95% amino acid identity with its rat orthologue [61]. The disruption in the MC4R gene in humans [71,79] as well as the MC4R in mice [11,13,33] produces a severe obesity syndrome. It is also worth noting that in humans, mutations in the MC4R are considered one of the most common genetic causes of severe early-onset obesity, accounting for approximately 6% of all cases [79]. Just like the MC3R knockout mice, MC4R knockout

mice provide physiological evidence for the role of MC4R in energy homeostasis. Nonetheless, there are subtle differences between the MC3R and MC4R knockout mice. MC4R null mice develop maturity onset obesity, hyperphagia, hyperinsulinemia, insulin resistance, hyperglycemia, have increased linear growth and hyperleptinemia [33]. The differences between the MC3R and MC4R null mice seem to suggest that the MC3R may regulate feed efficiency and partitioning of nutrients into fat, while the MC4R may regulate food intake and energy expenditure [46]. Mice deficient in both MC3R and MC4R are significantly heavier than their single knockout littermates [14], which again may suggest varying roles in energy balance. Additional information on the varying roles these receptors have in energy intake and expenditure can be gleaned from taking a closer look at the ligands that bind to the MC3R and MC4R receptors.

### Receptor Ligands

All four of the melanocortin peptides derived from POMC bind to the MC3R and MC4R, but the primary melanocortin known to be responsible for the regulation of energy balance through these two receptors is alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH).  $\alpha$ -MSH is a nonselective agonist at the receptors MC1 and MC3 – MC5, however there is no evidence that MC1R and MC5R play a role in energy homeostasis [46].  $\alpha$ -MSH binds with high affinity to both the MC3 and MC4 receptors [21], but reduces food intake by primarily activating on the MC4R [47]. When peripherally administered in diet-induced obese mice, it can reduce food intake, elevate several metabolic functions, and reduce body weight gain [29]. On the other hand, receptor antagonist, agouti-related protein (AgRP), blocks the binding of  $\alpha$ -MSH to the MC3 and

MC4 receptors, and after binding to the receptors it increases food intake, reduces metabolic functions, and promotes weight gain [51,59].

Neurons that produce  $\alpha$ -MSH and AgRP can be found in the ARC with projections into other areas of the hypothalamus [59]. Most AgRP neurons are colocalized with NPY neurons, while the POMC gene that  $\alpha$ -MSH is derived from is colocalized with another anorexic peptide cocaine amphetamine-regulated transcript (CART) [16,46]. These neurons are important targets for insulin and leptin signaling [6].

MTII and SHU9119 are synthetic agonist and antagonist of  $\alpha$ -MSH and AgRP respectively, and are commonly used to study energy regulation. MTII works as an agonist [10], while SHU9119 acts as antagonists [31]. The measured outcomes after the injection of these ligands has confirmed that the activation of the MC3R regulates energy balance a little differently than the MC4R [13,14,68].

The  $\alpha$ -MSH pathway leading to stimulation of MC4R is more involved in reducing food intake than MC3R. The evidence to support this idea is found in the research with MTII. Prior to studies on MC3R and MC4R with MTII, this synthetic receptor agonist was found to potently inhibit food intake in rats given an injection into the paraventricular nucleus of the hypothalamus (PVN) [25] and intracerebroventricularly (ICV) [10]. In a study shortly after those results, MC4R was noted to be the primary receptor for inhibiting food intake since there was no measured inhibition after MC4R null mice were injected with MTII into the ICV at a dose that inhibited feeding in wild-type mice [47].

AgRP has been shown to potently increase food intake when injected into the PVN [37,58] and ICV [58]. This is also seen in with injections of its analog SHU9119 [25,58]. AgRP is thought to have additional hypothalamic targets other than MC4R

because injections of AgRP and SHU9119 in MC4R null mice still influences food intake [58].

### Long Term Regulation of Energy Balance

The long term regulation hormone leptin acts as an adiposity signal upstream from the melanocortin system in the brain [52,63]. Leptin has shown to enter the arcuate nucleus of the hypothalamus and bind to receptors located on neurons that code for the melanocortin peptides, AgRP and  $\alpha$ -MSH [35]. During a state of positive energy balance, there is an increase in leptin in the circulation due to the increase in adiposity. When the melanocortin system receives information of the increase in leptin, there is an increase in the catabolic effector,  $\alpha$ -MSH, and a down-regulation in AgRP [35]. This, along with the activation of other catabolic pathways, aids in decreasing food intake and promoting weight loss. Conversely, adipose tissue reduces in a state of negative energy balance and less leptin is secreted into circulation. With a reduction of leptin passing into the brain, there is a measurable decrease in  $\alpha$ -MSH and an increase in AgRP [66]. An increase in this anabolic effector, as well as the activation of other anabolic pathways, leads to an increase in food intake and weight gain.

### Gene Expression Changes

Research literature indicates that there are hypothalamic gene expression changes within the melanocortin system during states of both positive and negative energy balance [9,48,62,69]. During states of negative energy balance induced by fasting, POMC mRNA and its peptide  $\alpha$ -MSH have been shown to decrease [9,48,62,69], while AgRP mRNA and its peptide increase during negative balance [9]. Unfortunately, the evidence for gene expression changes in this system during positive energy balance is not

as clear, because of the numerous ways researchers can manipulate diet-related factors involved in inducing a state of positive energy balance. Macronutrient composition [70,72], the predisposition of obesity Torri [32,70], and length of high fat diet exposure [70,80] are usually highly variable between studies. These differences present challenges in data interpretation and designing experiments. In the literature, for example, the length of time animals are exposed to a high fat diet varies from 12 hours to 22 weeks. This is because some studies are interested in short-term effects of a high fat diet, while other studies are interested in the longer effects, such as diet-induced obesity (DIO) effects. In addition, methods used to analyze gene expression (ex. in-situ hybridization vs. RT-PCR) and type of rodent used in the experiment make it difficult to make comparisons between studies. Despite the challenges of interpretation in current literature, there are some interesting trends worth focusing on in future studies.

When studying the development of obesity there are several diet-related factors that have been shown to alter POMC and AgRP mRNA expression. In a study by Wang et al [72], they investigated the effects of altering the type of fat in the diet. They looked at a low-fat diet (10% of calories from fat), and 3 high fat diets of varying fat type (saturated fat, omega-3 or omega-6 polyunsaturated fat). The results of this study revealed that the diet high in saturated fat potently decreased ARC AgRP mRNA expression and this decrease was seen before the detection of significant elevations of circulating leptin levels. Additionally, the confirmation that fat type played a role on determining gene expression in this study was demonstrated when changing the saturated fat group to omega-3 polyunsaturated diet reversed the change in ARC AgRP mRNA expression. This influence of dietary composition on gene expression was also seen in a

study by Torri et al [70]. Sprague-Dawley rats were given two different diets high in fat that also differed in carbohydrate and protein composition. The rats in the first experiment were fed a cafeteria diet (caf) and the rats in the second experiment were fed a high-fat diet (hif). Both the caf diet and the hif diet provided approximately 4-5 times higher fat content than the standard chow diet given to the control animals. The rats in the caf diet and hif diet were sacrificed at 2 months and 1.5 months respectively, which were the first time points where there was a significant weight difference between the experimental rats and control rats. The percent weight difference between groups was not provided in the study. They found that POMC mRNA increased, but only in the rats determined to be obese by the caf diet. There was no difference seen in the non-obese rats exposed to the caf diet or in any of the rats fed the hif diet [70]. Thus, not only did this study confirm the type of fat in expression, but they also found differences in gene expression between obese rats and rats resistant to obesity. The rats that developed obesity had changes in POMC, while the diet-obese resistant rats did not show a change in expression.

Huang et al also looked at difference in gene expression between diet-induced obese (DIO) mice and mice resistant to obesity (DR), but did not look at different fat diets [32]. In this study, 30 mice were randomized and 24 were placed on a high-fat diet (40%) and the remaining 6 were placed on a low-fat diet (10%). After 4 weeks the mice on the high-fat diet were divided based on weight. Six mice with the highest weight were designated as diet-induced obesity (DIO), six mice with the lowest weight were designated as diet resistant (DR). Mice were sacrificed after 22 weeks of feeding. The

results found the DIO mice had a significantly lower level of ARC POMC than the DR and low fat diet mice, but also a lower level of ARC AgRP mRNA expression [32].

Huang et al was one of only two studies that measured MC4R mRNA expression in different sites of the brain. They found that the DIO mice compared to the low fat controls had significant increases in MC4R mRNA in the ventromedial hypothalamic nucleus (VMH), medial amygdaloid nucleus (MePD), but no significant differences were found between any of the groups in the paraventricular nucleus (PVN), posteromedial part of amygdalohippocampal area (AHiPM) or hippocampus (Hi). Although this study did not find a change in PVN MC4R mRNA at 22 weeks [32], Archer et al found an increase in PVN MC4R mRNA after 24 hours on a high fat diet [2]. Archer et al concluded that MC4R gene expression may be required to mount an acute homeostatic response to a moderate change in dietary fat content [2], which they found consistent with another study that measured MC4R binding [12].

POMC and AgRP have also been measured at the initial physiological and neuroendocrine response of obesity-susceptible strains of high-fat feeding. Ziotopoulou et al picked four time points at the onset of a high fat diet, 24 hours, 48 hours, 7 days and 14 days [80]. There was no significant difference in AgRP and POMC mRNA between high fat and low fat groups at 24 hours, but at 48 hours there was a significant reduction in hypothalamic AgRP mRNA with still no change in POMC. At 7 days, AgRP returned to baseline levels, and at 14 days there was a significant increase in POMC mRNA [80]. Although these changes occur at different points in time, their effect would theoretically be to counter the development of positive energy balance.

Another approach to study gene expression changes in the melanocortin system involves looking at changes over the development of obesity. Currently there is only one study that has evaluated the effects of diet-induced obesity over the early, middle and late stages of the development of obesity. Lin et al [44] randomly divided 36 mice into two groups, one on a high-fat diet (58.7%) and one on a low-fat diet (9.7%). Then 6 mice were sacrificed after 1, 8, and 19 weeks of feeding and POMC mRNA expression and other parameters were measured. Unlike the other studies mentioned, Lin et al reported no significant changes in the level of ARC POMC mRNA between the high-fat and low-fat group at weeks 1 and 8. However, at week 19 the high fat group had significantly reduced POMC mRNA [44].

In summary, POMC and AgRP mRNA expressions have been shown to change in response to fat composition in the diet and whether an animal is prone or resistant to obesity. A diet higher in saturated fat has been shown to decrease AgRP mRNA in the ARC, while a cafeteria-type diet has shown to increase POMC mRNA in animals prone to an obese phenotype. mRNA expression also seems to change depending on how long rodents are exposed to a high fat diet. An increase in POMC mRNA has been measured at 2 weeks [80], while later a reduction in POMC mRNA was seen at 19 and 22 weeks [32,44]. Unfortunately, there are only two studies that have measured the effects of a diet-induced obesity on hypothalamic MC4R expression [2,32]. One of the studies found an increase in PVN MC4R expression after 1 day on a high fat die [2], while the other study found no change in PVN MC4R mRNA but an increase in MC4R in other areas of the hypothalamus after 22 weeks of high fat feeding [32]. Thus, changes that may occur in MC4R mRNA in animals fed a high fat diet are still unknown, while many

discrepancies have transpired regarding the change in AgRP and POMC mRNA expression. The main objective of this study is to further assess long-term changes in hypothalamic expression of AgRP, POMC and MC4R mRNA in Sprague Dawley rats fed a high fat diet.

## CHAPTER 3

### MATERIALS AND METHODS

Sixty-six male Sprague-Dawley rats (Harlan, Madison, WI) weighing 250-300 grams were individually housed in conventional hanging cages with a 12 h light/12 h dark photoperiod (lights on at 07.00) in a temperature controlled room (21-22°C). On arrival rats were fed Purina<sup>®</sup> Rodent Chow (Diet 5012; Lab Diet, Purina Mills, MO) ad libitum for the first week for adaptation to new environment. After one week of adaptation, six rats were sacrificed by decapitation for the collection of baseline measurements. The remaining sixty rats were divided into two weight-matched groups, one group being the control and the other being the experimental. The control group was placed on a low-fat diet containing 10% calories from fat, 70% calories from carbohydrate, 20% calories from protein (Diet 12450B; Research Diets Inc., New Brunswick, NJ) and the experimental group received a high-fat diet containing 45% calories from fat, 35% calories from carbohydrate, 20% calories from protein (Diet 12451; Research Diets Inc., New Brunswick, NJ). Due to the higher fat content, the high fat diet contained more calories per gram than the low fat diet (4.7 calories/gram vs. 3.8 calories/gram). Food intake was then measured and recorded at the same time every morning for 12 weeks. Food intake was measured by calculating the amount of food each rat consumed in a 24 hour period, which included a correction for spillage (food intake = total grams of food – leftover grams of food – spillage). Food spillage was collected and weighed from the absorbent paper placed beneath the hanging cages of

each rat. In addition to food intake, body weight was also measured every 48 hours in the morning during the same time food was collected and weighed.

Rats were sacrificed by decapitation using a guillotine in groups of 20 (10 from the control group and 10 from the high fat diet) at three different time points over the course of 12 weeks. The first 20 rats were randomly selected and sacrificed at week 2, the second group of 20 was randomly selected and sacrificed at week 7 and the remaining rats were sacrificed at week 12 (week 1-2, n=30/group; week 3-7, n=20/group; week 8-12, n=10/group). All rats were sacrificed in the morning (between 9am -12pm), after food was removed earlier in the morning. At time of sacrifice, including rats sacrificed at baseline, brain tissue and trunk blood was collected, and the epididymal fat pad on the left side of each animal was removed and weighed. Tail blood was collected at baseline from each rat before being placed on their respective diets and also from rats that were not sacrificed at week 2 and week 7. Serum from trunk and tail blood was taken for measurement of insulin (RIA kit #RI-13K, Linco Research, Missouri) leptin (RIA kit #RL-83K, Linco Research, Missouri), glucose (Glucose Oxidase Set #G7519-500, Pointe Scientific, Inc.) and triglycerides (Lipid Control Set #L7580-18, Pointe Scientific, Inc.).

#### Statistical Analysis

Calorie intake, body weight and fat/body weight ratio were all compared using a two-way ANOVA (treatment x time). Insulin, leptin, glucose and triglyceride levels were also compared using a two-way ANOVA (treatment x time). Differences between specific groups were identified by least square means test (Statistical Software: SuperANOVA).

### Brain Tissue Extraction

Each rat brain was quickly removed after decapitation and placed into ice-cold sterile saline, to firm the tissue for better sectioning. After cooling, the brain was placed ventral side up on the iced positioning stage of a Stoelting Tissue Slicer apparatus. The brain was perpendicular to the blade and positioned to slice through the supraoptic decussation line as a ventral surface landmark (Paxinos), which produced a coronal section. Once the sections of interest were sliced from the brain (2 mm each), the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of the hypothalamus were punched out, placed into RNAase/DNAase free microcentrifuge tubes and immediately frozen in liquid nitrogen for measurement of gene expression.

### RNA Extraction and Analysis

Total RNA was extracted from each brain tissue sample with TRIzol<sup>®</sup> Reagent (Cat. No. 15596-026, Invitrogen<sup>™</sup>) using the protocol adapted from Chomczynski et al [15]. The integrity of the RNA extracted was verified on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc) using the RNA 6000 Nano LabChip<sup>®</sup> Kit and quantitated using RNA 6000 ladder by Ambion (Cat. No. 7152).

### Real time RT-PCR

Due to the expense and newness of the real time RT-PCR method in our lab, this method was only performed on thirty rats in the study, 5 picked randomly from each diet group at week 2, week 7 and week 12. The total RNA from thirty rats was reversed transcribed using the ABI complementary DNA (cDNA) archive kit (ABI No. 4322171). This random primed cDNA synthesis followed the manufacture's protocol using MultiScribe Reverse Transcriptase<sup>™</sup>. Reactions were incubated at 25°C for 10 minutes

and subsequently at 37°C for 120 minutes. The reverse-transcribed cDNA was used as a template for real time-polymerase chain reaction (RT-PCR).

#### *Real Time Polymerase Chain Reaction Analysis*

cDNA from each sample was diluted 1:10 before being used as PCR template. 1 µl of diluted cDNA was used in a 12.5 µl PCR reaction in a 384 well optical plate from ABI. Gene expression quantitation was performed as the second step in the two-step RT-PCR on the Applied Biosystems (ABI) Prism<sup>®</sup> 7900 Sequence Detection System Instrument. Taqman<sup>®</sup> gene expression assays were designed for the detection and quantitation of POMC ( $\alpha$ -MSH), AgRP and MC4R gene sequences (Taqman<sup>®</sup> MGB probes, FAM<sup>™</sup> dye-labeled; Applied Biosystems). All assays were optimized to work with Taqman<sup>®</sup> Universal PCR Master Mix (P/N 4304437). The cycle conditions were set as a preincubation step at 95 °C for 15 minutes for Taq DNA polymerase activation, followed by 40 two-steps cycles of 15 seconds at 95 °C for template denaturation and 1 minute at 60 °C for primer annealing. The ABI 7900HT version 2.2.1 Sequence Detection Systems (SDS) was the software used to analyze data collected from the PCR.

#### *Statistical Analysis for RT-PCR*

Once cycle thresholds (Ct) were calculated, the  $\Delta\Delta$ Ct method was used to determine the relative gene expression level (SDS 2.2.1 software). Relative gene expression levels were corrected for 18S (internal control) and then normalized to mRNA expression of the control group (calibrator), which was considered to be 1. Expression levels for each group levels were compared using a two-way ANOVA. Differences between specific groups were identified by least square means test (Statistical Software: SuperANOVA).

## CHAPTER 4

### RESULTS

#### Calorie intake of all rats in study

The calorie intake from week 1 through week 7 was significantly higher in the high fat diet (HF) group than in the control group (Figure 1:  $p < 0.001$ ; week 1-2,  $n = 30/\text{group}$ ; week 3-7,  $n = 20/\text{group}$ ).

#### Body weight and fat pad/body weight ratio of all rats in study

The body weight of the HF group was significantly higher than the control group at week 5 (mean  $\pm$  S.E.M.: HF =  $385.3 \pm 4.9$  vs. control =  $372.2 \pm 5.4$ ;  $p < 0.05$ ,  $n = 20/\text{group}$ ) and week 7 (mean  $\pm$  S.E.M.: HF =  $422.6 \pm 5.7$  vs. control =  $405.2 \pm 5.6$ ;  $p < 0.008$ ,  $n = 20/\text{group}$ ). There was no significant difference in body weight at any other time points. The epididymal fat pad was divided by total body weight to produce a fat pad (g) to body weight (g) ratio. The ratio was significantly higher in the HF group than in the control group at week 2 (mean  $\pm$  S.E.M.: HF =  $0.54 \pm 0.03$  vs. control =  $0.42 \pm 0.01$ ;  $p < 0.005$ ,  $n = 10/\text{group}$ ), week 7 (mean  $\pm$  S.E.M.: HF =  $0.56 \pm 0.03$  vs. control =  $0.41 \pm 0.03$ ;  $p < 0.0005$ ,  $n = 10/\text{group}$ ) and week 12 (mean  $\pm$  S.E.M.: HF =  $0.58 \pm 0.05$  vs. control =  $0.42 \pm 0.02$ ;  $p < 0.0002$ ,  $n = 10/\text{group}$ ).

#### Serum glucose, insulin, triglyceride and leptin levels of all rats in study

There was no significant difference in glucose or insulin levels between the diet groups at any particular time point, however circulating glucose at week 12 was significantly less than week 2 in both diet groups ( $p < 0.05$ ). Triglyceride significantly

decreased over time in the control group (Figure 2:  $p < 0.002$ ) and in the HF group (Figure 2:  $p < 0.02$ ), and the control group had a significantly greater triglyceride level than the HF group at week 2 (Figure 2:  $p < 0.01$ ,  $n = 30/\text{group}$ ). Leptin was significantly higher in the HF group than in the LF group at week 2 ( $p < 0.03$ ), week 7 ( $p < 0.008$ ) and week 12 ( $p < 0.002$ ) (Figure 2 & 3: week 2,  $n = 30/\text{group}$ ; week 7,  $n = 20/\text{group}$ ; and week 12,  $n = 10/\text{group}$ ).

#### Calorie intake of the sub-group of rats utilized for real time RT-PCR

The calorie intake results from the sub-group of rats utilized for real time RT-PCR were similar to the results from the total group. The calorie intake from week 1 through week 7 was significantly higher in the HF group than in the control group (Figure 4:  $p < 0.001$ ,  $n = 5/\text{group}$ ).

#### Body weight and fat/body weight ratio of rats utilized for real time RT-PCR

While the body weight of the HF group was significantly higher than the control group at week 7 (Figure 5:  $p = 0.05$ ,  $n = 5/\text{group}$ ), no significance was found at week 2 or week 12. The fat pad (g)/body weight (g) ratio was significantly higher in the HF group than in the control group after week 2, week 7 and week 12 of feeding (Figure 6:  $p < 0.001$ ,  $n = 5/\text{group}$ ).

#### Serum triglyceride, glucose, insulin and leptin levels of rats utilized for real time RT-PCR

There were no significant differences in serum triglyceride and glucose in rats utilized for real time RT-PCR. After 2 and 7 weeks of HF feeding, no significant differences were found in insulin or leptin between HF and control groups. However, after 12 weeks of feeding, insulin levels were significantly higher in the HF group than in

the control group (Figure 7,  $p < 0.002$ ,  $n = 5/\text{group}$ ) and leptin levels were significantly high in the HF group than in the control group (Figure 8,  $p < 0.002$ ,  $n = 5/\text{group}$ ).

#### AgRP mRNA expression

ARC AgRP mRNA expression levels were significantly higher in the HF group than in the LF group after 7 weeks of feeding ( $6.425 \pm 1.03$ ,  $p = 0.001$ ) with no significant difference between groups at week 2 or week 12 (Figure 9). No significant difference between the HF group and the LF group were found in the PVN after 2, 7 or 12 weeks of feeding (Table 1).

#### POMC mRNA expression

ARC POMC mRNA expression levels were significantly higher in the HF group than in the LF group after 7 weeks of feeding ( $4.804 \pm 1.06$ ,  $p = 0.013$ ) with no significant difference between groups at week 2 or week 12 (Figure 10). No significant difference between the HF group and the LF group were found in the PVN after 2, 7 or 12 weeks of feeding (Table 1).

#### MC4R mRNA expression

PVN MC4R mRNA expression levels were significantly higher in the HF group than in the LF group after 12 weeks of feeding ( $2.024 \pm 0.22$ ,  $p = 0.038$ ) with no significant difference between groups at week 2 or week 7 (Figure 11). No significant difference between the HF group and the LF group were found in the ARC after 2, 7 or 12 weeks of feeding (Table 2).

Table 1: Effects of a high fat diet on agouti-related protein (AgRP), pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN). Data are relative quantity  $\pm$  S.E.M (n=5/group).

The asterisk indicates a significant difference (p=0.0378).

<b>Time/Diet</b>	<b>AgRP mRNA</b>	<b>POMC mRNA</b>	<b>MC4R mRNA</b>
<b>2 weeks</b>			
High fat	0.407 $\pm$ 0.086	0.797 $\pm$ 0.329	1.472 $\pm$ 0.117
Low fat	1 $\pm$ 0.0	1 $\pm$ 0.0	1 $\pm$ 0.0
<b>7 weeks</b>			
High fat	1.587 $\pm$ 0.285	1.045 $\pm$ 0.108	0.989 $\pm$ 0.206
Low fat	1 $\pm$ 0.0	1 $\pm$ 0.0	1 $\pm$ 0.0
<b>12 weeks</b>			
High fat	1.164 $\pm$ 0.165	1.32 $\pm$ 0.157	<b>2.024 <math>\pm</math> 0.217*</b>
Low fat	1 $\pm$ 0.0	1 $\pm$ 0.0	1 $\pm$ 0.0

Table 2: Effects of a high fat diet on agouti-related protein (AgRP), pro-opiomelanocortin (POMC) and melanocortin 4 receptor (MC4R) mRNA expression in the arcuate nucleus of the hypothalamus (ARC). Data are relative quantity  $\pm$  S.E.M (n=5/group). The asterisks indicate significant difference (AgRP mRNA, p=0.001; POMC mRNA, p=0.013).

Time/Diet	AgRP mRNA	POMC mRNA	MC4R mRNA
<b>2 weeks</b>			
High fat	2.390 $\pm$ 0.186	3.399 $\pm$ 0.263	0.860 $\pm$ 0.078
Low fat	1 + 0.0	1 + 0.0	1 $\pm$ 0.0
<b>7 weeks</b>			
High fat	<b>6.425 <math>\pm</math> 1.027*</b>	<b>4.804 <math>\pm</math> 1.063*</b>	1.624 $\pm$ 0.394
Low fat	1 + 0.0	1 + 0.0	1 $\pm$ 0.0
<b>12 weeks</b>			
High fat	0.515 $\pm$ 0.107	1.018 $\pm$ 0.200	1.141 $\pm$ 0.170
Low fat	1 + 0.0	1 $\pm$ 0.0	1 $\pm$ 0.0

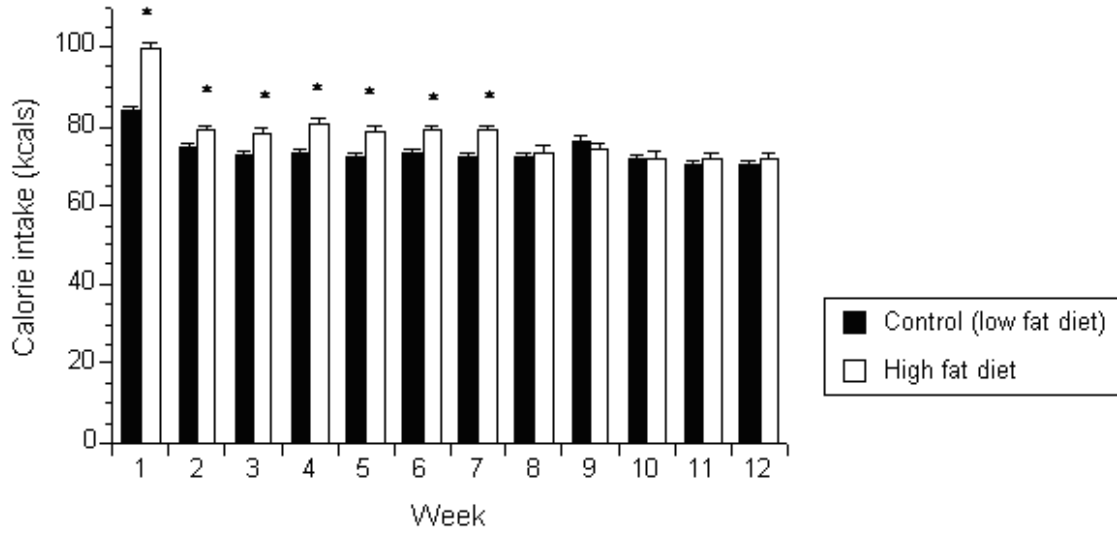


Figure 1: Effect of a high fat diet on calorie intake in male Sprague Dawley rats over a period of 12 weeks. Taking into account the different sample sizes across time, data are means  $\pm$  S.E.M for each week (week 1-2, n=30/group; week 3-7, n=20/group; and week 8-12, n=10/group). Asterisks indicate this significant difference ( $p < 0.001$ ).

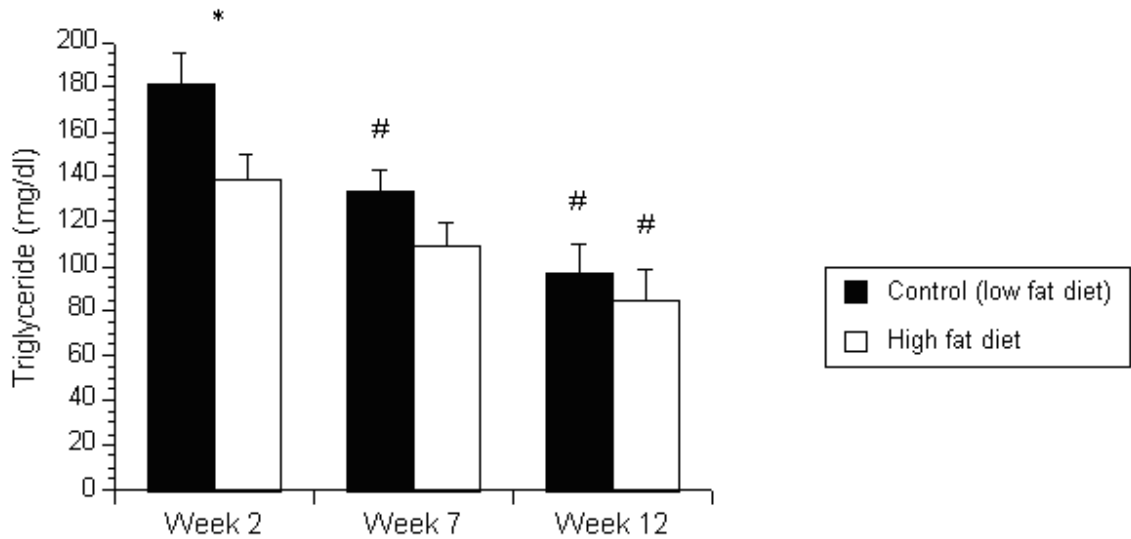


Figure 2: Effect of high fat diet on circulating triglyceride levels in male Sprague Dawley rats over a period of 12 weeks. Taking into account the different sample sizes across time, data are means  $\pm$  S.E.M for each week (week 2, n=30/group; week 7, n=20/group; and week 12, n=10/group). An asterisk indicates a significant difference in triglyceride levels at week 2 between the diet groups ( $p < 0.05$ ). #'s indicate significant decreases in triglyceride levels in the control group at week 7 (control:  $p < 0.006$ ) and in both groups at week 12 (control:  $p < 0.0002$ ; HF:  $p < 0.02$ ) compared with levels at week 2.

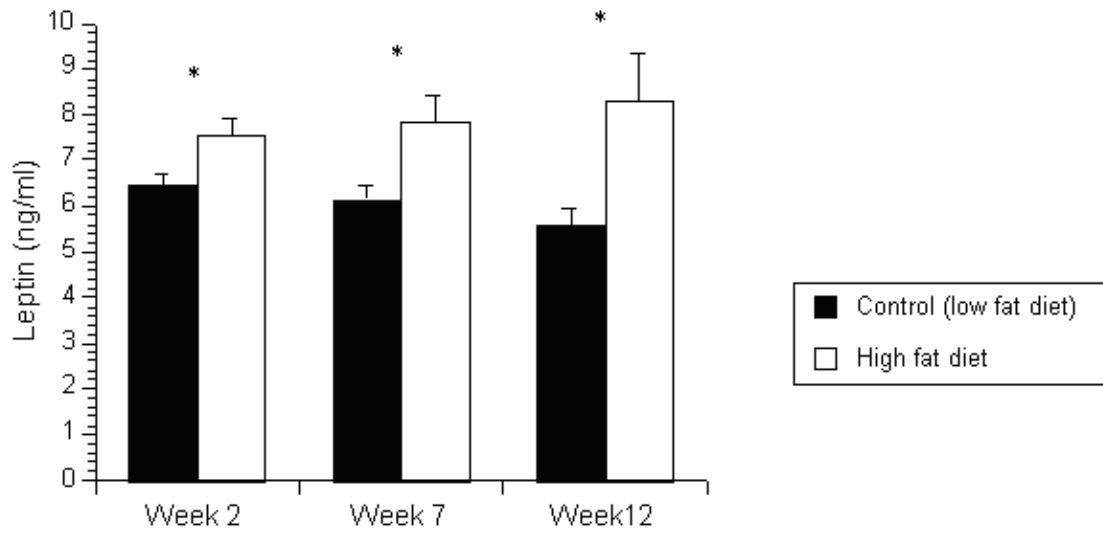


Figure 3: Effect of a high fat diet on circulating leptin levels in male Sprague Dawley rats over a period of 12 weeks. Taking into account the different sample sizes across time, data are means  $\pm$  S.E.M for each week (week 2, n=30/group; week 7, n=20/group; and week 12, n=10/group). Asterisks indicate a significant difference between groups at week 2 ( $p < 0.03$ ), week 7 ( $p < 0.008$ ) and week 12 ( $p < 0.002$ ).

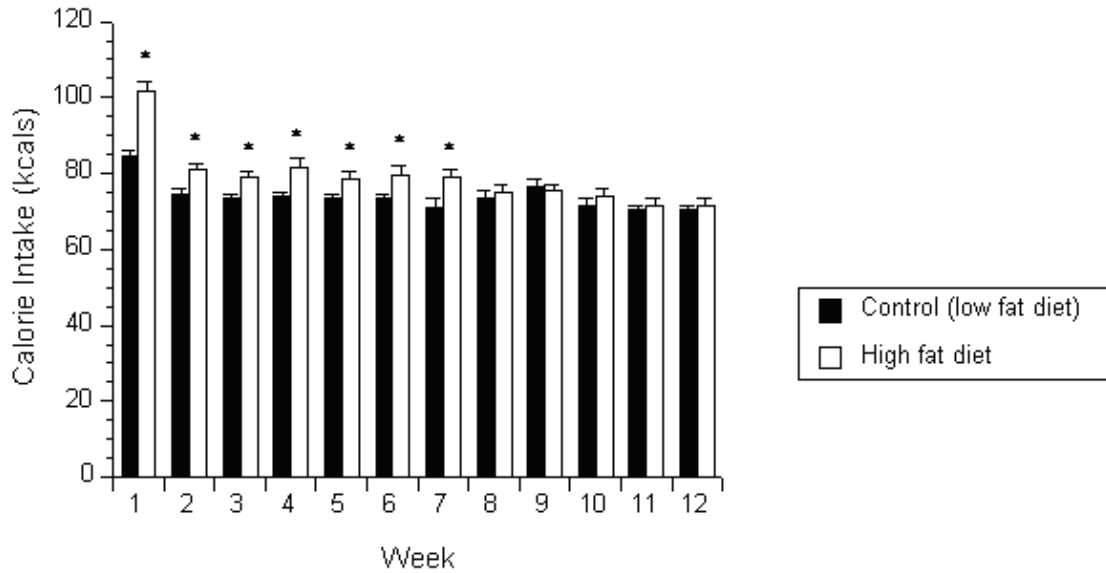


Figure 4: Effect of a high fat diet on calorie intake in a sub-group of male Sprague Dawley rats utilized for real time RT-PCR over a period of 12 weeks. Taking into account the different sample sizes across time, data are means  $\pm$  S.E.M for each week (week 1-2, n=15/group; week 3-7, n=10/group; and week 8-12, n=5/group). Asterisks indicate a significant difference between groups ( $p < 0.001$ ).

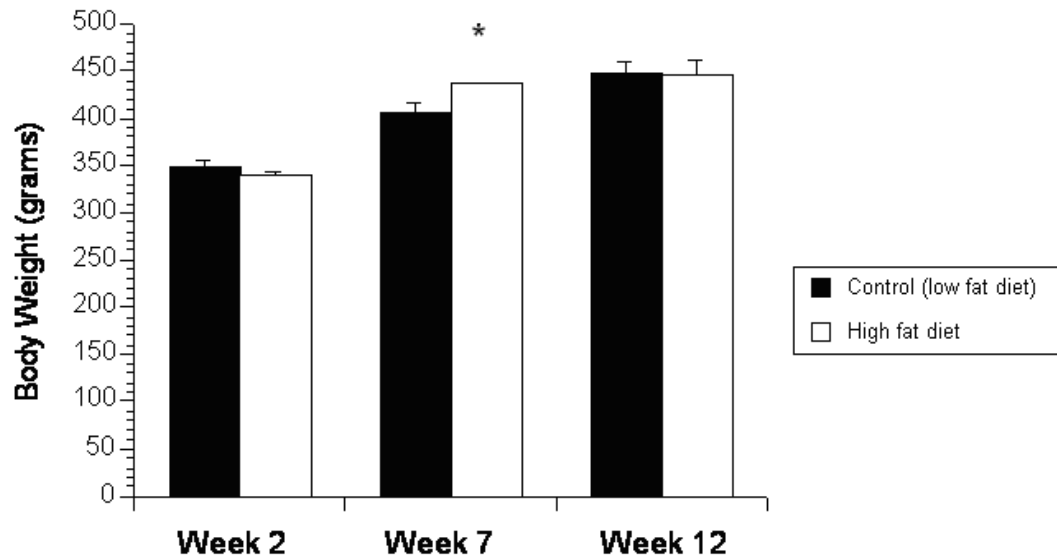


Figure 5: Effect of a high fat diet on body weight in a sub-group of male Sprague Dawley rats utilized for real time RT-PCR over a period of 12 weeks. Data are means  $\pm$  S.E.M for each week (n=5/group). An asterisk indicates a significant difference between the diet groups (p=0.05).

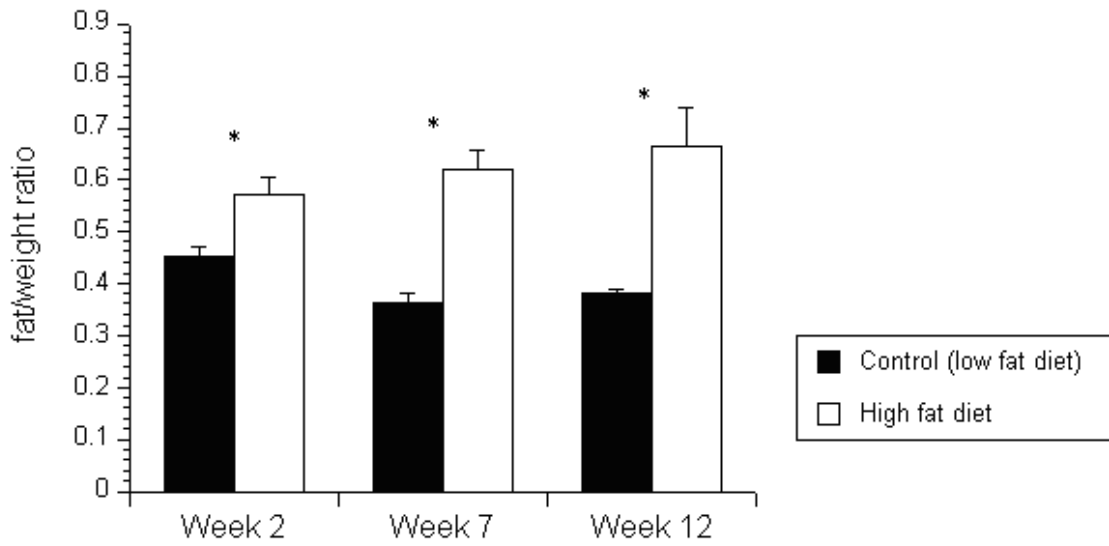


Figure 6: Effect of a high fat diet on fat mass in a sub-group of male Sprague Dawley rats utilized for real time RT-PCR over a period of 12 weeks. Data are means  $\pm$  S.E.M for each week (n=5/group). An asterisk indicates a significant difference between the diet groups ( $p < 0.001$ ).

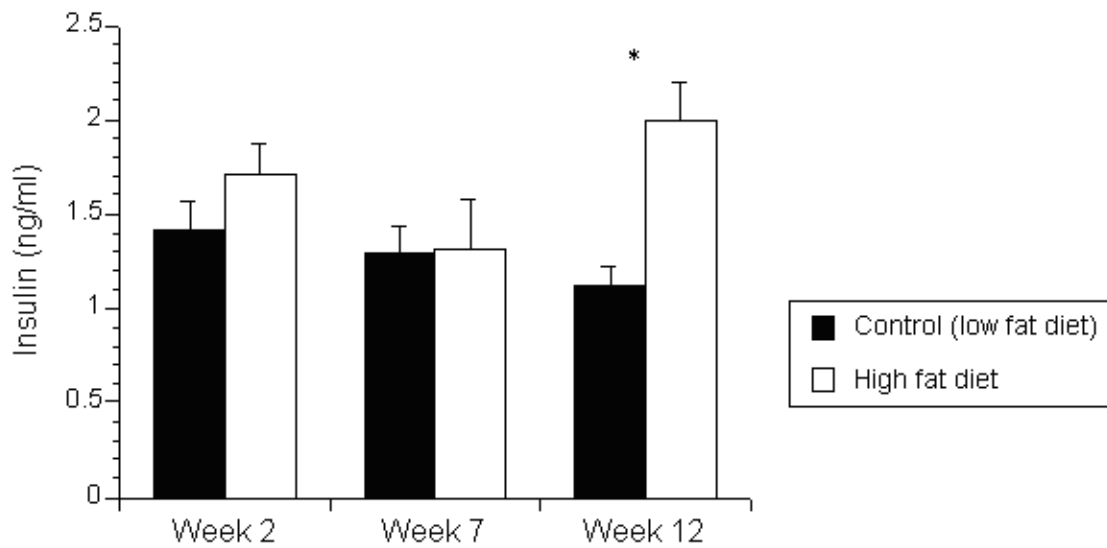


Figure 7: Effect of a high fat diet on circulating insulin levels (ng/ml) in a sub-group of male Sprague Dawley rats that were utilized for real time RT-PCR. Data are means  $\pm$  S.E.M (n=5/group). An asterisk indicates a significant difference between the diet groups (p=0.002).

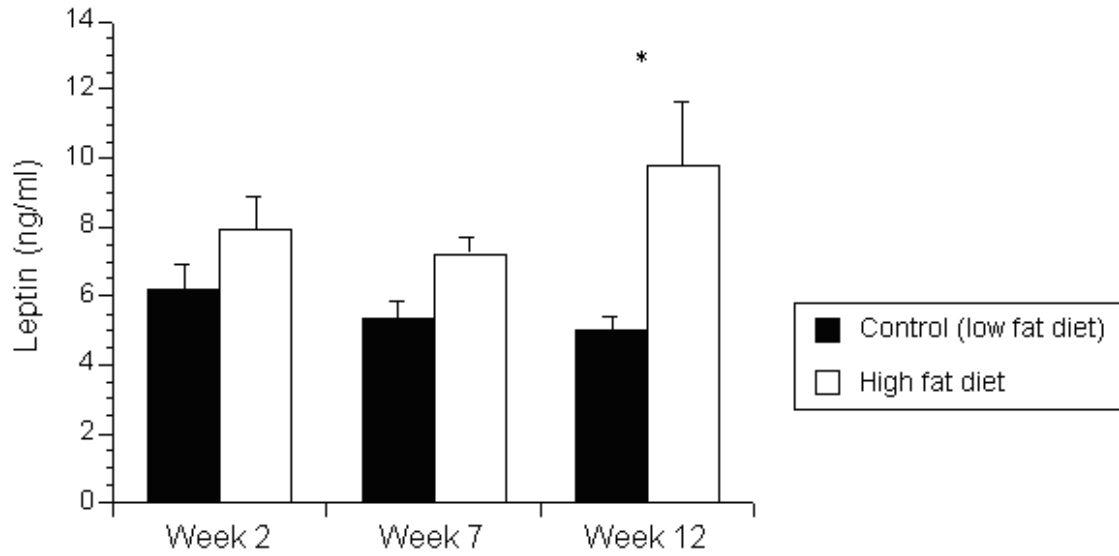


Figure 8: Effect of a high fat diet on circulating leptin levels (ng/ml) in a sub-group of male Sprague Dawley rats that were utilized for real time RT-PCR. Data are means  $\pm$  S.E.M (n=5/group). An asterisk indicates a significant difference between the diet groups ( $p < 0.002$ ).

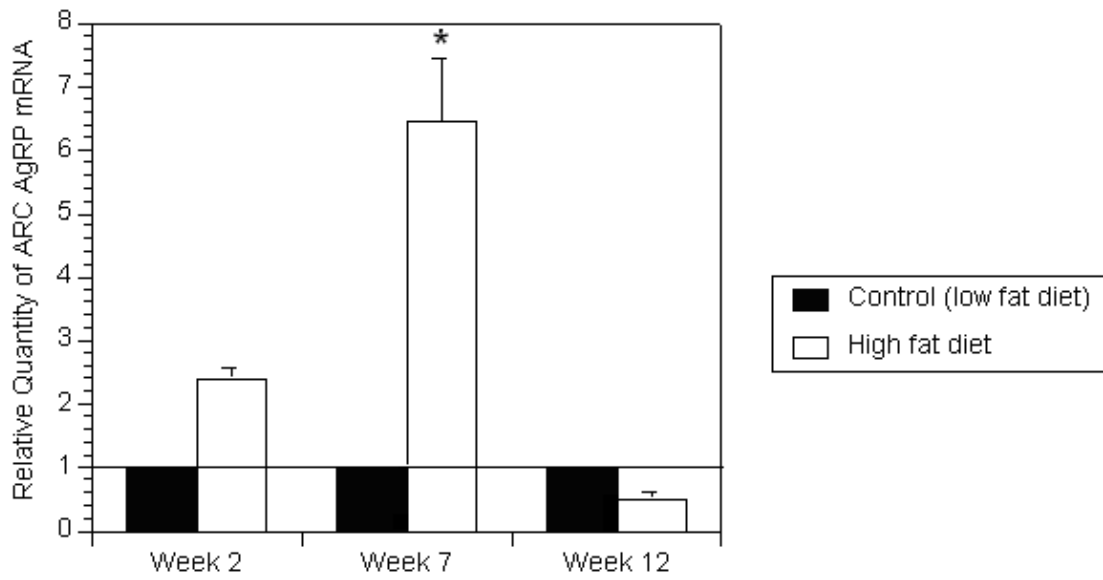


Figure 9: Effects of a high fat diet on agouti-related protein (AgRP) mRNA expression in the arcuate nucleus (ARC) of the hypothalamus in male Sprague Dawley rats (n=5/group). The asterisk indicates a significant difference between the diet groups (p=0.001).

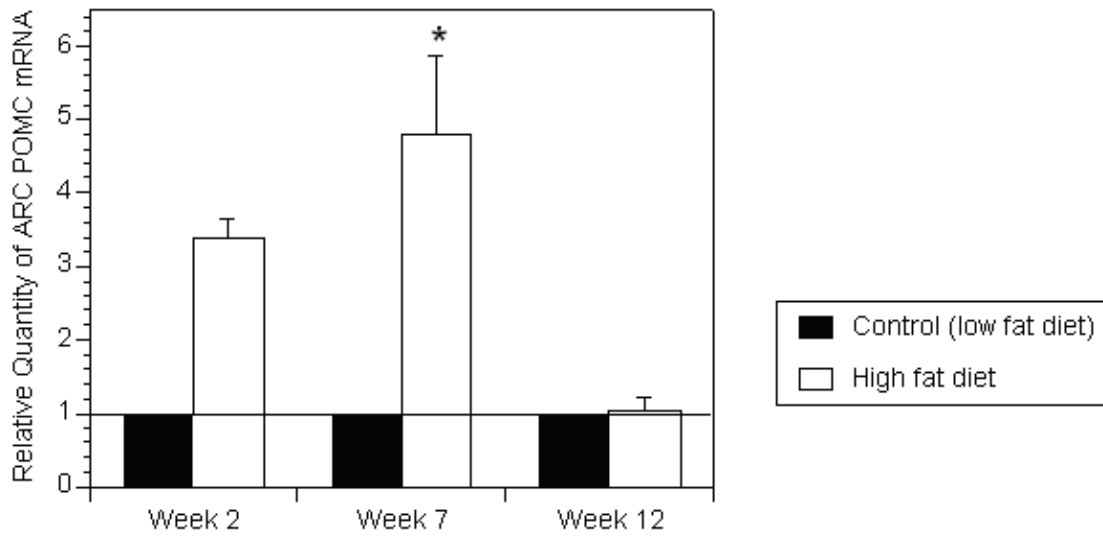


Figure 10: Effects of a high fat diet on pro-opiomelanocortin (POMC) mRNA expression in the arcuate nucleus (ARC) of the hypothalamus in male Sprague Dawley rats (n=5/group). The asterisk indicates a significant difference between the diet groups (p=0.013).

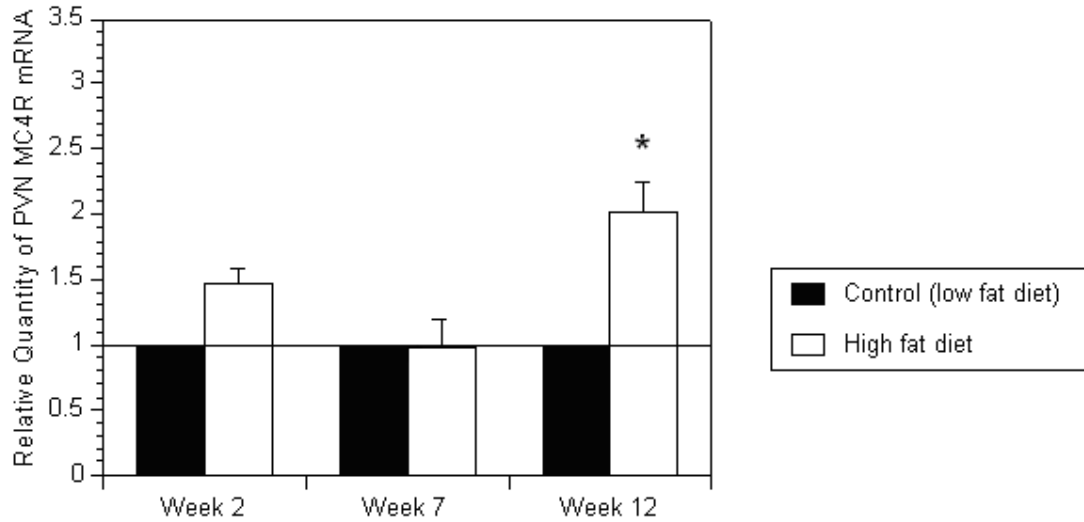


Figure 11: Effects of a high fat diet on expression of melanocortin 4 receptor (MC4R) mRNA in the paraventricular nucleus (PVN) of the hypothalamus in male Sprague Dawley rats (n=5/group). The asterisk indicates a significant difference between the diet groups (p=0.0378).

## CHAPTER 5

### DISCUSSION

The main findings of this study are that a high fat diet significantly increases both ARC AgRP and POMC mRNA expression after 7 weeks of feeding, while PVN MC4R mRNA expression increases after 12 weeks of feeding. This is the first study of a limited collection to report an increase in ARC AgRP and PVN MC4R mRNA expression induced by a high fat diet. While these findings seem to contradict the evidence found in some studies, they actually provide supporting evidence on how diet composition, genetic susceptibility to obesity and minor differences in experimental design can profoundly influence mRNA expression in the hypothalamus.

Diet-induced changes in AgRP and POMC mRNA expression have shown to vary across time and between studies. In this study, the increase of ARC AgRP after 7 weeks in the HF group was accompanied by a state of positive energy balance, which in this case was defined by a significant increase in calorie in relation to the LF control group. Thus, prolonged hyperphagia coupled with positive energy balance seems to be associated with increased ARC AgRP mRNA expression. It is interesting to note that the level of ARC AgRP was drastically reduced between week 7 and 12. This could have been due to a significant increase in circulating insulin and leptin in the HF group than in the LF group, since ARC AgRP expression has been shown to be inversely associated with the level of leptin in the blood. Obesity is commonly characterized by a significant increase in body weight and fat tissue stores, hyperinsulinemia, hyperleptinemia,

hypertriglycemia, and hyperglycemia [77]. The HF group in this study did show a significantly higher fat/body weight ratio than the LF control group at week 2, 7 and 12, but it was not until week 12 that the HF group had significantly higher circulating insulin and leptin levels than the LF control group.

ARC POMC mRNA expression can also be altered by diet composition just as ARC AgRP mRNA expression. An increase in ARC POMC was also reported in a study by Torri et al in rats fed a cafeteria type of fat diet after 2 months, while the same change in ARC POMC was reported after 1.5 months of a different diet composition of fat [70]. In our study, there was a significant increase in ARC POMC found at week 7. Since POMC is the precursor of  $\alpha$ -MSH, a potent inhibitor of food intake, an overexpression of POMC mRNA is thought to aid in defending body weight by resisting the effect of a high fat diet to induce hyperphagia. The mechanism for this increase in expression is unknown, but it has been speculated that increased levels of leptin in HF fed rodents may play a role since leptin receptors are found on neurons that express POMC. Diet resistance is another factor known to influence POMC mRNA expression in rodents fed a high fat diet. Bergen et al found increased levels of POMC mRNA in diet resistant mice after 14 weeks on a HF diet. At that same time point the diet-induced obese mice did not have increased POMC mRNA levels. It is possible that diet resistance may have played a role in the increased level of POMC mRNA in our study, since there was not a significant difference in leptin between diet groups at week 7 and Sprague Dawley rats are known to exhibit diet resistance [41,42].

Unfortunately, the effect of a high fat diet on MC4R mRNA in the hypothalamus is not as well known. In fact, this is one of three studies that have measured MC4R

mRNA in the PVN. In this study, we found an increase in MC4R mRNA in the PVN after 12 weeks of high fat feeding, while the other study found no change in the PVN after 22 weeks of high fat feeding [32]. However, this other study did report an increase in MC4R mRNA in other areas of the hypothalamus not measured in our study. It will be important for future experiments to measure MC4R mRNA in the PVN as well as other areas of the hypothalamus and brainstem during high fat feeding and over the development of obesity.

In summary, the response to high fat feeding in the hypothalamus is complex and changes in expression are influenced by numerous factors that are directly and indirectly associated to a diet high in fat. A high fat diet does change mRNA expression of ARC AgRP, ARC POMC and PVN MC4R over the development of obesity. This change can be directly influenced by the diet composition or indirectly by genetic susceptibility to obesity. Although the change in mRNA expression has been measured over the development of obesity, it is important that future studies address the link between mRNA expression and actual peptide release.

## CHAPTER 6

### CONCLUSION

The high fat diet used in this study increased both ARC AgRP and POMC mRNA expression after 7 weeks of feeding, while PVN MC4R mRNA expression increased after 12 weeks of feeding. No other expression changes were found to be significant. This is the first study of a limited collection to report an increase in ARC AgRP mRNA expression and PVN MC4R mRNA expression induced by a high fat diet. Further research is still required to understand why mRNA expression changes during a state of positive energy balance.

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