THE ROLE OF ENDOCANNABINOID RECEPTOR ACTIVITY IN YOUNG AND AGED RATS WITH HIGH-FAT FEEDING

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

To my patient, loving husband Michael Adam Judge
Adam, you are the icing on my cake.

ACKNOWLEDGMENTS

The research described in this dissertation was made possible through the help and support of University of Florida faculty, fellow graduate students, post-docs, scientists, and other collaborators of the Philip J. Scarpace laboratory. First, I would like to thank Dr. Scarpace for all his guidance and support. He has been integral in my scientific maturation and has provided me the opportunity to explore areas that interest me as well as attend important annual meetings in the field. It has been a pleasure to work with him for the last few years.

Each and every member of the Scarpace lab has contributed to these experiments as well as my development as a scientist. Without Michael Matheny, the lab would be a dull, dismal, quiet place. While giving endless scientific aid, Mike kept everyone's spirits up with singing, yodeling, excellent conversation, the occasional debate, and laughter. On a more personal note, Mike offered me much-needed emotional support when I felt like giving up on an assay or experiment. Jiejin Zhang continuously offered her scientific expertise during trouble-shooting or planning an experiment. I also want to thank her for helping to expand my small world by entertaining my many questions about the Chinese culture. Dr. Yi "Edi" Zhang has provided priceless experimental advice. Her wealth of knowledge in obesity research and science in general always amazes me. Kit-Yan "Thomas" Cheng has been a life-saver for me, weighing rats and helping in as many ways as possible. Thomas, a fellow soccer- and chocolate-lover, never raised the courage to yodel in lab, but I'm sure he practiced at home all the time. In Dr. Lourdes Andino, I found a kindred spirit. We immediately bonded through our love of baking and our experiences as newlyweds. Her advice during experiments and when preparing this dissertation has been greatly appreciated. Dr. Alexandra Shapiro

has provided much support on presentations, posters, and experiments. Simultaneously, she has served as a good example for me on how to successfully balance raising a family and pursuing a career.

I owe a debt of gratitude to many faculty members who gave advice and guidance through my years as a graduate student. As the coordinator of Women in Science meetings, Dr. Nihal Tumer has given me the opportunity to network with other women in the science field as well as hear their concerns and beliefs. Dr. Tumer has also participated in many lab meetings and provided valuable guidance on my experiments. Dr. Kathleen Shiverick, also a member of the Women in Science group, has helped open my eyes to the issues facing females in the scientific field. Dr. Neil Rowland has continually provided an intellectual challenge through his questions, comments, and courses taught. Dr. Christy Carter has an amazing knowledge of animal physiology, especially in aging models. Although I was unable to use it for my dissertation, Dr. Michael King kindly took the time to teach me immunohistochemistry. Dr. Timothy Garrett has provided invaluable resources for attempting to measuring hypothalamic endocannabinoid levels.

Above all else, though, I would like to thank my family and friends for providing a firm foundation of support throughout my life. I thank my husband Adam for being a quiet listener when I had to get something off my chest and helping me put things back into perspective. He has listened to countless PowerPoint presentations, though very little of which he fully understood. Through everything, he has been a shoulder to cry on, a hand to hold, and a best friend to laugh with. I want to thank my parents, Bob and Sue, for their unconditional support and love throughout my life. They have always been my biggest (and loudest) fans at sporting events, formal ceremonies, and in every-

day life. My sister, Robin, a member of People for the Ethical Treatment of Animals (PETA), also deserves a special note of gratitude as it was particularly hard for her to know I worked with rodents. We had an unspoken agreement that I would not tell her about my experiments and she would not bombard me with PETA propaganda. However, it was hard to resist not throwing a couple of teasing comments her way every once in a while. I also thank my grandparents – both paternal and maternal. They were always interested in how my research was progressing and how many rats I had to feed every day. Throughout my life, my grandparents have supported every decision I made and believed I could accomplish anything I set my mind to.

Lastly, I thank the members of my “Girls’ Night” group for helping me escape from the world of science every once in a while. Even though the conversation often turned back to science during our outings, it was nice to vent out frustrations and celebrate accomplishments with a good group of friends.

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2-AG	2-arachidonoylglycerol
AEA	andandamide; N-arachidonylethanolamine
AgRP	agouti-related protein
Arc	Arcuate nucleus
BAT	brown adipose tissue
BBB	blood brain barrier
BMI	body mass index
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CART	cocaine- and amphetamine-regulated transcript
CB1	cannabinoid receptor-1
CB2	cannabinoid receptor-2
CRH	corticotropin-related hormone
CSF	cerebrospinal fluid
DIO	diet-induced obese
DMH	dorsomedial hypothalamus
DR	diet resistant
EC	endocannabinoid
ECS	Endocannabinoid System
ERK	extracellular signal-related kinase
EWAT	epididymal white adipose tissue
FAAH	fatty acid amide hydrolase
F344xBN	Fischer344xBrown Norway

GABA	γ-aminobutyric acid
GI	gastrointestinal
HF	high-fat
i.p.	intraperitoneal
JAK	janus kinase
LCD	liquid crystal display
LHA	lateral hypothalamic area
MAPK	mitogen-activated protein kinase
MGL	monoglycerol lipase
MSH	melanocyte stimulating hormone
NPY	neuropeptide Y
PDE3B	phosphodiesterase 3B
PFA	perifornical area
PI3K	phosphatidylinositol 3-kinase
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
PWAT	perirenal white adipose tissue
RTWAT	retroperitoneal white adipose tissue
SOCS3	suppressor of cytokine signaling 3
STAT3	signal transducer and activator of transcription 3
TD-NMR	time domain nuclear magnetic resonance
THC	(-)-Δ9-tetrahydrocannabinol
UCP-1	uncoupling protein
VMH	ventromedial hypohtalamus
WAT	white adipose tissue

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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By

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December 2009

Chair: Philip J. Scarpace
Major: Medical Sciences – Physiology and Pharmacology

Two-thirds of adult Americans are overweight or obese, which increases the risk of developing other serious diseases. Leptin is a hormone produced in adipose tissue that acts within the hypothalamus to increase energy expenditure and decrease food intake. In contrast, endocannabinoids are produced on-demand and act on central cannabinoid-1 (CB1) receptors to stimulate food intake and fat storage. This dissertation examined leptin and CB1 antagonist responses in both young adult and aged rats with or without high-fat (HF) feeding in order to better understand the dysregulation of these two signaling systems in the aged and/or obese state.

First, we demonstrated that all aged rats, as opposed to only some young adult rats, are susceptible to the detrimental effects of a HF diet. When given ad libitum access to a HF diet, aged rats display exacerbated hyperphagia and body weight gain, characterized by a disproportionate gain in fat versus lean mass. Additionally, we demonstrated that young adult rats display dose-dependent reductions in food intake and body weight in response to peripheral leptin infusions while aged rats remain completely unresponsive to the exogenous leptin.

Next, we showed that daily i.p. administration of AM251, a CB1 antagonist, reduced the intake of the highly palatable HF diet to a greater extent than normal chow during short-term exposure do the diets. AM251 stimulated greater anorectic responses, characterized by decreases in caloric intake and body weight gain, in aged rats, and this effect was further enhanced with short-term HF feeding. In accordance with the decrease in body weight, AM251 treatment induced a reduction in adiposity and serum leptin levels in young adult and aged rats. However, AM251 was unable to change the palatability or preference of the diets tested.

After characterizing AM251 responsiveness during short-term diet exposure, we investigated responsiveness after long-term exposure. Again, AM251 induced greater anorectic effects with age and HF-feeding, measured by increased sensitivity and maximal efficacy. These results appeared to be related to the diet and established obesity as well as the development of leptin resistance. Further studies are needed to confirm the connections between leptin resistance and the dysregulation of the endocannabinoid signaling system that is believed to occur in aged and/or obese states.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

The Obesity Epidemic

Overweight and obesity are defined as the accumulation of fat that may cause impaired health (4). Physicians often use body mass index (BMI), measured by a person's weight in kilograms divided by his/her square of the height in meters, to measure an individual's adiposity level, where a BM1 of 25-29.9 is considered overweight and a BMI of 30 or higher is obese (4). According to the World Health Organization (WHO), approximately 1.6 billion adults were overweight and at least 400 million adults were obese in 2005. These figures are expected to further increase to 2.3 billion overweight and 700 million obese by 2015. At least 20 million children less than 5 years old were overweight in 2005, and these numbers are expected to increase as well (164). In addition, some believe that the current economic recession may exacerbate these epidemic trends because when finances are tight, people often choose less nutritional, calorically dense fast foods over healthy supermarket purchases and are forced to give up memberships at gyms and sports clubs (95).

Health Consequences Associated with Obesity

Obese individuals suffer both emotional, caused by social prejudices, and physical consequences (4). In fact, obesity is likely caused by multiple factors like diet, genes, and psychological factors (122). Many diseases are believed to be secondary to the establishment of obesity, including type 2 diabetes, uterine cancer, gallbladder disease, osteoarthritis, stroke, hypertension, coronary heart disease, breast cancer, and colon cancer (4).

Whole Body Energy Homeostasis

Obesity results from an imbalance between energy intake and energy expenditure. A positive energy balance is characterized by a change in energy intake without a compensatory and parallel change in energy expenditure. For example, it can be caused by an increase in energy intake without a corresponding increase in energy expenditure. Similarly, a positive energy balance can also be caused by a decrease in energy expenditure, as with aging, without a corresponding decrease in energy intake. Moreover, a prolonged positive energy balance may result in increased body weight gain and adiposity.

HF Diets

The ever-spreading obesity epidemic is often attributed to lifestyle changes, especially in Western societies (129). The typical Western diet is often characterized by expanding portion sizes and relatively inexpensive foods high in fat and sugar (129). When provided a highly palatable HF diet, rats display diet-induced hyperphagia, which increases caloric intake and promotes body weight gain (41). Moreover, chronic HF feeding can alter the hormonal signaling involved in regulating energy homeostais, whether the rats become obese or not (50, 172).

Complex Mechanisms for Energy Homeostasis

Under homeostatic conditions, energy intake is metabolized to fulfill the body's fuel needs, and excess energy is stored as fat for later use (88). Thus, the body requires a signaling system to assess the nutritional state of the animal and adjust energy intake/output as necessary, including the inhibition of food intake when fuel requirements are met or the increase energy expenditure with excess food consumption. This system is often called the homeostatic regulation of body weight and

involves interacting signals from both the central nervous system (CNS) and peripheral organ systems (88). Nutrient sensing begins with oral taste receptors and continues in the gastrointestinal (GI) tract, pancreas, liver, muscle, and adipose tissue, which all involve crucial communication systems with the brain. For example, ghrelin is secreted in the mucosa of an empty stomach in preparation of food intake, but its secretion is abruptly inhibited after ingestion (88). In contrast, pancreatic insulin secretion is increased after a meal, and the adipocyte-produced leptin, which is released as a signal of the body's adiposity level, acts in the brain to reduce energy intake (156). White adipose tissue (WAT) has also become widely accepted as an important endocrine organ, secreting adipokines like adiponectin, resistin, adipsin, visfatin, and leptin. Studies in rodents have shown that peripheral administration of adiponectin doesn't alter food intake, but decreases body weight by increasing energy expenditure (123). Although conflicting evidence exists as to whether resistin levels increase, decrease, or remain the same in obesity and type II diabetes, stimulation of macrophages with an endotoxin stimulates resistin production and release, suggesting that resistin is a key mediator in the insulin resistance associated with certain inflammatory conditions (45). Adipsin, which is primarily expressed by adipocytes in mice and monocytes-macrophages in humans, is decreased in murine models of obesity and increased or unchanged in human obesity (45). Visfatin, the most recently discovered adipokine, is primarily produced in visceral white adipose tissue and binds to the insulin receptor to produce insulin-like effects (45). However, since its discovery in 1994, leptin has been the most actively investigated adipokine.

Leptin

Role of Leptin in Energy Regulation

Leptin, a 16 kD hormone, is produced by the *obese* (ob) gene and is named for the Greek word *leptos*, meaning thin (53). It is primarily produced in adipose tissue and circulates in proportion to whole body adiposity, but it is also synthesized in placenta, gastric fundic mucosa, skeletal muscle, and mammary epithelium (2). Human and rat leptin are 83% homologous (2). Leptin appears to be involved in both short- and long-term regulation of energy homeostasis. In rodents, leptin levels increase within hours after ingestion, and in humans, leptin levels are increased days after overeating. However, leptin levels normalize hours after fasting begins in both species (2). In addition, leptin is rapidly depleted from the stomach of rodents after a meal, suggesting some activity in the short-term regulation of food intake (53).

Congenital Leptin Deficiency

Mice with homozygous mutations in the *ob* gene are characterized by early onset obesity. These ob/ob mice lack leptin, causing them to be hyperphagic, hypothermic, and obese in addition to other metabolic and neuroendocrine abnormalities (2). While congenital leptin deficiencies in humans are rare, a few cases have been reported. Two Pakistani cousins, who were severely obese at an early age although the family history included no obesity, were found to be homozygous for a frame-shift mutation that resulted in the deletion of a guanine nucleotide in codon 133 and produced a premature stop codon in the leptin message. As a result, the truncated leptin protein was not properly secreted, and the children had undetectable serum leptin levels (105). Another mutation, involving a single nucleotide substitution in codon 105 of the leptin gene, was found in a Turkish family. The affected individuals were characterized by obesity,

hyperphagia, hypogonadism and low serum leptin levels (148). Daily subcutaneous administration of leptin in these individuals caused a dramatic reduction in body weight, 98% of which was adipose tissue, attenuation of hyperphagia, and a steady reduction in the levels of plasma insulin, triglycerides, and serum cholesterol (47).

Other Physiological Functions of Leptin

In addition to its energy homeostasis roles, leptin is an active participant in many other physiological functions, including reproduction, bone growth, metabolism, immunity, angiogenesis, and blood pressure regulation. Mutations in the ob and db genes in rodents and humans result in hypogonadism, but administration of leptin restores puberty and fertility (23, 47). Leptin deficient ob/ob mice have various skeletal bone abnormalities, including decreased bone length and increased spongy bone mass, when compared to wild-type controls, but this condition is rectified when leptin is administered to these ob/ob mice (78). Leptin decreases both glucose and insulin levels in ob/ob mice before weight loss occurs and stimulates lipolysis and fatty acid synthesis in the liver (3, 26). It has recently been shown to have many actions related to immune responses, including direct actions on T-cells, a regulatory action on natural killer cells, and induction of cytokines in macrophages (114). Leptin stimulates angiogenesis and has been found in the placenta, a highly angiogenic tissue (124). Regional sympathetic outflow is stimulated by endogenous leptin, even in the obese state, but short-term subcutaneous infusions of low doses of leptin have been shown to significantly reduce blood pressure in rats (82).

Leptin Receptor

Isoforms

The leptin receptor, originally cloned in 1995, is a single membrane-spanning receptor, a member of the class I cytokine receptor family, and has multiple isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf) (53, 149, 150). These isoforms result from alternative RNA splicing at the most C-terminal coding exon, meaning that all isoforms are identical throughout the entire extracellular portion and the differences in isoforms arise from differing lengths and sequencing compositions in the intracellular portions (149). The long-form of the receptor, Ob-Rb, has an intracellular domain of 303 amino acids and is capable of intracellular signal-transduction (149). This isoform is expressed predominantly in the hypothalamus, but lower levels can be found in other brain regions, testes, and adipose tissue (86, 149). The short-form of the receptor, Ob-Ra, which contains only a small portion of the intracellular domain, has been implicated in participating in transporting leptin from the blood into the cerebrospinal fluid (CSF) (149). Ob-Re, the soluble leptin receptor, contains no intracellular portion and thus circulates in the bloodstream (86). Expression of Ob-Re has been located in the hypothalamus, testes, heart, and adipose tissue (86).

Leptin Expression in the Brain

Leptin receptors are expressed throughout the brain, but patterns of expression appear to be isoforms-specific. The short form of the receptor has been localized to the choroid plexus and moreover, the microvessels, which make up the blood brain barrier (BBB) (11). This evidence supports the possibility that these Ob-Ra receptors function, at least partly, in transporting leptin across the BBB through a high-affinity, saturable transport system (178). The long form of the receptor is primarily located in the

hypothalamus and cerebellum. In fact, cerebellar expression, which is believed to be involved in somatic motor activity, muscle tone regulation, and equilibrium maintenance, surpasses expression in all other brain regions (11). Within the hypothalamus, the highest concentrations of the leptin receptor are found in the arcuate nucleus (Arc), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), and ventral premamillary nucleus (43, 90). These nuclei are all associated with feeding centers in the brain, providing further evidence for leptin action in the hypothalamus. The leptin receptor is only moderately expressed in the paraventricular nucleus (PVN) but highly expressed in brain regions that project to the PVN (i.e. DMH, VMH, and Arc), suggesting that leptin activation of the PVN is both direct and through innervation (43).

Leptin-Leptin Receptor Binding

All of the alternative splicing products of the leptin receptor have the same extracellular and transmembrane domains, but the mechanism of leptin binding to its receptor is still largely unknown (13). The extracellular portion of the receptor is made up of 820 amino acids and contains two immunoglobulin-cytokine receptor homologous regions (CRH) separated by an Ig-like domain and followed by two fibronectin III-like domains (34, 118). The CRH domain closest to the membrane has been identified as the high affinity binding site on the receptor (52, 118). The Ig-like and two fibronectin III-like domains are not integral for leptin binding, but are required for leptin receptor activation (52, 118). Experiments based on quantitative bioluminescence resonance energy transfer (BRET) suggest that two leptin molecules bind to a pre-existing receptor dimer in a 2:2 ratio to induce a receptor conformational change and stimulate downstream signaling. In contrast, the short form of the receptor primarily exist as monomers (31). This finding was supported by Devos, et al., who further described the

leptin receptors as homodimers, rather than heterodimer complexes where the different isoforms interacted (34).

Intracellular Domain of the Leptin Receptor

The isoforms of the leptin receptor have a long (Ob-Rb), short (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-RF) or no (Ob-Re) intracellular domain (86). The membrane-bound isoforms share a common 29 amino acid sequence called “Box 1,” which is also highly conserved among other members of the cytokine receptor family (13). The second motif, called “Box 2,” is usually located within the first 50-60 amino acids in the cytoplasmic domain of cytokine family receptor members, and is only found in the Ob-Rb leptin receptor isoforms (13). These motifs are necessary for the intracellular interaction of the leptin receptor with tyrosine kinases. Because all other forms of the leptin receptor, aside from Ob-Rb, do not contain both of these motifs, they are signaling inactive and are considered incapable of mediating downstream signaling (13, 31, 86).

Leptin Receptor Deficiency

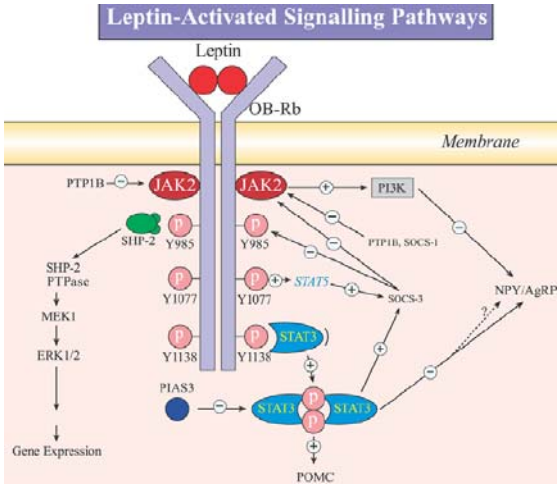
Leptin receptor mutations result in a phenotype characterized by obesity, hyperphagia, hypercholesterolemia, hyperlipidemia, and hyperglycemia (48, 119). In C57BL/K db/db mice, the mutation is caused by a single nucleotide substitution, which introduced a premature stop codon in the cytoplasmic region. This causes a replacement of Ob-Rb with Ob-Ra in these rodents (86). In Zucker fatty (fa/fa) rats, a missense mutation causes a Glutamine to be replaced with a Proline on codon 269 of the extracellular domain (69). Surprisingly, these rats are still capable of binding leptin, suggesting that the mutation does not allow for proper dimerization of the leptin receptors, thus inhibiting intracellular downstream signaling (119). However, mutations

in the leptin receptor are extremely rare in humans. Clement, et al. studied a family with a strong prevalence of morbid obesity and found a single nucleotide substitution on exon 16. Both parents and 4 children were heterozygous for this allele, two additional unaffected children were homozygous for the wild type allele, and the remaining 3 children were homozygous for the mutation. The heterozygous children displayed normal BMIs, eating behavior, growth patterns, and sexual maturation, suggesting that one functional copy of the leptin receptor gene seems to be enough for normal physiology (25).

Leptin Receptor Signal Transduction

The leptin receptor is similar to all other members of the class 1 cytokine receptor family in that it has no intrinsic kinase activity. Instead, it is dependent on cytoplasmic-associated Janus kinases (JAKs) for intracellular signaling to occur (173). Leptin binding to the long form of the leptin receptor stimulates activation of JAK2 by cross-phosphorylation, which then phosphorylates specific tyrosine residues (Tyr 985, 1077, and 1138) on the intracellular domain of the receptor (173). Leptin receptor activation stimulates many different intracellular signaling pathways.

Leptin Intracellular Signaling Cascade:



JAK2-STAT3 Pathway

Tyrosine 1138 phosphorylation recruits signal transducer and activator of transcription 3 (STAT3) and acts as a docking site, where the STAT3 molecules are phosphorylated by JAK2. Then the activated STAT3 proteins dimerize and translocate to the nucleus where they bind to DNA to regulate gene transcription (175). This JAK2-STAT3 pathway is crucial for leptin regulation of energy homeostasis. Bates, et al. created mice with a homozygous substitution of a serine residue at the Tyr1138 site. These mice (s/s) were hyperphagic and obese. Physiologically, the *s/s* mice were very similar to *db/db* mice except the *db/db* mice were infertile, short, and diabetic while the *s/s* mice were fertile, long, and less hyperglycaemic (7). This suggests that while the regulation of feeding behavior is dependent on this pathway, reproduction, fertility, and glucose homeostasis are regulated via another pathway.

ERK Pathway

Phosphorylation on Tyr985 recruits the tyrosine phosphatase SHP-2, which is then phospohorylated and activates the extracellular signal-related kinase (ERK) signaling pathway (163). The ERK pathway entails a set of serine/threonine kinases that are involved in cellular physiology and gene transcription regulation (6). Phosphorylation at Tyr985 also increases the levels of c-fos, which may be responsible for activation of neurons in the arcuate nucleus, and suppressor of cytokine signaling 3 (SOCS3), which acts as a negative regulator of leptin receptor-mediated signaling (6). Mice with a homologous leucine substitution at Tyr985, which blocks SHP2/SOCS3 recruitment, have lower energy intake levels, lower adiposity, and increased sensitivity to leptin. These results, in addition to those obtained from cultured cells, confirm a role of Tyr985 phosphorylation in the inhibition of leptin receptor signaling (14).

PI3K-cAMP Pathway

Another leptin signaling pathway is mediated by JAK2 phosphorylation, independent of leptin receptor intracellular tyrosine phosphorylation. JAK2 phosphorylation leads to the phosphorylation of the insulin receptor substrate (IRS) protein. This recruits phosphatidylinositol 3-kinase (PI3K), which activates phosphodiesterase 3B (PDE3B) and decreases cyclic adenosine monophosphate (cAMP) levels (68). Central administration of a PDE3B inhibitor or cAMP blocks leptin's anorectic effects and stimulates feeding, respectively (176). These experiments suggest that the PI3K-PDE3B-cAMP pathway is integral for leptin's hypothalamic control of energy balance.

Tyr1077-STAT5 Pathway

Of the three intracellular tyrosines phosphorylated on the leptin receptor, the least is known about Tyr1077. However, recent evidence shows that phosphorylation at this site is necessary for phosphorylation and transcriptional activation of STAT5 (55, 61). While STAT5 signaling is used by many growth factors and cytokines, it may also play an important role in leptin-mediated energy homeostasis. Mice with whole-body knockout of the STAT5 protein display many severe phenotypes due to the versatility of this signaling protein, but mice with only a central nervous system knockout of STAT5 are relatively normal besides a case of moderate obesity and elevated leptin levels (2, 12).

Negative Regulators

SOCS3. Of the many negative regulators of the leptin receptor-signaling cascade, SOCS3 activity is the most studied and characterized. SOCS3, which contains an SH2 domain, inhibits specific signal transduction pathways, either by targeting the complex

for degradation or by direct inhibition of JAK2 activity (12). SOCS3 is both induced by STAT3 signaling and inhibits it by binding to Tyr985 via its SH2 domain (107). SOCS3 knockout mice displayed greater anorectic responses to exogenously administered leptin. In addition, when these knockout mice were challenged with a HF diet, they consumed less food and gained less weight than wild-type controls (177).

PTP1B. Another negative regulator of leptin signaling is protein tyrosine phosphatase 1B (PTP1B), which binds directly to and dephosphorylates JAK2. Mice without PTP1B display increased leptin sensitivity, increased energy expenditure, and resistance to both HF diet-induced weight gain and increasing triglyceride levels (88).

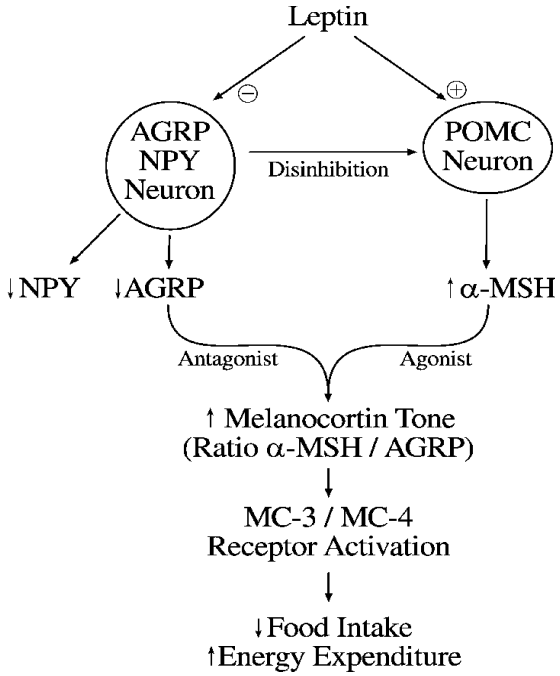
Downstream Leptin Signaling & Neuropeptide Regulation in the Hypothalamus

Within the hypothalamus, the arcuate nucleus (ARC) is believed to be the primary integration site of various peripheral and central nutritional signals (2, 88, 156). The long form of the leptin receptor is coexpressed with two subpopulations of neurons in the arcuate nucleus (88). The first subpopulation of neurons releases the orexigenic (appetite-stimulating) peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) (88). Leptin acts to inhibit the synthesis and release of these peptides (50). Ablation of the AgRP neurons in adult mice causes severe self-starvation and death (53). Ob/ob mice have increased NPY RNA, but the obesity and other detrimental physiological characteristics of ob/ob mice are improved with NPY knockout (88).

The second subpopulation of neurons in the arcuate nucleus coexpresses the anorexigenic (appetite-suppressing) peptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (121). Leptin acts on these neurons to increase the synthesis of these peptides. The POMC peptide is further processed to create adrenocorticotrophin, β-endorphin, and α-, β-, and γ-melanocyte-

stimulating hormones (MSH). The POMC-derived peptides activate the melanocortin receptors to produce a wide range of biological responses, including roles in skin pigmentation, adrenal steroidogenesis, thermoregulation, and appetite regulation (167). The most important of these peptides with respect to obesity is α-MSH. Specifically, α-MSH binds to downstream melanocortin-3 and -4 receptors to decrease food intake (50). Melanocortin-4 receptor knockout mice are obese and resemble leptin deficient mice (88).

Leptin-Mediated Neuropeptide Regulation in the Hypothalamus:



The NPY/AgRP and POMC/CART respond to circulating leptin, glucose, fatty acids, and amino acids, and their output signals on food intake interact both directly and on downstream neurons (88). NPY neurons can directly inhibit POMC neurons through local axon collaterals. However, the POMC neurons lack a reciprocal inhibitory axon to NPY, which suggests that appetite stimulation may be the fail-safe default action (16, 35, 88).

Secondly, the neurons have overlapping projections to second-order neurons located in the paraventricular nucleus (PVN), zona incerta, perifornical area (PFA), dorsomedial nuclei (DMH), and the lateral hypothalamic area (LHA). Each of these areas has been implicated in regulating feeding (133).

Brown Adipose Tissue (BAT) and Uncoupling Protein 1 (UCP1)

Whole body energy expenditure is comprised of obligatory expenditure, which is required for normal cellular function, physical activity, and adaptive thermogenesis. It entails the conversion of substrates (i.e. food, stored fat, stored protein, or stored glycogen) and oxygen to carbon dioxide, water, and energy (i.e. heat or work). Leptin-containing hypothalamic nuclei have sympathetic neural connections to BAT, white adipose tissue (WAT), and muscle. Lipolysis in these tissues frees fatty acids to act, in turn, as substrates in BAT and muscle for adaptive thermogenesis, which is one mechanism for regulating body temperature and weight after cold exposure and hyperphagia, respectively (171). BAT contains uncoupling protein-1 (UCP1) that is expressed in the intermitochondrial membrane and act as proton transporters, allowing protons to leak across the membrane and leading to a loss of the electrochemical gradient the mitochondria usually use to create adenosine triphosphate (ATP) (171). This UCP1-mediated uncoupling of cellular respiration from ATP formation produces heat (171). The BAT receives sympathetic simulation from brain regions that act on β-adrenergic receptors on surface of the BAT cells increasing cAMP levels in BAT. The increased cAMP levels, through a complex mechanism apparently involving free fatty acids, activate UCP1 to produce heat (109).

Leptin Resistance

Leptin Treatments in Human Obesity

With the discovery of leptin in 1994, scientists and physicians had high hopes that exogenously administered leptin would induce satiety and weight loss in obese humans. Indeed, leptin treatment improved adiposity levels and satiety in leptin deficient rodents and humans (109). In addition, leptin treatment was efficacious in humans with lipodystrophy and eating disorders, conditions characterized by hyperphagia, low body fat content, and low leptin levels (109). However, these conditions represent only a small percentage of the population. In contrast, the majority of obese humans have elevated leptin levels and a condition known as leptin resistance, where they are not responsive to endogenous or exogenously administered leptin (42, 136, 137).

Mechanisms

The underlying mechanisms of leptin resistance are still under debate, but it is believed to be multifactorial. Two of the dominant hypotheses are the inability of leptin to reach leptin receptors in the brain because of its limited transport across the blood brain barrier (BBB) and impaired central leptin signal transduction. Central and, moreover, peripheral leptin administration produces blunted anorectic responses in rodent models of diet-induced and aged-related obesity (136, 137). Thus, it has been proposed that leptin resistance has a central and a peripheral component, where peripheral leptin resistance develops first. Studies have shown that both diet-induced and age-related obese rodents display reduced STAT3 phosphorylation, possibly due to diminished leptin receptor number or reduced affinity of leptin for its receptor (109). In addition, elevated leptin levels in obese rodent models stimulate SOCS3, which further attenuates leptin signaling (2).

Role of Elevated Leptin in Leptin Resistance

Leptin levels in the blood and cerebrospinal fluid (CSF) rise with increasing adiposity in both humans and rodents (99). Evidence suggests that chronically administered exogenous leptin may lead to the development of leptin resistance. For example, male Long-Evans rats given a continuous subcutaneous leptin infusion for 21 days lost the anorectic response to leptin 2 weeks into the infusion period and remained resistant to exogenous leptin during a subsequent peripheral high-dose challenge (127). In another experiment, chow-fed, male Sprague-Dawley rats were centrally infused with leptin. The leptin treatment initially reduced food intake by 50-60%, but food intake levels began gradually increasing on Day 4 until the leptin-treated and vehicle-treated rats were isocaloric on Day 13 (58, 132, 159, 165). Interestingly, both diet- and age-related obesity are associated with elevated leptin levels and leptin resistance (139, 140). Collectively, this suggests that the elevated leptin levels may significantly contribute to the development of leptin resistance in these animals.

This is further supported by studies in young adult and aged rats inducing central leptin resistance by leptin overexpression through gene delivery in the brain in the absence of obesity or elevated serum leptin levels. In these experiments, the leptin-induced anorectic effects, decrease in adiposity, and increase in oxygen consumption completely attenuated over time, and the rats were completely unresponsive to a central challenge of a supra-physiological dose of leptin (143). Thus, central leptin overexpression can induce leptin resistance without influences from obesity or elevated serum leptin levels. This suggests that elevated central leptin is a causative factor in the development of leptin resistance.

In addition, studies have recently discovered a link between fructose intake and leptin resistance (66, 87, 106, 125). While acute fructose ingestion failed to stimulate leptin release, chronic fructose intake in both rats (85) and humans (131) is accompanied by an increase in plasma leptin levels, which is often a predictor of leptin resistance (87, 106, 125). In fact, this increase in plasma leptin levels may precede the development of obesity (42, 169). Although the mechanism is still unknown, researchers have implicated both defective central leptin signaling and the inability of leptin to cross the BBB as possible contributors.

Leptin resistance is a trademark of diet-induced obesity in rats and occurs naturally in obese humans. Diet-induced leptin resistance is associated with diminished hypothalamic leptin signaling and reduced hypothalamic leptin receptor levels (42, 83, 155). This reduced signaling capacity may be a result of the inability of peripheral leptin to cross the BBB and/or diminished STAT3-leptin receptor binding within the hypothalamus (174).

The condition of leptin resistance predisposes rats to display exacerbated hyperphagia and body weight gain on a HF diet. When young adult rats are provided a HF diet ad libitum, they immediately experience an increase in caloric intake that normalizes in approximately 6 days (130, 143). The restoration of caloric intake to pre-HF diet levels is dependent on leptin receptor activity. For example, when young adult rats are centrally infused with a leptin receptor antagonist, they are unable to normalize the HF diet-induced hyperphagia. This suggests that leptin resistance in rats results in an exacerbated hyperphagia during the introduction of a HF diet that may, in turn, cause an exaggerated body weight gain. In fact, similar studies in rats with central leptin

overexpression, aged-related obesity, and fructose-induced leptin resistance show that these leptin resistant animals display an elevated caloric intake and body weight gain when exposed to a HF diet (93).

Age-Related Obesity and Leptin Resistance

In both rodents and humans, body weight and adiposity steadily increase with age until early senescence, which is then followed by a decline later in life (93, 131). Twenty-four month old male F344xBN rats have 3-4 times greater serum leptin levels than 3 month-old rats (131). This increase in body fat with age cannot be accounted for by an increase in food intake, nor is it due to deficient leptin synthesis or peripheral serum leptin levels. In fact, there is an increase in leptin with age that should normally serve to lower body weight. But despite their elevated leptin levels, obesity continues and worsens in these rats, suggesting the relationship between leptin, adiposity, and food intake is altered with age (20). In addition, aged rats typically demonstrate impaired physical performance and activity (131) and have impaired leptin modulation of NPY and AgRP expression. After central or peripheral administration, the aged rats display blunted responses, as measured by food intake and energy expenditure (140). Similarly, leptin gene therapy produces modest and transient effects on food and body weight in aged rats (169).

Caloric Restriction Reverses Leptin Resistance

In rats with diet-induced obesity, unstimulated leptin signaling, measured by hypothalamic STAT3 phosphorylation levels, is approximately 3 times higher than chow-fed controls. However, maximal signaling is greatly diminished in these obese rats which is accompanied by a parallel reduction in leptin receptor expression level (49, 169). Interestingly, caloric restriction increases hypothalamic leptin receptor expression

and improves leptin signaling capacity in young adult diet-induced obese and aged-obese rats (49). In fact, caloric restriction dramatically decreases serum leptin levels but only modestly reduces adiposity levels in rats (28). Further research may help clarify whether it is the reduction in serum leptin or adiposity, or both, that helps restore leptin responsiveness in these rats.

Summary of the Leptin Signaling System

Leptin, produced in peripheral adipose tissue, crosses the BBB to act on hypothalamic satiety centers, inducing an increase in energy expenditure and a decrease in food intake. Age-related, diet-induced, and genetic models of obesity are associated with leptin resistance, where neither endogenous nor exogenous leptin is able to produce its effects on energy homeostasis. Because proper leptin receptor function is required for the normalization of caloric intake upon HF diet initiation, this predicts that aged leptin resistant animals will display an exaggerated and prolonged caloric intake when introduced to a HF diet. As a result of this prolonged hyperphagia, these rats will likely experience a dramatic increase in adiposity and body weight. Testing these predictions in aged rats is one objective of this thesis.

The Endocannabinoid System (ECS)

For centuries, Cannabis sativa and (-)-Δ9-tetrahydrocannabinol (THC) have been used to increase appetite, particularly for sweet and palatable foods. However, pharmacological exploitation of the ECS has been largely ignored until the 19th century (65). To date, two cannabinoid receptors have been cloned and identified where the differences between the two receptors include their signaling mechanisms and tissue distributions (32, 40, 168). Current therapeutic uses for cannabinoid agonists include reducing nausea in cancer patients, preventing weight loss in AIDS patients, and

treating pain, asthma, glaucoma. In contrast, cannabinoid antagonists have been investigated for the treatment of cardiac disease and obesity (65).

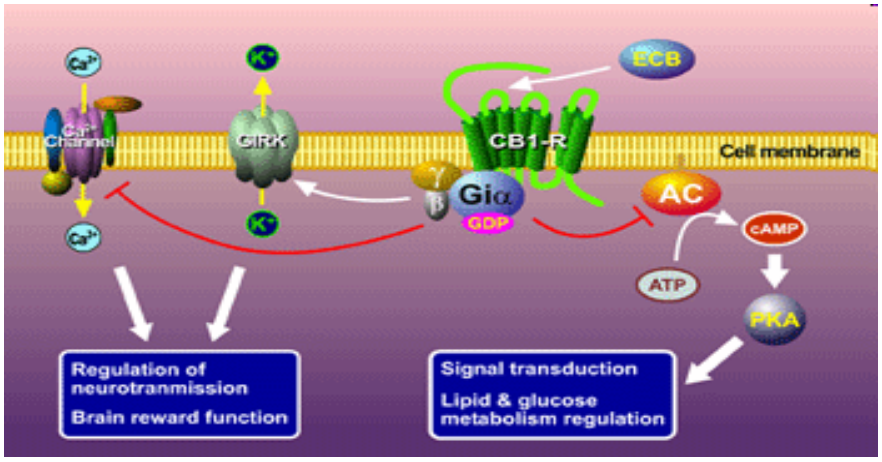
Cannabinoid-1 (CB1) Receptors

Both of the cannabinoid receptors have seven transmembrane domains and are coupled to $G_{i/o}$ proteins, positively to mitogen-activated protein kinase (MAPK) and negatively to adenylate cyclase (65). In addition, CB1 receptors are coupled through $G_{i/o}$ proteins to certain potassium and calcium channels and through G_s proteins to activate adenylate cyclase (142). Within the brain, CB1 receptors are located in the presynaptic membrane to inhibit release of neurotransmitters, including dopamine, noradrenaline, serotonin, glutamate, and γ-aminobutyric acid (GABA) (116).

Cloned in 1990, CB1 receptors were originally believed to be found only in the central nervous system, but they have recently been located in many peripheral tissues. CB1 receptors have been located in humans and rodents in adipose tissue, liver, skeletal muscle, the GI tract, and the pancreas (116). CB1 receptors are the most abundantly expressed G-protein-coupled receptors in the mammalian brain, expressed in areas like the olfactory bulb, cortical regions (neocortex, pyriform cortex, hippocampus, and amygdala), parts of the basal ganglia, cerebral cortex, brainstem, and many thalamic and hypothalamic nuclei (40).

In fact, the ECS-mediated influence on energy homeostasis may occur by regulation of the expression of hypothalamic anorexic and orexigenic mediators. CB1 receptors co-localize with corticotropin-releasing hormone (CRH), melanin-concentrating hormone, and pre-pro-orexin in the PVN, LHA, and VMH, respectively (29). CB1 receptor knockout mice express elevated levels of CRH, implicating ECS inhibition of this anorectic mediator (63). CB1 receptor activation sensitizes orexin-1

receptors when the two are located within the same cell and may enhance the action of orexins (38). In contrast, CB1 receptors were not found to co-localize with NPY, but CB1 activation downstream of NPY mediates some of its orexigenic effects, which were blunted with both genetic and pharmacological inhibition of CB1 signaling. However, CB1 antagonists are equally effective as anorectic agents in both wild-type and NPY-deficient mice (108). Collectively, these data suggest that the stimulation of food intake by endocannabinoid (EC) action is not mediated by NPY, and the normal food intake observed in NPY-deficient mice is not due to an EC compensatory mechanism.



Cannabinoid-2 (CB2) Receptor

The second cannabinoid receptor was cloned in 1993, and it has 68% homology to the CB1 receptor within the transmembrane domains and 44% homology throughout the total receptor (65, 110). It is primarily expressed in immune tissues but has recently been discovered in brain microglial cells, especially in stress and immune response conditions (17). In fact, CB2 receptors have been observed both pre- and postsynaptically in rodent striatum, midbrain, hippocampus, brain stem, cerebellum, and substantia nigra (80, 145). Some of these regions also express high levels of CB1

receptors. Thus, it is plausible that these CB1 and CB2 receptors work both independently and/or cooperatively in the various neuronal populations.

Mice with genetic deletion of the CB2 receptor have reduced bone mass, and humans with CB2 gene polymorphisms have been associated with osteoporosis and autoimmune disorders (111). Pharmacological activation of the CB2 receptor reduces locomotor activity, and pharmacological blockade inhibits food intake in some, but not all rodent strains (72). In addition, CB2 receptor gene expression is reduced in rodents with developed alcohol preference (72). Acute administration of alcohol also downregulates CB2 receptor gene expression in the ventral midbrain of mice (28). These data, combined with data about CB1 receptors, suggest that CB1 receptors directly regulate food intake by affecting the desire for the food while the CB2 receptors indirectly regulate food intake, possibly by altering the activity of the digestive system during stress, addition, or immune response.

ECs

The best characterized endogenous cannabinoids are N-arachidonyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). AEA, which consists of an amide tail on arachydonic acid, was discovered in 1992 and named for the Sanskrit word ‘ananda’ that means bliss (28). 2-AG, characterized by an est\er tail on arachydonic acid, was discovered in 2001 and is the most abundant EC in the brain (28). ECs are produced on-demand and are released from neurons after membrane depolarization and Ca^{2+} influx to act by retrograde signaling on presynaptic receptors (39). After acting on the cannabinoid receptors, AEA and 2-AG are rapidly internalized and degraded by fatty acid amide hydrolase (FAAH) and monoglycerol lipase (MGL),

respectively (32). AEA and 2-AG have different affinities and concentrations in different tissues, but they generally cause similar reactions in response to receptor binding (32).

Central ECs

ECs and their receptors are found throughout the brain, including areas like the hypothalamus that regulates energy homeostasis and the limbic forebrain which evaluates the hedonic value of food. Generally, the central ECS is believed to have an anabolic tone, in that ECS activation in the brain stimulates energy intake and storage (81). Hypothalamic and limbic forebrain EC levels fluctuate with nutritional status. For example, 2-AG levels are increased during fasting and normalize shortly after refeeding (38). CB1-deficient mice consume significantly less food than wild-type controls after a period of fasting (27, 59, 73, 81, 126, 144, 166). Both central and peripheral administration of CB1 agonists induces feeding in rodents. In fact, administration of a CB1 receptor antagonist reduces food intake of both palatable and normal food in ad libitum-fed animals but only normal diet in food-restricted animals (38, 67). In addition, the ECS interacts with neurotransmitters, hormones, and neuropeptides involved in energy regulation. For example, while EC production in the hypothalamus is inhibited by leptin, the ECs themselves inhibit orexin neurons and stimulate melanin-concentrating hormone (154). These data suggest that central CB1 receptors act by 1) reinforcing the motivation to find and consume palatable foods, possibly through interactions with the mesolimbic reward pathway and 2) transiently regulating the levels and actions of other energy homeostasis regulators to induce appetite. Furthermore, it suggests that hyperactivity within the ECS may contribute to obesity and other symptoms associated with the metabolic syndrome.

Peripheral ECs

Chronic peripheral administration of a CB1 receptor antagonist causes a transient reduction in food intake, but the metabolic effects (body weight loss, adiposity reduction, decreased triglyceride levels, improved glucose and insulin sensitivity, and increased adiponectin) continue for several weeks (15, 37). This suggests that the peripheral ECS, which is likely hyperactive during obesity, plays an important role in energy homeostasis.

EC levels are elevated in plasma and adipose tissue of obese animals and humans, but these levels normalize after weight loss (29, 79, 101, 115). In fact, CB1 receptors have been found in many peripheral tissues, including adipose tissue, liver, skeletal muscle, the GI tract, and the pancreas. The receptor expression level changes with nutritional status and obesity in each tissue. Adipose tissue contains all the essential ECS elements, including the ECs, CB1 receptors, and the enzymes that degrade the ECs. Stimulation of the adipose tissue CB1 receptors induces formation and storage of triglycerides, downregulates adiponectin expression, and increases glucose uptake (112, 113). In the liver, CB1 receptor activation stimulates the activity of lipogenic factors, causing an increased fatty acid synthesis and the development of fatty liver (94). Pharmacological blockade of CB1 receptor activation in skeletal muscle increases the rate of glucose uptake in mice (19, 36). CB1 receptors in the GI tract and vagal nerves are believed to be involved in relaying satiety signals from the gut to the brain. For example, CB1 receptor activation reduces satiation caused by cholecystokinin and enhances the ghrelin-induced stimulation of food intake (101). Both cannabinoid receptors are expressed in the pancreas, and ECs are negatively regulated by insulin (76). Studies have shown that the ECS becomes hyperactive during

hyperglycemic periods, which may aid in the development of hyperinsulinemia that is a common characteristic of obesity (38).

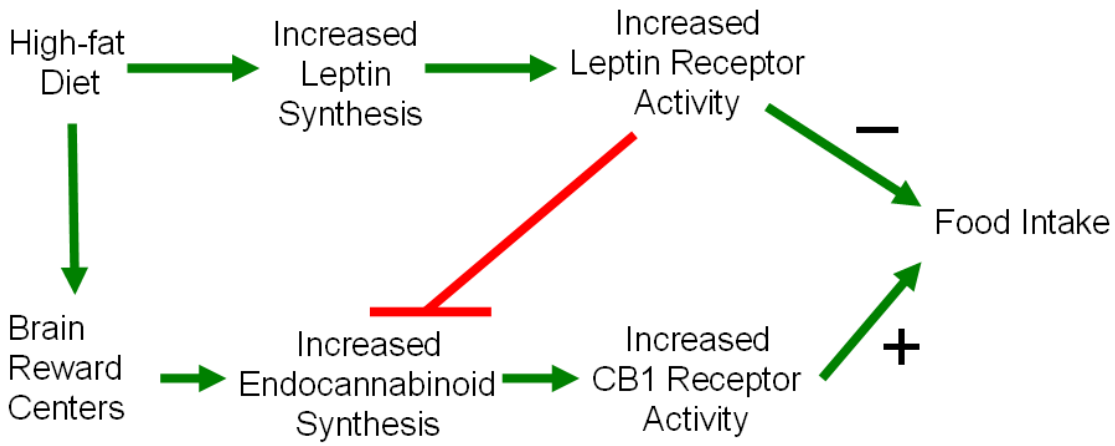
Interactions of Leptin and the ECS

Rodents with genetic leptin or leptin receptor deficiencies (obese Zucker rats, db/db mice, and ob/ob mice) display elevated hypothalamic EC levels, which are drastically reduced in ob/ob mice with acute intravenous leptin administration (75, 97). Studies suggest that this negative regulation occurs through post-synaptic leptin receptor action downregulating EC biosynthesis (18). In fact, Buettner *et al*. recently discovered that central administration of leptin is able to downregulate EC levels in peripheral WAT (39, 81). Interestingly, the authors found that this downregulation is independent of STAT3 signaling. Instead, they found that FAAH, the enzyme primarily involved in metabolizing anandamide, is induced by central leptin administration and is, therefore, likely responsible for the peripheral reduction of ECs.

Changes in hypothalamic EC levels and blood leptin levels during nutritional fluctuations are inversely correlated. For example, hypothalamic 2-AG is elevated during fasting and reduced after refeeding while blood leptin levels are decreased during food deprivation and increased after food intake (39). Di Marzo, *et al*. propose that these fluctuations in ECs occur as a consequence of the presence of leptin. They suggest that as leptin levels decrease during fasting, ECs are allowed to be elevated because of the lack of leptin-mediated downregulation of EC synthesis (84). This is confirmed by studies in genetically obese rodents, in which administration of a CB1 receptor antagonist reduced food intake, suggesting that enhanced EC activity contributed to the hyperphagia normally seen in these animals (147).

HF feeding, which is known to increase circulating leptin levels, also generally increases EC levels in many different tissues. For example, Starowicz et al., reported elevated pancreatic AEA and 2-AG levels in mice fed with a HF diet compared with lean controls, and suggested that this was due to the dysregulation of the biosynthetic and degradative enzymes of ECs (112). Similarly, reduced FAAH expression with HF feeding has been implicated in elevated liver EC levels in mice and in circulating EC levels in obese humans (102). Matias et al., showed that onset, duration and extent of EC dysregulation is dependent on the type of HF diet, specifically its fatty acid composition (10). These data are supported by data showing increased brain AEA levels in piglets fed a HF diet rich in long-chain polyunsaturated fatty acids (101). HF-fed mice have 2.5-fold higher epididymal white adipose tissue (EWAT) EC levels compared with lean mice (101). Interestingly, the ECS is upregulated immediately before adipocyte differentiation in fat cells. It is proposed that the ECS is then turned off by leptin, which is produced from the matured adipocytes (128).

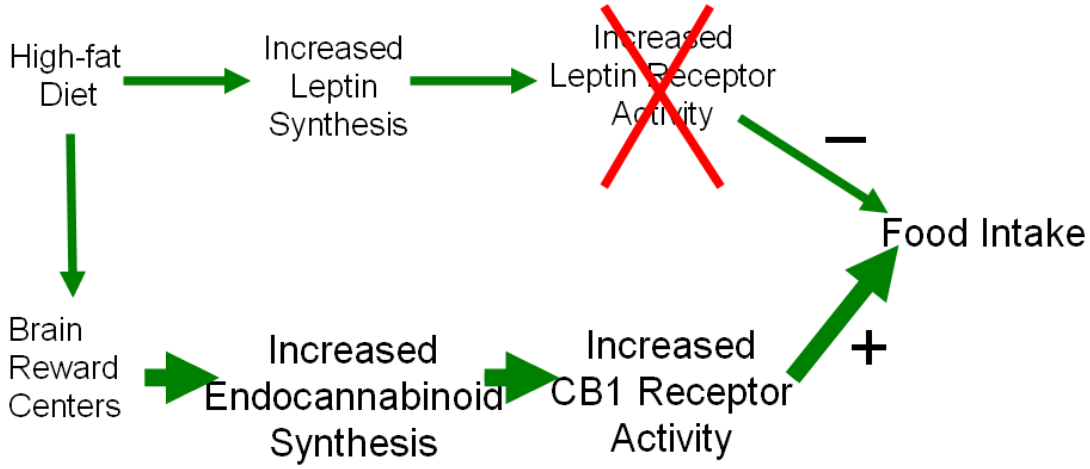
Model of ECS and Leptin Interactions in a Normal, Leptin Responsive Animal:



HF feeding stimulates leptin synthesis and leptin receptor activity to inhibit food intake. In a parallel, and possibly interacting pathway, the HF diet stimulates brain reward

centers, which increases EC synthesis and CB1 receptor activity to stimulate food intake. In a normal, leptin responsive animal, the increased leptin receptor activity should downregulate EC synthesis to prevent the ECS-mediated increase in food intake.

Model of ECS and Leptin Interaction in a Leptin Resistant Animal:

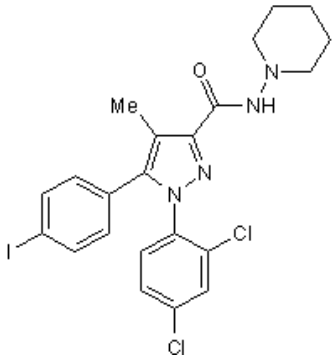


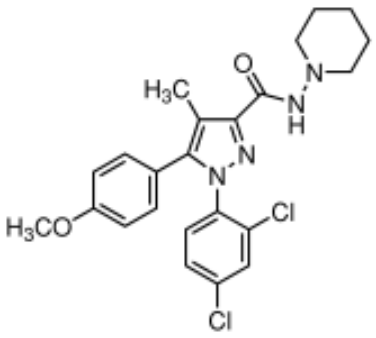
However, in a leptin resistant animal, this increased leptin receptor activity is likely unable to downregulate EC synthesis. Therefore, the HF diet may stimulate brain reward centers, which causes a further increase in EC synthesis and CB1 receptor activity to further stimulate food intake. The dysregulation of the ECS may contribute to diet-induced hyperphagia and obesity.

CB1 Receptor Antagonists & the Treatment of Obesity

Many CB1 receptor antagonists have been shown to have a high binding affinity for the CB1 receptor, a small degree of selectivity for CB1 over CB2, and act as inverse agonists upon binding to CB1 receptors (21, 64, 154). Specifically, peripheral or oral administration of both SR141716 (rimonabant) and AM251 in rodents stimulates gastric

motility, suppresses food intake, and induces long-term body weight loss that continued even after food intake resumed normal levels (33, 120, 141, 157, 158). Several double-blind clinical trials, Rimonabant in Obesity, have been conducted with chronic administration of rimonabant in obese humans with or without type 2 diabetes or hyperlipidemia (32). Daily treatment with 20 mg rimonabant, combined with a 600-calorie-deficient diet and increased physical activity for one year, caused increases in high-density lipoprotein cholesterol and adiponectin; reduced body weight, waist circumference, plasma triglycerides, fasting insulin; and improved glucose tolerance when compared with placebo-treated subjects. At the end of the first year, the rimonabant-treated subjects were re-randomized to either continue to receive 20 mg rimonabant or begin to receive placebo for one year. While the placebo-treated group experienced weight regain, continued rimonabant treatment at this dose produced sustained body weight reductions and improvements in many metabolic parameters. However, the rimonabant-treated subjects displayed a 2-fold increase in the risk of psychiatric adverse events, including depression, sleep disturbances, and anxiety. For this reason, the Food and Drug Administration recently declined to approve rimonabant for the treatment of obesity, and the European Medicines Agency also acknowledged that rimonabant treatment is contraindicated in patients with depression (93).





AM251 **SR141716**

Summary of the ECS

In contrast to leptin, ECs bind to CB1 receptors to increase food intake and fat storage, and obesity is often associated with an overactive ECS. Leptin downregulates both central and peripheral EC levels. This suggests that in a state of leptin resistance, the leptin negative modulation of EC levels will be ineffective, allowing the ECS to become overactivated and induce an increase in food intake as well as body weight and adiposity. This may cause the CB1 receptors to become hyper-responsive to antagonism. Because aged rats are leptin resistant, this predicts that they will display enhanced responsiveness to a CB1 antagonist.

Central Hypothesis

The major objective in this doctoral dissertation was to further understand the role of leptin and the ECS in energy regulation in the context of both aging and HF feeding. The development of leptin resistance with both aging and HF feeding continues to puzzle researchers. Specifically, we investigated the possible link between HF induced and age-related leptin resistance and the ECS. Therefore, we put forth three major hypotheses. First, the leptin resistance associated with age-related obesity results in a prolonged hyperphagia during HF feeding, contributing to exacerbated weight gain. Second, the overactive ECS associated with obesity and likely with age-related obesity contributes to the exacerbated hyperphagia and weight gain observed in aged-obese rats during HF feeding. Third, long-term HF-feeding or aging leads to leptin resistance, therefore it is likely that the leptin negative modulation of CB1 receptor activity is blunted or absent in obese rodent models. As a result, we predict that CB1 receptor antagonism will be enhanced in young adult rats with diet-induced obesity due to long-term HF

feeding and this exacerbated CB1 receptor antagonism will be further enhanced in long-term HF fed aged rats. The goals of this dissertation are to, first, characterize physiological responses to a HF diet in rats of varying ages; second, to examine the effects of a CB1 receptor antagonist on body weight and caloric intake in young adult and aged rats with or without HF feeding – both short- and long-term.

CHAPTER 2
GENERAL METHODS AND MATERIALS

Experimental Animals

The F344xBrown Norway (F344xBN) rat, whose weight gain with age parallels that observed in humans, is a rodent model for late-onset obesity, whose weight gain with age parallels that observed in humans. This rat strain demonstrates a steady gain in adiposity into early scenescence, (approximately 24 months of age), followed by a decline beginning at 30 months that continues into late life (93). This increase in body fat with age cannot be accounted for by an increase in food intake, nor is it due to deficient leptin synthesis or peripheral serum leptin levels (136). In fact, there is an increase in leptin with age that should normally serve to lower body weight. But despite their elevated leptin levels, these rats become obese, suggesting the relationship between leptin, adiposity, and food intake is altered with age. These rats experience a reduced responsiveness to leptin, including an impaired anorexic response and little increase in energy expenditure, indicative of leptin resistance (151). Rats of ages 3 to 6 months are considered young adult, 12 to 18 months are adult, and 24 to 30 months are aged. The median age of this rat strain is 34 months of age (US National Institutes of Health National Insitute on Aging www.nia.nih.gov Aged Rodent Colony Handbook). For the purpose of this dissertation, all male F344xBN rats were purchased from the National Institute on Aging.

General Experimental Design

Rats were housed in individual unventilated cages with corncob bedding under a light-dark cycle of 12 hours of light and 12 hours of darkness with an ambient temperature of 22^{o}C. Food intake (Teklad Rat Chow or Research Diets D12492 60%

HF Diet) and animal weight was measured daily. At the conclusion of each experiment, rats were sacrificed under a Xylazine/Ketamine (75mg/kg ketamine plus 7mg/kg xylazine) anesthesia cocktail. The hypothalamus, cerebellum, interscapular BAT, and epididymal, retroperitoneal and peenal white adipose tissue were removed and weighed at animal sacrifice. Adiposity was assessed by 3 methods: 1) adiposity index (the sum of the weights of the three WAT depots divided by the rat body weight multiplied by 100) at sacrifice, 2) Time-Domain Nuclear Magnetic Resonance (TD-NMR, MiniSpec, Bruker Optical) on a weekly basis, and 3) serum leptin levels in blood collected from the tail during the experiment and by heart puncture at sacrifice.

Experimental Diets

All animals were fed either a chow or HF diet as specified for each experimental design. The chow and HF diets were purchased from Harlan Teklad and Research Diets, respectively. Both diets were provided to the rats at room temperature and replaced every 7 days.

Harlan Teklad 7012 Chow Diet:

Guaranteed Analysis		
Crude Protein	(Min.)	19.0%
Crude Fat	(Min.)	5.0%
Crude Fiber	(Max.)	5.0%
Average Nutrient Composition		
Protein	%	19.92
Fat	%	5.67
Fiber	%	4.37
Ash	%	6.48
Nitrogen-Free Extract	%	53.66
Gross Energy	Kcal/g	4.05
Digestible Energy	Kcal/g	3.75
Metabolizable Energy	Kcal/g	3.41
Linoleic Acid	%	2.90
Amino Acids		
Arginine	%	1.33
Methionine	%	0.40
Cystine	%	0.34
Histidine	%	0.48
Isoleucine	%	1.01
Leucine	%	1.80
Lysine	%	1.15
Phenylalanine + Tyrosine	%	1.65
Threonine	%	0.81
Tryptophan	%	0.25
Valine	%	1.05

Minerals		
Calcium	%	0.98
Phosphorus	%	0.66
Sodium	%	0.32
Chlorine	%	0.54
Potassium	%	0.83
Magnesium	%	0.25
Iron	mg/Kg	284.11
Manganese	mg/Kg	93.34
Zinc	mg/Kg	63.59
Copper	mg/Kg	23.06
Iodine	mg/Kg	2.61
Cobalt	mg/Kg	0.72
Selenium	mg/Kg	0.16
Vitamins		
Vitamin A	IU/g	29.63
Vitamin D_3	IU/g	2.39
Vitamin E	IU/Kg	114.36
Choline	mg/g	2.23
Niacin (Nicotinic Acid)	mg/Kg	100.11
Pantothenic Acid	mg/Kg	86.66
Pyridoxine (Vitamin B_6)	mg/Kg	16.94
Riboflavin (Vitamin B_2)	mg/Kg	13.68
Thiamine (Vitamin B_1)	mg/Kg	95.28
Menadione (Vitamin K_3)	mg/Kg	8.37
Folic Acid	mg/Kg	6.70
Biotin	mg/Kg	0.77
Vitamin B_{12} (Cyanocobalamin)	mcg/Kg	91.00
Vitamin C	mg/Kg	----

Kcal Comparison of Chow and HF Diet:

Macronutrient Information*	
7012 Teklad LM-485 Mouse/Rat Sterilizable Diet	
Crude Protein	19.9%
Crude Oil (Fat)	5.7%
Crude Fiber	4.4%
Metabolizable Energy	3.1 kcal/g
Calories from Protein	25%
Calories from Fat	17%
Calories from Carbohydrate	58%

Product #		D12492	
		gm%	kcal%
Protein		26.2	20
Carbohydrate		26.3	20
Fat		34.9	60
	Total		100
	kcal/gm	5.24	
Ingredient		**gm**	**kcal**
Casein, 80 Mesh		200	800
L-Cystine		3	12
Corn Starch		0	0
Maltodextrin 10		125	500
Sucrose		68.8	275.2
Cellulose, BW200		50	0
Soybean Oil		25	225
Lard*		245	2205
Mineral Mix, S10026		10	0
DiCalcium Phosphate		13	0
Calcium Carbonate		5.5	0
Potassium Citrate, 1 H2O		16.5	0
Vitamin Mix, V10001		10	40
Choline Bitartrate		2	0
FD&C Blue Dye #1		0.05	0
Total		**773.85**	**4057**

Subcutaneous Leptin Infusion

Rats were anesthetized with 5% isoflurane inhalation and maintained on 2.5% isoflurane when surgical plane of anesthesia was reached. An osmotic minipump (model 2001, Durect, Cupertino, CA) containing either murine recombinant leptin or saline was implanted in a subcutaneous pocket on the dorsal surface of the rat, and the incision was closed with sutures.

Intraperitoneal (i.p.) CB1 Antagonist Administration

Rats were held with gentle restraint without the use of anesthesia. The syringe needle was inserted in the abdominal area approximately one inch from the base of the right hind leg. Care was taken to ensure that no organs or veins were struck with the needle. Either vehicle (6% DMSO, 5% Tween 80, and 89% Saline) or varying doses of AM251 in the vehicle were administered by i.p. injection.

Body Composition Measurement

Body composition was determined by time domain-nuclear magnetic resonance measurements on restrained but awake and alert animals (TD-NMR, Minispec, Bruker Optics, The Woodlands, TX, USA). The MiniSpec provides three components of body composition (fat, free body fluid, and lean tissue) by acquiring and analyzing TD-NMR signals from all protons in the sample area. Validation of TD-NMR methodology has been provided (20).

Serum Leptin

Blood was collected by tail nick and a gentle milking motion in restrained rats without anesthesia or by cardiac puncture in rats under anesthesia at sacrifice. Total blood samples were centrifuged at 12,000g for 10 minutes and serum was frozen at -80^{o}C until ready to be analyzed. Serum leptin was measured using rat radioimmunoassay (RIA) kits (Linco Research).

Physical Performance Tests

Forelimb grip strength was measured using an automated grip strength meter (Columbus Instruments, Columbus, OH) as described previously (20). Data were expressed as kilograms of force/kilograms of body weight. Muscle tone and endurance were determined by use of an inclined plane as described previously (24). Data are presented as latency time/kg body weight.

Wheel Running

Voluntary wheel running was measured automatically on Nalgene Activity Wheels (1.081 meters circumference; Fisher Scientific, Pittsburgh, PA) by a magnetic switch and counter with liquid crystal display (LCD). Data are presented as meters ran per day for each age group.

Tissue Harvesting and Preparation

Rats were killed between 9:00AM and 4:00PM by thorocotomy under anesthesia (ketamine (75 mg/kg)/xylazine (7 mg/kg cocktail) or 5% isofluroane inhalation). Blood samples were collected by cardiac puncture, and serum was separated by centrifugation in serum separator tubes. The circulatory system was then perfused with 30 ml of cold saline. The epididymal, perirenal, and retroperitoneal white adipose tissues (EWAT, PWAT, and RTWAT, respectively), hypothalamus, and BAT were excised. For removal of hypothalamus, an incision was made medial to the piriform lobes, caudal to the optic chiasm and anterior to the cerebral crus to a depth of 2-3 mm. The hypothalamus was sonicated in 10 mM Tris-HCl (pH 6.8), 2% SDS, and 0.08 μg/mL okadaic acid plus protease inhibitors. Protein concentrations were determined using the DC protein assay kit (Bio-Rad, Hercules, CA). BAT samples were prepared using a similar protocol but were filtered through a 0.45-μm syringe filter (Whatman, Clifton, NJ) to remove lipid particles before measuring the protein concentration.

Western Analysis

Homogenate samples were boiled and separated on Tris-HCl polyacrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. Immunoreactivity was assessed on separate membranes with antibodies specific to hypothalamic STAT3, both unphosphorylated and phosphorylated (Cell Signaling), and BAT UCP-1 (Linco Research). Immunoreactivity was visualized by a chemiluminescent detection system (GE Healthcare, Piscataway, NJ) and quantified by ImageQuant TL (GE Healthcare)

RNA Isolation and Reverse Transcription

Cellular RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO), using a modified method originally published by Chomczynski (137). The integrity of the

RNA was verified by running it in a 1% agarose gel stained with ethidium bromide and quantified using multiple dilutions of each sample by spectrophotometric absorption at 260 nm. The RNA was then treated with a DNA-free kit (Ambion, Austin, TX), and a first-strand cDNA was synthesized from 1 ug RNA using random primers (Invitrogen) containing 200 units of M-MLV reverse transcriptase (Invitrogen).

Relative-Quantitative PCR

Using a Quantum RNA 18S Internal Standard kit (Ambion), relative quantitative PCR was completed by multiplexing target gene primers and 18s primers and coamplifing for a specific number of cycles in the linear range of the target. For example, the primer sequences for SOCS-3 are sense 5′-ACCAGCGCCACTTCTTCACA-3' and antisense 5'-GTGGAGCATCATACTGGTCC-3'. The optimum ratio of 18S primer to competimer was 1:9, and the primers used were sense PCR was performed at 94^{o}C denaturation for 90 s, 59^{o}C annealing temperature for 60 s, and 72^{o}C elongation temperature for 120 s for 28 cycles. The PCR product was then electrophoresed on a 5% Tris-Borate EDTA acrylamide gel (BioRad) and stained with SYBR green (Molecular Probes). Gels were scanned using a STORM fluorescent scanner (GE Healthcare) and quantified using ImageQuant (GE Healthcare).

Statistical Analysis

Data were analyzed by one-way and two-way ANOVA. When the mean effect was significant, a post-hoc test (Newman-Keuls, Bonferroni, or Dunnett) was applied to determine individual differences between the means. Body composition values were analyzed by paired and non-paired T-tests. A value of $p < 0.05$ was considered significant.

For all objectives, the decision to assign 6 animals per group was based on experience using a statistical power analysis from preliminary studies in our laboratory. In the preliminary data, the ratio of difference to standard deviation was always greater than 2.5. Therefore, statistical power analysis indicates the number of animals needed for significance at $\alpha = 0.05$ (two-tailed) and $\beta = 0.01$ is 6 rats per group. In experiments with aged rats, extra animals may be included in each group to account for natural death or disease-onset that is typical with senescence.

CHAPTER 3
UNEXPECTED PROLONGED HYPERPHAGIA WITH HIGH-FAT FEEDING CONTRIBUTES TO EXACERBATED WEIGHT GAIN IN RATS WITH ADULT-ONSET OBESITY

Introduction

The typical western diet contains an excess of fat, and this is believed to be one contributor to the prevalence of obesity (130). Dietary obesity in adult humans and adult rats has been the subject of intense research, but the role of a HF diet with aging has largely been ignored. In rodents, upon initiation of a high fat diet, there is a transient increase in caloric intake that returns to pre-treatment levels usually within a week despite continuation of the HF diet (174). One factor necessary for this normalization of caloric intake after high fat feeding is leptin receptor activity (174). We previously established that blockade of the leptin receptor with a specific leptin receptor antagonist prevents the normalization of caloric intake after HF feeding (130, 139, 140). Furthermore, rats made leptin resistant by central overexpression of leptin also display a prolonged hyperphagia with HF feeding and an exaggerated weight gain compared with leptin responsive rats fed a HF diet (93). Collectively, these studies suggest that the normalization of caloric intake is mediated by leptin action.

The F344xBrown Norway (F344xBN) rat, whose weight gain with age parallels that observed in humans, is a rodent model for late-onset obesity. This rat strain demonstrates a steady gain in adiposity into early senescence, (approximately 24 months of age), followed by a decline beginning at 30 months that continues into late life (93). This increase in body fat with age can not be accounted for by an increase in food intake, nor is it due to deficient leptin synthesis or peripheral serum leptin levels (136). In fact, there is an increase in leptin with age that should normally serve to lower

body weight. But despite their elevated leptin levels, these aged rats become obese, suggesting the relationship between leptin, adiposity, and food intake is altered with age. These rats experience a reduced responsiveness to leptin, including an impaired anorexic response and little increase in energy expenditure, indicative of leptin resistance (136).

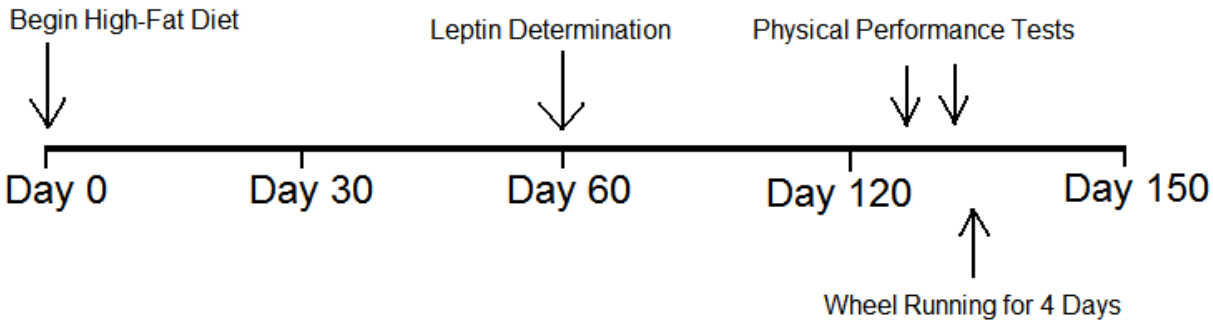
Because aged-obese rats are leptin resistant (89), these data predict that they will have a delayed normalization of caloric intake and exaggerated weight gain when provided a high fat diet. In order to evaluate this hypothesis, we HF-fed rats of various ages and examined caloric intake and body weight over a 5-month period.

Experimental Design

Six- (n=5), twelve- (n=5), eighteen- (n=5), twenty-four- (n=7), and thirty-month old rats (n=5) were provided a HF diet (60% fat; 5.2 kcal/g D12492; Research Diets, New Brunswick, NJ, USA) ad libitum for up to 5 months, and an additional twenty-four-month old rats (n=7) were fed a standard rat chow (15% fat; 3.3 kcal/g diet 2018; Harlan Teklad, Madison, WI, USA) ad libitum. Body weight and food intake were recorded daily. A separate group of three- (n=32) and thirty-month-old (n=23) rats were placed on either a standard chow or HF diet for 60 days and killed for assessment of serum leptin levels, hypothalamic PTP1B levels, hypothalamic SOCS-3 mRNA expression levels, BAT UCP-1 levels, and fat depot sizes.

Body composition (fat/lean mass) was determined before noon prior to initiation of the experimental diets and periodically thereafter. Towards the end of the 150-day experimental period, physical performance tests and voluntary wheel running were assessed.

Additional groups of three- (n = 36) and thirty-month-old rats (n =24) on a chow diet were used to examine a 7-day dose response to peripheral leptin infusion.



(Body composition evaulated every 2 weeks.)

Results

Body Weight Change with HF-Feeding

Normally, when young adult rats are provided with a HF diet, they spontaneously divide into two groups, diet-induced obese (DIO) prone and diet resistant (DR), based on the amount of weight gained (46, 89, 91). This phenomenon has been well described in the Sprague-Dawley rat strain (170), and recently described in the F344xBN rat strain (2). In the present study, the 3-month-old rats fed the HF diet spontaneously divided in two distinct groups, consisting of the top 75% of weight gainers (DIO prone), and the lowest 25% (DR, Figure 3-1, A), which were more similar to the chow-fed as measured by the maximized value of the log likelihood. Surprisingly, this phenomenon was not seen in the aged rats. All of the aged rats provided the HF diet gained similar and considerable amounts of weight, and thus in comparison to young adult rats, aged rats are all susceptible to the weight-gaining effects of HF feeding (Figure 3-1, B).

This age-related susceptibility to HF feeding was also evident in the body weight gain across different rat ages. In particularl, age exacerbated the initial body weight gain seen with HF feeding (Figure 3-2). Both the six-month-old young adult rats and the twenty-four-month-old mature rats steadily gained weight on the HF diet and continued to gain weight throughout the experimental period, but the weight gain in the 24-month-old rats was significantly greater throughout the study period except during the last 2 weeks. This diet- and age-related weight gain was most pronounced in the oldest age group, especially during the initial phase of HF feeding. In the first 30 days of HF feeding, the 30-month-old rats gained the most weight, but this increase in body weight reached a plateau by day 30 and at approximately 31 months of age.

It should be noted that initial body weights, prior to initiation of HF-feeding were greater with increasing age (3-month-olds: 324.35 ± 3.68 g, 6-month-olds: 385.26 ± 11.28 g, 12-month-olds: 435.58 ± 14.24 g, 18-month-olds: 500.86 ± 13.47 g, 24-month-olds: 551.23 ± 9.47 g, 30-month-olds: 568.33 ± 9.17 g).

Caloric Intake with HF-Feeding

The disproportionate age-related increase in initial weight gain suggests that this may be related to the hyperphagia normally observed after introduction of a HF diet. When three-, six-, and thirty-month old rats were provided the HF diet, all ages experienced an immediate increase in daily caloric intake that gradually normalized to basal levels, or near basal level in the case of the oldest age group, over time (Figure 3-3, A). For ease in analyzing the data, the daily caloric intake was divided into two phases, with phase 1 representing the peak caloric intake upon initiation of the HF diet, and phase 2 being the days necessary to normalize the elevated caloric intake back to levels prior to HF feeding. It is important to note that the animals of all ages consumed

a similar amount of calories on the regular chow diet prior to the introduction of the HF diet (Figure 3-3, A, data points prior to Day 0).

The peak daily caloric intake with HF feeding increased with age (R^2 = 0.85, P = 0.009), as is evident in a comparison of Phase 1 across ages (Figure 3-3, B). The three-month-old rats reached a peak caloric intake of 100 kcal. The six-, twelve-, and eighteen-month old rats all reached a peak of approximately 115 kcal, and the twenty-four-month-old rats reached a peak caloric intake of 125 kcal. The thirty-month-old rats, the oldest age tested in this experiment, reached a maximal caloric intake peak of 142 kcal.

A similar trend was seen in Phase 2 (Figure 3-3, C), where the days required to normalize caloric intake to basal levels increased with age (R^2 = 0.93, P = 0.002). In this F344xBN rodent strain, the 3-month-old rats experienced a complete normalization of caloric intake by day 6. The 6-month-old rats and the 12-month-old rats required 9 days and 17 days, respectively, whereas, the 18- and 24-month-old rats did not experience normalization until day 25. In contrast to the younger ages, the 30-month-old rats were unable to normalize to pre-HF caloric intake, but stabilized at a new slightly elevated plateau by day 29.

Body Composition

Absolute fat and lean body mass were greater in the 30-month-old rats compared with the 3-month-old rats at the beginning of the study (fat mass: 175.67±4.38 g vs. 73.56±1.86 g, P < 0.0001; lean mass: 339.78±5.33 g vs. 199.05±4.17 g, P < 0.0001). By examining the change in body composition, the increase in fat and lean mass was significantly greater in both young adult and aged HF-fed groups compared to their age-matched chow-fed controls (Figure 3-4, A & B). In addition, the HF-fed aged rats

gained significantly more fat mass than the HF-fed young adult rats. Conversely, the HF-fed young adult rats gained significantly more lean mass than the HF-fed aged rats. This resulted in a significantly larger fat-to-lean mass ratio in the aged, HF-fed rats compared to all other age/diet groups (Figure 3-4, C). In contrast, the ratio of fat-to-lean mass was relatively constant over time in the chow-fed aged rats (Figure 3-4, C).

Serum Leptin and Adiposity

With HF feeding in young adult rats, by day 60, serum leptin levels rose by more than two-fold compared with the chow-fed counterparts. Interestingly, after 60 days of HF feeding in the young adult, serum leptin reached the same levels as the aged chow-fed rats (Figure 3-5, A). Similar to the young adult, the HF-fed aged rats had serum leptin levels more than two-fold greater than the aged chow-fed rats by day 60. Serum leptin levels at Day 60 paralleled that of the amount white adipose tissue (sum of PWAT, RT WAT, and EWAT) present in each group at sacrifice (Figure 3-5, B). The young adult HF-fed and aged chow-fed rats had the same amount of white adipose tissue, while the young adult chow-fed rats had significantly less and the aged HF-fed rats had significantly more. This is consistent with previous reports that leptin circulates in proportion to whole body fat depots (170, 174).

Wheel Running & Physical Performance Tests

Two to 5 months after HF or chow feeding, 3 performance tests were administered - wheel running and two measures of fore-limb grip strength. Each age group was provided access to running wheels for 4 consecutive days, but the rats were not provided any training or encouragement to run. The youngest chow-fed rats ran greater than 1000 meters/day (Figure 3-6). In contrast, the oldest chow-fed rats ran 8-fold less, only 136 meters/day during the same period. HF feeding also impacted wheel running,

but not to the same degree as age. The youngest HF-fed rats ran two-fold less than the corresponding chow-fed. With increasing age and HF feeding, wheel running declined with the oldest HF-fed rats running only 81.6 meters per day (Figure 3-6).

Because wheel running may be dependent on muscle strength, we subjected the twenty-four-month-old rats to a grip strength and inclined plane test at approximately day 130 on the standard chow or HF diet. There was no significant difference in either grip test or ability to perform in the inclined plane test between the diet groups in the 24-month old rats (data not shown).

Hypothalamic Measures of Leptin Action

We examined two factors that participate in leptin signaling, SOCS-3, a negative regulator of leptin signaling, and PTPIB, a phosphatase that dephosphorylates activated components in the leptin signaling cascade. Consistent with models of leptin resistance, both SOCS-3 mRNA levels and PTP1B protein levels were significantly increased in aged rats compared to young adult rats (Table 3-1). In addition, both hypothalamic SOCS-3 expression and PTP1B levels are significantly elevated with HF feeding (Table 3-1).

BAT UCP1 Levels

The induction of UCP1 in BAT is a marker for enhanced thermogenesis in rodents, and is often used as an indicator of energy expenditure. Consistent with our previous findings (42, 92, 130), HF feeding increased UCP1 protein levels in BAT in young adult rats (Table 3-1). Surprisingly, the HF diet also induced increased UCP1 protein levels in BAT in the aged-leptin resistant rats.

Dose Response to Peripheral Leptin Infusion

Because previous data suggest that leptin resistance prolongs or prevents the renormalization of caloric intake following HF feeding, we evaluated the responsiveness to leptin prior to initiation of the HF feeding in both young adult and aged rats. The doses used were 0.03, 0.05, 0.07, 0.1, and 0.5 mg/day (0.1, 0.167, 0.233, 0.33, and 1.67 mg/kg/day, respectively) in the young adult rats and 0.05, 0.1, and 0.5 mg/day (0.091, 0.182, and 0.909 mg/kg/day) in the aged rats. In the young adult rats (initially 3 months old and weighing 308.79 ± 3.19 g), all doses greater than 0.03 mg/day of peripheral leptin significantly increased serum leptin levels compared with saline-infused rats (Table 3-2). The leptin-infused rats displayed a dose-dependent body weight and food intake reduction in response to a peripheral leptin up to a dose of 0.07 mg/day, above which there was no additional effect (Figure 3-7, A and B, respectively). The decrease in body weight for each of the leptin-infused groups was significantly greater than that of the saline-infused group. In addition, the reduction in cumulative food intake due to leptin infusion was greater with each leptin dose except the lowest dose compared to the saline-infused group. The anorectic and weight reduction responses to doses of 0.07, 0.1, and 0.5 mg/day were not statistically different from each other and appear to represent the maximum response to leptin.

In contrast, the aged rats (initially 30 months old and weighing 553.39 ± 5.48 g) did not respond to even the largest dose of leptin. In particular, cumulative food consumption was unchanged across leptin doses (Figure 3-8, B). All of the aged rats lost weight, likely in response to the detrimental effects of surgery (Figure 3-8, A). However, the leptin-infused rats did not differ from the saline-infused rats with respect to either body weight or food intake reduction, even though both the doses of 0.1 and 0.5

mg/day of leptin significant increased serum leptin compared to saline-infused rats (Table 3-2).

Discussion

Rodents provided with a highly palatable calorically dense diet initially consume an elevated level of calories, but within several days adjust their total food consumption, such that their diet becomes isocaloric to that of chow-fed groups (174). Leptin resistant animals, however, display impaired normalization of caloric intake with HF feeding (130). Because aged-obese rats are also leptin resistant (70, 71), we predicted that aged rats would also fail to properly normalize caloric intake after exposure to HF feeding. The present investigation confirms this hypothesis by examining the physiological effects of a HF diet on rats of various ages between 3 and 33 months of age, and provides several salient findings.

First, our data are consistent with several previous studies indicating that aging increases the susceptibility to obesity and fat storage (70). Iossa, et al. (1999) showed that young male Wistar rats that are naturally growing to maturity have the ability to store both proteins and lipids. However, as the rats age, from 1 month to 6 months old, the protein deposition eventually becomes almost nonexistent and all excess energy consumed is stored as fat (70, 71). They propose that this is one mechanism underlying age-associated obesity. Moreover, these adult rats were more prone to obesity when fed a HF diet than younger counterparts (170). It is reasonable that these trends toward obesity continue as rats age even further. Supporting this hypothesis is our demonstration that aged rats do not divide into DIO and DR, as previously and currently seen in young F344xBN rats (57, 134, 175). Whereas young rats are either susceptible or resistant to weight gain, all aged rats are susceptible to this negative

effect of a HF diet. Moreover, body composition analysis indicates that the older rats gain a disproportionate amount of body fat compared with younger counterparts when provided a HF diet. Hence, these data support the concept that energy storage shifts towards fat deposition with aging, implicating one mechanism underlying age-related obesity. Moreover, aged F344xBN rats, in our case both 24- and 30-month old rats, provided a HF diet gain more weight than correspondingly fed young rats. Together, these data indicate that *all* aged rats, compared to only some young rats, are prone to develop obesity on a HF diet, and furthermore the degree of weight gain is greater in the older rats.

Second, our data indicate that the nature of the transient increase in caloric intake upon initiation of HF feeding is dependent on age. Both the peak increase in caloric intake upon initiation of HF feeding and the time to normalization increase with age. Moreover, this hyperphagia was a specific result of the HF diet: prior to initiation of the HF diet, all rats, regardless of age, consumed the same amount of chow diet, confirming earlier studies (174).

Thus, the greater initial body weight gain in the 24- and 30-month-old rats appears to be a consequence of this failure to normalize caloric intake after initiation of HF feeding, and we suggest the latter is a direct result of leptin resistance in these aged animals. Previous experiments in younger animals demonstrated that the simultaneous administration of a leptin receptor antagonist along with HF feeding prevents the normalization of caloric intake after HF feeding, indicating that leptin receptor activity is necessary for this normalization (137). Older rats have reduced numbers of leptin receptors and diminished leptin signaling (138). In addition, in the present study, we

found increases with age in both SOCS-3, a negative regulator of leptin signaling, and PTPIB, a phosphatase that dephosphorylates activated components in the leptin signaling cascade. These changes with age likely impair the native responses to the endogenous elevation in leptin triggered by HF feeding. We examined the status of leptin resistance at the point prior to initiation of the HF feeding by assessing the leptin dose response decrease in food consumption and body weight over the course of seven days in young adult and aged rats. As expected, the young adult rats responded in a dose-response fashion, whereas there were no responses in the aged rats, thus confirming that prior to initiating HF feeding the aged rats were unresponsive to leptin. As such, these leptin resistant animals display a delayed normalization of caloric intake on a HF diet, strongly suggesting that pre-existing leptin resistance is causal to the exacerbated weight gain with age. It should be noted, however, that these measures of leptin responsiveness were examined only in the 3- and 30-month-old rats. While they demonstrate impaired leptin responsiveness by 30 months of age, we cannot dismiss the possibility that the leptin resistance may be fully manifested prior to this age. If this is the case, the leptin resistance may be only one factor in the progressive exacerbated weight gain with age to HF feeding.

Our data indicating that leptin receptor activity is necessary for the normalization of caloric intake predict that any impaired normalization should be proportional to the degree of leptin resistance and thus may never occur in aged rats that are fully leptin resistant. Our data support this prediction. The delay in normalization is proportional to advancing age with the oldest group achieving only a partial normalization. Similarly, leptin resistance is greater in 30-month-old rats compared with 18 months (152). We

suspect the partial normalization in the oldest age group represents some residual leptin receptor activity or compensation by another anorexic pathway.

Subsequent to the caloric normalization, rats of all ages continued to gain weight suggesting that energy expenditure must be diminished. However, any such decrease in energy expenditure does not appear to be related to the thermic effect of food, because both the young adult and aged HF-fed rats responded equally with an increase in UCP1 protein level in BAT. In addition to thermogenesis, an important component of energy expenditure is physical activity levels. There is an inverse relationship between body weight and physical activity (152), and a decrease in locomotor activity, including volitional activity, may be an important contributor to age-related obesity. Voluntary wheel running is one form of volitional activity involving motivational, exploratory, muscular, age, and body size components (152). Data indicate that locomotor activity declines both with age and obesity (22). We hypothesized that both age and HF feeding would impact voluntary wheel running, and this hypothesis proved correct, wheel running activity declined with both age and HF feeding, the latter especially in young adult rats. It has previously been reported that Sprague Dawley and S5B/P1Ras rats on a high carbohydrate diet voluntarily run more than those fed a HF diet, but there was no comparison to rats on a standard chow diet (41). Another group showed that the introduction of sweet milk plus standard chow decreased voluntary wheel running in female rats, but not male rats (30). Research with hamsters indicated that aged hamsters run significantly less than young ones (77). These data are consistent with our findings that aged F344xBN rats run significantly less than young adult F344xBN rats on a standard chow diet. In addition, HF feeding can further reduce voluntary

wheel running activity in young adult rats. Interestingly, the aging and HF feeding suppressive effect on voluntary wheel running do not appear to be independent; for instance, in the oldest age group, that ran the least, HF feeding had little additional suppressive effect. Collectively, these data suggest that the propensity for inactivity with age may be one contributory factor in age-related obesity, and the inactivity with HF feeding may accelerate the rate of diet-induced obesity.

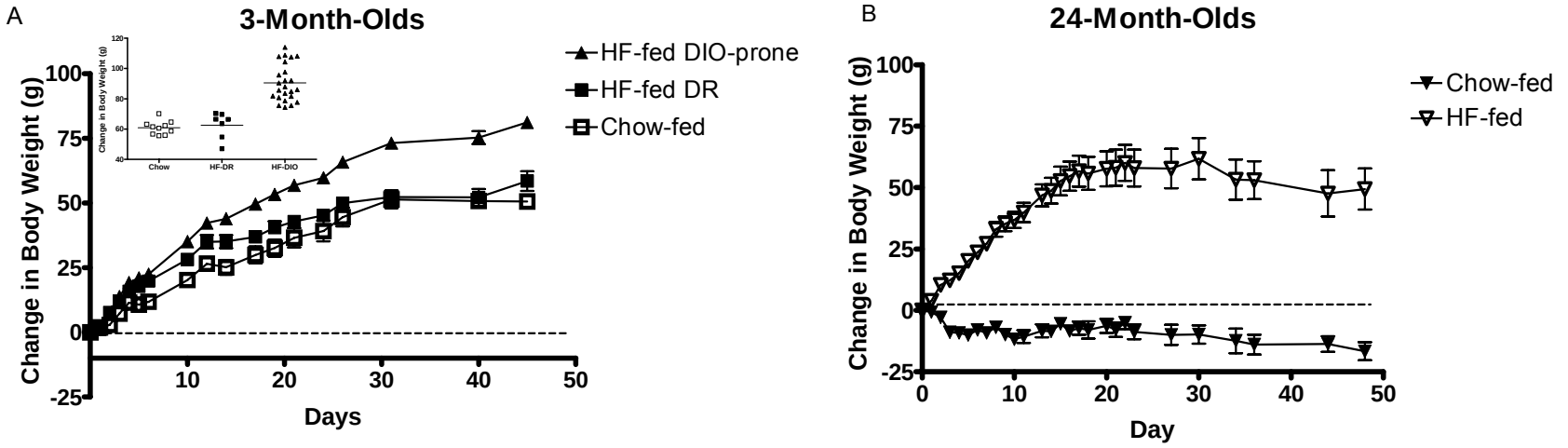


Figure 3-1. A) Body weight gain in young adult rats on chow (open symbols) or HF (closed symbols) diet. Data represent mean ± s.e.m. of HF-fed DIO-prone (n=24, triangles), HF-fed DR (n=8, closed squares), Chow-fed (n=10, open squares). INSET: Young adult rats were either prone or resistant to the effects of the HF diet. We used the maximized value of the log likelihood as a measure of model fit (larger values indicate better fit). When we assumed one normal distribution for the chow-fed together with the HF-fed DR rats, and a separate normal distribution for the HF-fed DIO-prone, the model-fit yielded a log likelihood value of -530 compared with -542 when one normal distribution was assumed for the chow-fed rats and one normal distribution for the all the HF-fed rats (DIO-prone plus DR). This suggests that HF-fed DR rats are more similar to the chow-fed rats than the HF-fed DIO-prone rats, and thus, the latter should be considered a separate group. By day 3 on the diet, the body weight gain in HF-fed DIO-prone animals was significantly greater than that of the Chow-fed group ($P < 0.0001$ by t-test). The change in body weight was significant between the two HF-fed groups by day 15 ($P < 0.05$ by ANOVA). B) Body weight change in aged rats on either chow (solid triangles) or HF diet (open triangles). Data represent mean ± s.e.m. of chow-fed (n=5) and HF-fed (n=7). All aged rats were susceptible to the detrimental effects of the HF diet. By day 1 on the diet, the HF-fed aged animals had experienced a weight gain significantly greater than their chow-fed counterparts ($P < 0.05$ by ANOVA).

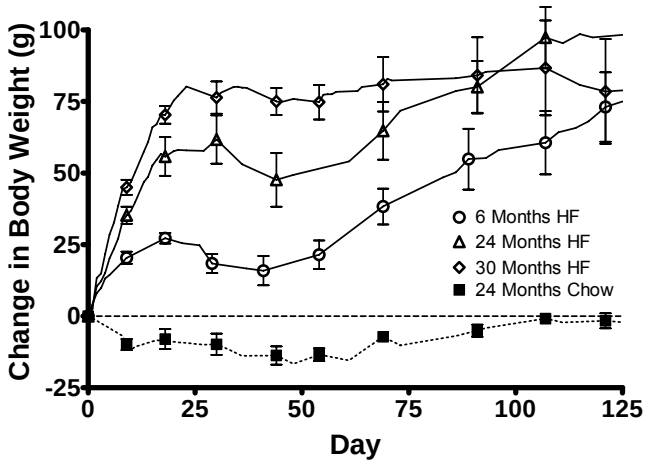


Figure 3-2. Body weight change in rats of differing ages on chow (dotted line) or HF (solid lines) diet. Data represent mean ± s.e.m. of 6-month old HF-fed rats (n=5, circles), 24-month old HF-fed rats (n=7, triangles), 30-month old HF-fed rats (n=5, diamonds), and 24-month old Chow-fed rats (n=7, squares). The 30-month-old rats fed a HF diet experienced a body weight gain greater than the 6 and 24-month-old rats fed a HF diet by day 3 ($P < 0.01$, one-way ANOVA) and 5 ($P < 0.01$), respectively. The change in body weight was no longer significantly different between the 24- and 30-month-old rats fed a HF diet by day 69 ($P > 0.05$). The change in body weight in the 3-, 24-, and 30-month-old rats fed a HF diet was no longer significantly different by day 89 ($P > 0.05$) and for the duration of the experiment.

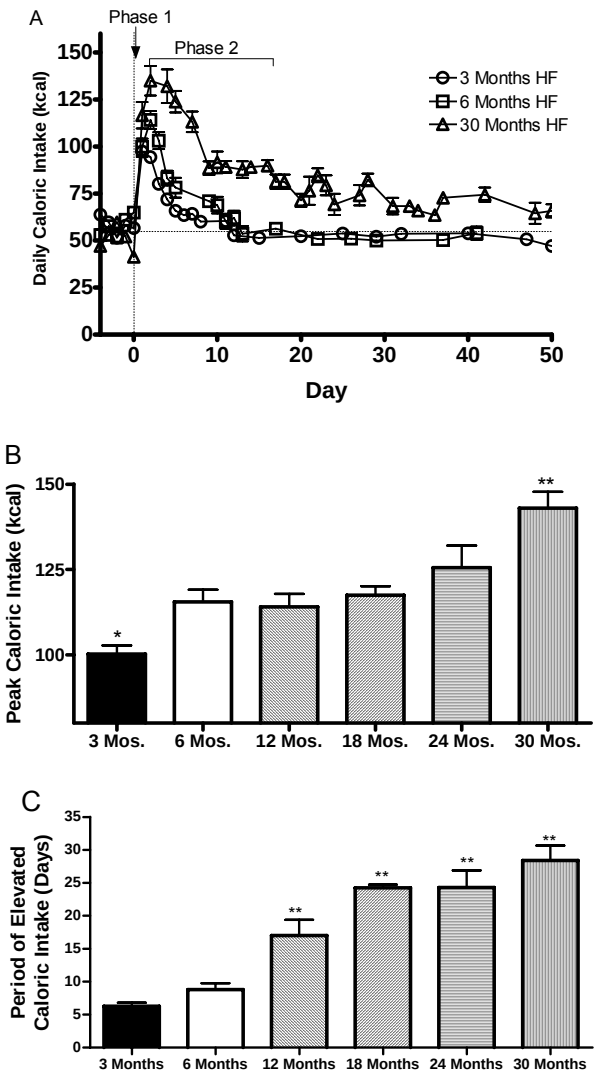


Figure 3-3. A) Daily caloric intake of 3-month-old (circles), 6-month-old (squares), and 30-month old (triangles) rats on a HF diet. Data represent mean ± s.e.m. of 3 Months HF (n=9), 6 Months HF (n=5), and 30 Months HF (n=5). B)The peak caloric intake after the initiation of HF feeding. Data represent mean ± s.e.m. of 3-month old HF-fed (n=24), 6-month old HF-fed (n=5), 12-month old HF-fed (n=5), 18-month old HF-fed (n=5), 24-month old HF-fed (n=7), and 30-month old HF-fed (n=5) rats. $P < 0.0001$ for difference with age by one-way ANOVA. *$P < 0.05$ for the difference between 3-month old rats and all other ages by post-hoc analysis. **P-value < 0.001 for the difference between the 30-month-old rats and all other ages by post-hoc analysis. C) The days required to normalize the elevated caloric intake following HF feeding. Data represent mean ± s.e.m. of 3-month old HF-fed (n=11), 6-month old HF-fed (n=5), 12-month old HF-fed (n=5), 18-month old HF-fed (n=4), 24-month old HF-fed (n=7), and 30-month old HF-fed (n=5) rats. $P < 0.0001$ for difference with HF feeding by one-way ANOVA. **P-value < 0.001 for the difference compared to 3-month-old rats by one-way ANOVA and post-hoc.

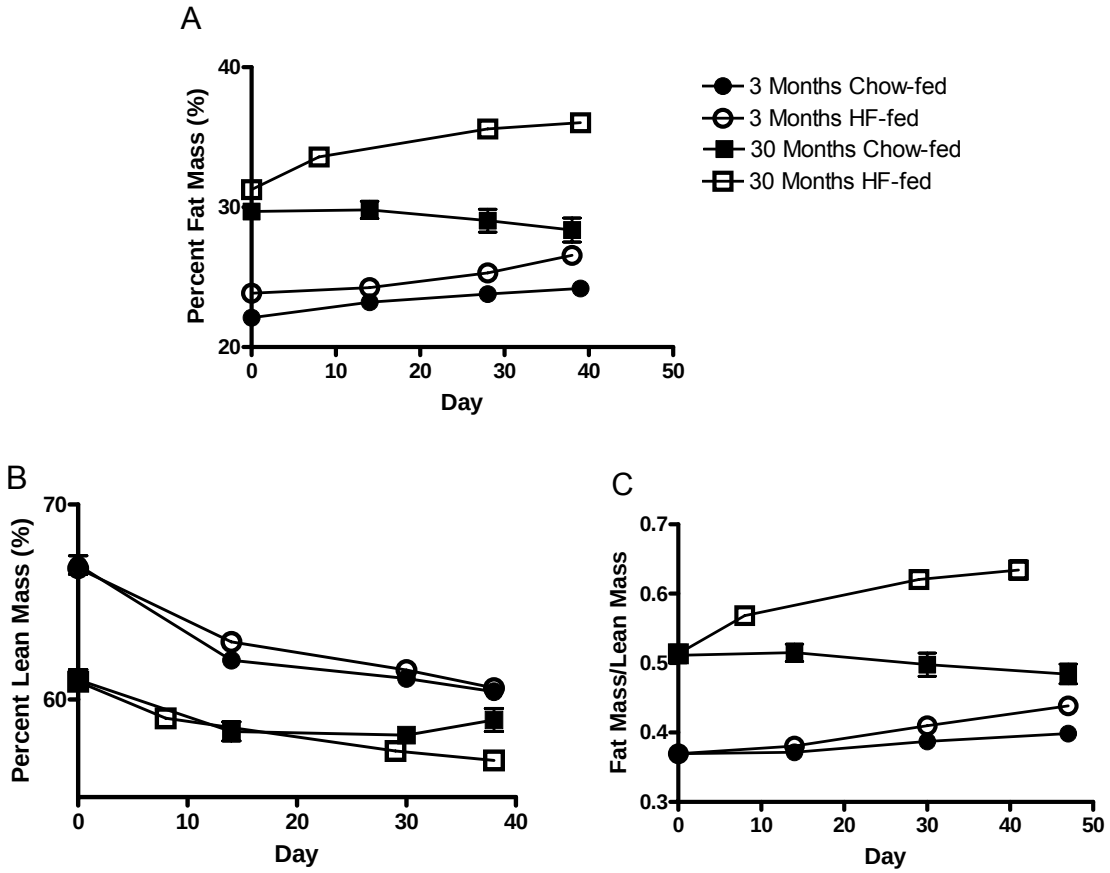


Figure 3-4. Percent fat mass (A), percent lean mass (B), and the ratio of fat-to-lean mass (C) over time in 3- (circles) and 30-month-old (squares) rats on either a HF (open symbols) or a standard chow (closed symbols) diet. Statistical analysis performed by comparing the data collected on Day 38. Data represent mean ± s.e.m. of 3-month old chow-fed (n=10), 3-month old HF-fed (n=12-32), 30-month old chow-fed (n=4-6), and 30-month old HF-fed (n=5) rats. A) $P < 0.0001$ for the interaction; $P < 0.001$ for he difference with HF feeding and the difference with age by two-way ANOVA. B) $P = 0.0003$ for the interaction; $P < 0.01$ for the difference with age, regardless of dietary treatment; $P < 0.001$ for the difference with HF feeding in aged rats only by two-way ANOVA. C) $P = 0.0003$ for the interaction; $P < 0.0001$ for the difference with HF feeding and difference with age by two-way ANOVA.

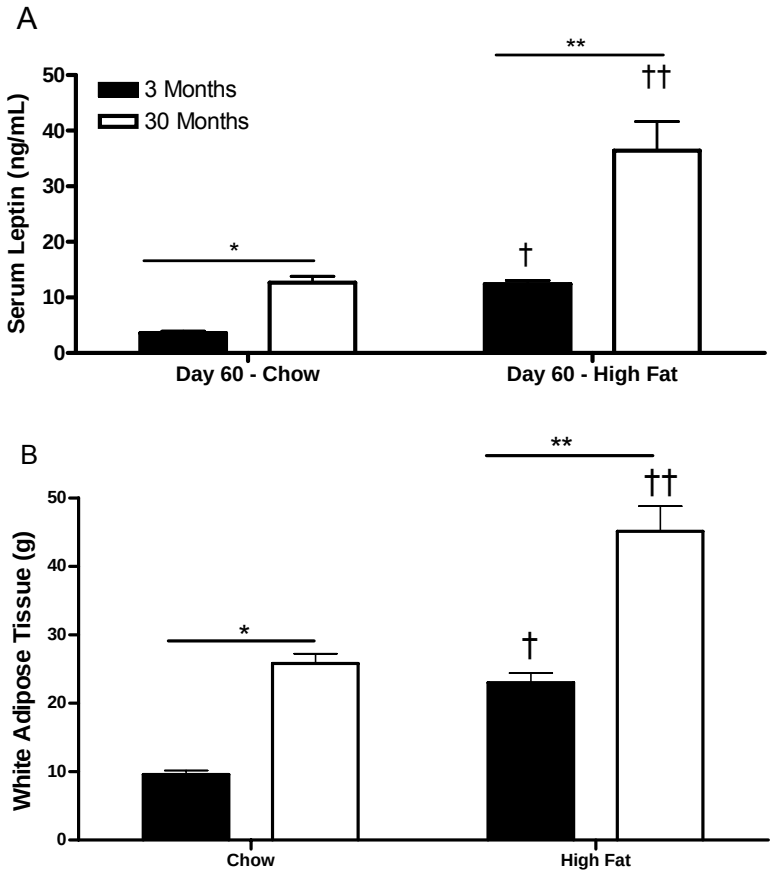


Figure 3-5. A) Serum leptin at day 60 in 3- and 30-month-old rats on chow or HF diets. Ages represent the age of the animal when HF or chow feeding was begun. Assessments were determined 60 days later. Data represent mean ± s.e.m. of 3-month old rats (n = 9-10) and 30-month old rats (n = 9-13). $P < 0.0001$ for the difference with HF feeding and difference with age by two-way ANOVA; $P = 0.0041$ for the interaction. *$P < 0.05$ for the difference between chow-fed young adult and aged rats by post-hoc analysis. **$P < 0.001$ for the difference between HF fed young adult and aged rats by post-hoc analysis. †$P < 0.05$ for the difference between chow-fed and HF fed young adult rats by post-hoc analysis. ††$P < 0.001$ for the difference between chow-fed and HF fed aged rats by post-hoc analysis. B) White adipose tissue mass at sacrifice from three- and thirty-month-old rats following chow or HF feeding. Ages represent the age of the animal when HF or chow feeding was begun. Assessments were determined 60 days later. Data represent mean ± s.e.m. of 3-month old (n=10-12) and 30-month old (n=8-14) rats. $P < 0.0001$ for the difference with HF feeding and difference with age by two-way ANOVA. *$P < 0.001$ for the difference between chow-fed young adult and aged rats by post-hoc analysis. **$P < 0.001$ for the difference between HF fed young adult and aged rats by post-hoc analysis. †$P < 0.001$ for the difference between chow-fed and HF fed young adult rats by post-hoc analysis. ††$P < 0.001$ for the difference between chow-fed and HF fed aged rats by post-hoc analysis.

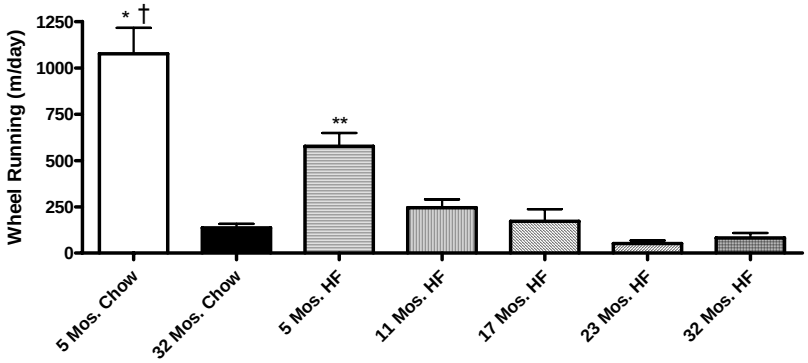


Figure 3-6. Voluntary wheel running over a 4-day period. WR activity was determined 2 months after HF or chow feeding for rats initially of 3 or 30 months of age and after 5 months of HF or chow feeding in rats initially of 6, 12 or 18 month of age. Ages represent the age at the time of WR. Data represent mean ± s.e.m. *P < 0.001 for the difference between five- and thirty-two-month-old chow-fed rats. †P < 0.001 for the difference between five-month-old chow-fed and five-month-old HF fed rats. **P < 0.01 for the difference between five-month-old HF fed and all other HF fed groups by post-hoc analysis.

Table 3-1. Hypothalamic PTP1B protein levels, hypothalamic SOCS-3 mRNA levels, BAT UCP-1 protein levels, NPY mRNA levels, and POMC mRNA levels at sacrifice from 3- and 30-month old rats following chow or HF feeding.

	Young Adult (3-month old)		**Aged (30-month old)**	
	Chow-Fed	***HF Fed***	***Chow-Fed***	***HF Fed***
PTP1B protein (Arbitrary units)	1.00 ± 0.04	1.25 ± 0.06	1.31 ± 0.04*	1.27 ± 0.09
SOCS-3 mRNA (Arbitrary units)	1.00 ± 0.04	1.27 ± 0.09**	1.20 ± 0.04	1.41 ± 0.08
BAT UCP-1 protein (Arbitrary units)	1.00 ± 0.14	2.53 ± 0.40**	1.30 ± 0.25	3.19 ± 0.48**
NPY mRNA	1.00 ± 0.04	1.01 ± 0.05	1.29 ± 0.07*	1.02 ± 0.04
POMC mRNA	1.00 ± 0.07*	0.93 ± 0.06	0.80 ±0.12	0.84 ± 0.09*

Data represent mean ± s.e.m. of 3-9 rats per group. Levels in young adult chow fed rats are set to 1.0 and s.e.m adjusted accordingly. **PTP1B protein measured by Western analysis**: $P = 0.017$ for difference with HF feeding and $P = 0.038$ for difference with age by two-way ANOVA. *$P < 0.05$ for the difference between chow-fed three- and thirty-month-old rats by post-hoc analysis. **SOCS-3 mRNA measured by RT-PCR**: $P = 0.006$ for difference with HF feeding and $P = 0.022$ for difference with age by two-way ANOVA. **$P < 0.05$ for the difference between three-month-old HF-fed and three-month-old chow-fed. **BAT UCP-1 protein measured by Western analysis**: $P < 0.0001$ for the difference with HF feeding by two-way ANOVA. **$P < 0.01$ for the difference with HF feeding in both three- and thirty-month-old rats by post-hoc analysis. **NPY mRNA measured by RT-PCR**: $P = 0.02$ for the interaction; $P < 0.01$ for the difference with age in chow-fed rats by two-way ANOVA. **POMC mRNA levels measured by RT-PCR**: $P < 0.0001$ for the interaction; $P < 0.001$ for the difference with age on chow diet; $P < 0.001$ for the difference with HF feeding in aged rats by two-way ANOVA.

Table 3-2. Serum leptin levels in young adult and aged rats on a chow diet after 7-day saline or leptin infusion.

Dose (mg/day)	Young Adult Rats (ng/ml)	Aged Rats (ng/ml)
Control	6.91 ± 1.12	13.25 ±2.00
0.03	10.10 ± 0.47	
0.05	14.05 ± 0.86*	19.56 ±1.34
0.07	19.75 ± 1.38*	
0.1	20.16 ±1.08*	25.38 ± 2.86**
0.5	22.23 ± 2.15*	24.62 ± 2.26**

Data represent mean ± s.e.m. of 7-9 rats per group. Young Adult Rats: $P < 0.0001$ for the difference with leptin infusion. *$P < 0.01$ for the increase in serum leptin levels compared with controls. Aged rats: $P=0.0008$ for the difference with leptin infusion. **$P < 0.01$ for the increase in serum leptin levels compared to control values by post-hoc analysis.

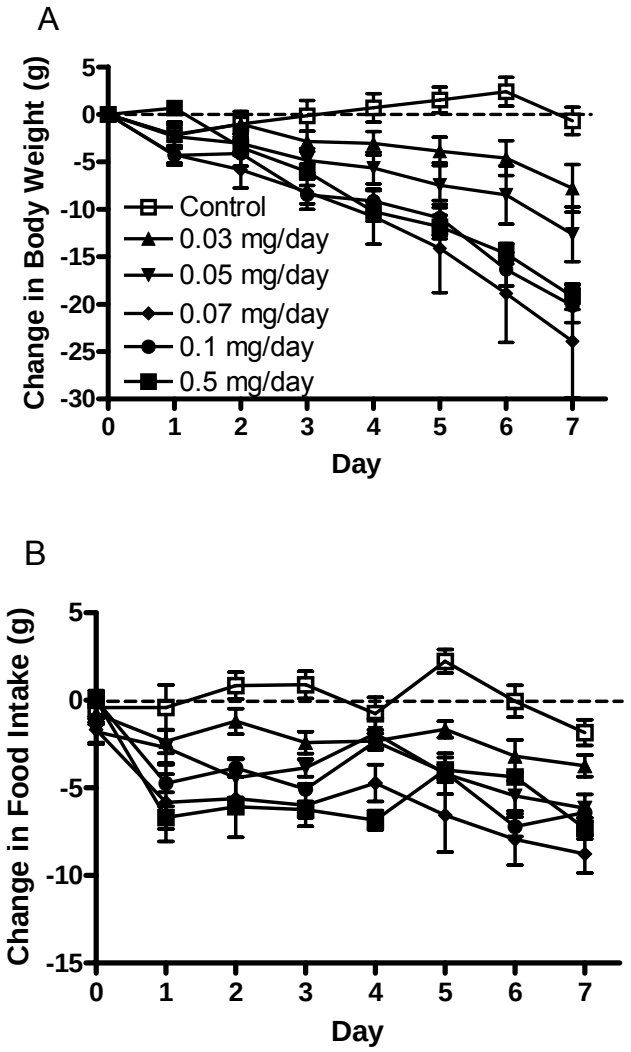


Figure 3-7. A) Change in body weight during a 7-day peripheral leptin infusion in 3-month-old chow-fed rats. Data represent mean ± s.e.m. of 7 rats per group. $P < 0.0001$ for the difference with leptin treatment by one-way ANOVA. Each individual dose is significantly different from control ($P < 0.0002$ for the difference in slope). B) Change in food intake in young adult rats during a 7-day peripheral leptin infusion. Data represent mean ± s.e.m. of three-month-old chow-fed (n=7 per group) rats. $P < 0.0001$ for the difference with leptin treatment by one-way ANOVA. $P < 0.01$ for the difference between control and each dose at Day 7 and between control and each dose for cumulative food intake.

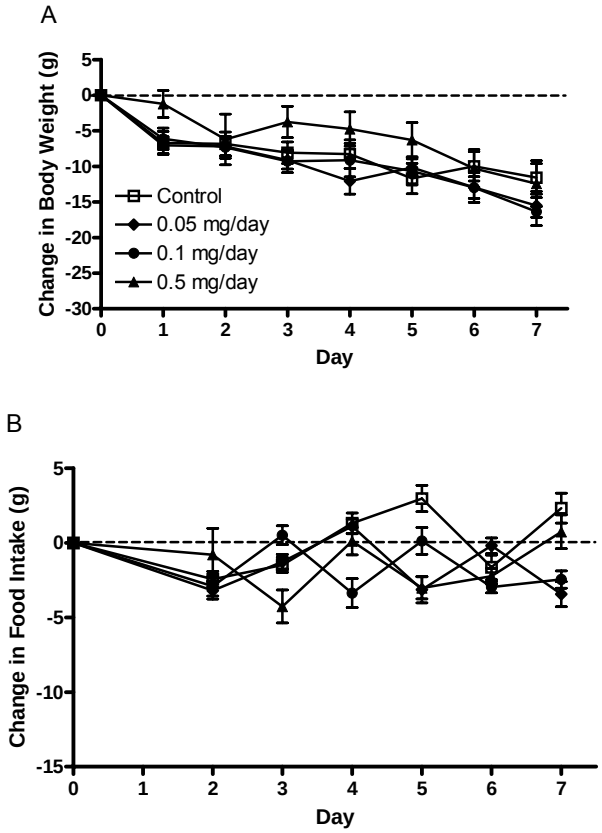


Figure 3-8 A) Change in body weight during a 7-day peripheral leptin infusion in 30-month-old chow-fed rats. Data represent mean ± s.e.m. of 7-8 per group. P = 0.9975 for the difference with leptin treatment by one-way ANOVA. B) Change in food intake in aged rats during a 7-day peripheral leptin infusion. Data represent mean ± s.e.m. of 30-month-old chow-fed (n=7-8 per group) rats. There was no difference (P = 0.087) in cumulative food intake with leptin treatment.

CHAPTER 4
A NOVEL STUDY OF HIGH-FAT DIET-INDUCED HYPERPHAGIA AND RESPONSES TO CB1 ANTAGONIST, AM251, IN YOUNG AND AGED RATS

Introduction

When laboratory rats are introduced to a HF diet with ad libitum access, they experience an immediate and spontaneous increase in caloric intake (77). As discussed in Chapter 3, the peak and duration of this increased hyperphagia escalates with age. For example, 3-month-old male F344xBN rats were previously found to have a peak caloric intake of approximately 100 kcal and required one week to return to basal levels while 30-month-old rats had a peak caloric intake of 140 kcal and required at least one month to return to pre-HF diet levels (174). Normalization of this HF diet-induced increased caloric intake is dependent upon leptin receptor signaling. When young adult rats are infused with a leptin receptor antagonist, they are unable to normalize this elevated caloric intake level (35, 51, 98). Leptin is a peptide hormone that is produced primarily in white adipose tissue and circulates in proportion to whole body adiposity. In a normal, leptin responsive animal, leptin travels across the blood brain barrier and acts on hypothalamic leptin receptors to decrease food intake and increase energy expenditure (51). Leptin resistance, however, is generally characterized by the lack of responsiveness to endogenously or exogenously administered leptin (51). The mechanisms for developing or treating leptin resistance are not fully understood, but the condition is often associated with genetic, diet-induced, and adult-onset obesity (9, 15, 37, 38).

Another dysregulated pathway that is becoming increasingly associated with obesity is the ECS (117). EC action on energy homeostasis is mediated through central CB1 receptor activation, which results in increased food intake and decreased energy

expenditure (38). Hypothalamic EC levels are elevated in db/db mice which lack functional leptin receptors and in obese Zucker rats and ob/ob mice which lack leptin (38). Leptin action in the hypothalamus down-regulates EC activity, although the mechanism is still unclear (38). In fact, when leptin is administered intravenously in rats, a 40-50% reduction in hypothalamic EC levels is observed (15, 37, 38). Presumably, rats with leptin resistance or dysfunctional leptin signaling may lack the leptin-mediated down-regulation of hypothalamic EC levels (5, 64, 144, 160). This interaction has been examined through limited studies in genetically obese rodent models that are either completely leptin deficient or are characterized by impaired leptin signaling. To date, no studies have been reported comparing young adult, leptin responsive and aged-obese, leptin resistant rats, especially with respect to HF diet-induced hyperphagia.

Administration of a CB1 antagonist in normal animals has been shown to decrease food intake, especially on a highly palatable diet, and body weight in many different experimental designs (162). However, the responsiveness to a CB1 antagonist during the period immediately after introducing a HF diet (the HF-diet-induced hyperphagic period) has not been examined in aged rats compared with young adult rats. To this end, we administered two doses of AM251 by daily i.p. injection in young adult, leptin responsive and aged, leptin resistant rats during chow or acute HF feeding periods. We hypothesize that, similar to genetically obese rodent models (77), our age-related obese rats will be hyper-responsive to the anorectic effects of the CB1 antagonist. Additionally, we sought to determine if the CB1 antagonist changes the rodent's preference for the chow vs. a highly palatable HF diet.

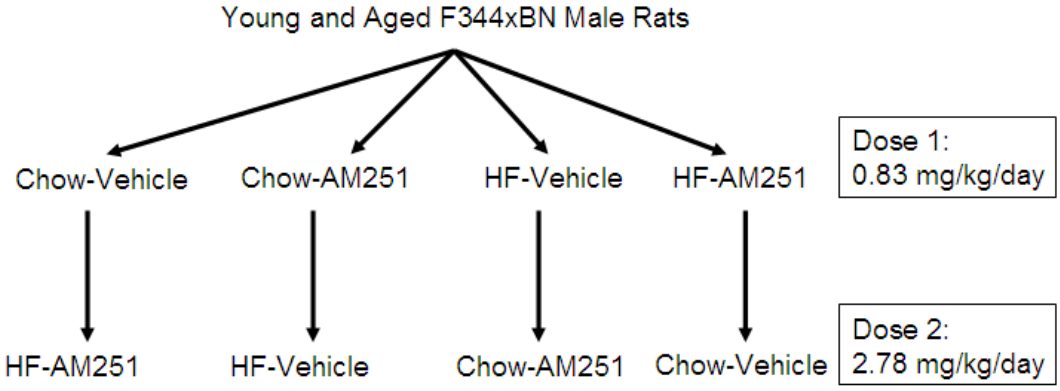
Experimental Design

Experiment 1

Young adult (n = 24, 4 months of age) and aged (n = 30, 29 months of age) rats initially weighing 305.85 ± 6.1 g and 551.70 ± 7.3 g, respectively, were provided a chow (15% fat; 3.3 kcal/g diet 2018; Harlan Teklad, Madison, WI, USA) diet ad libitum until the experiment began. Approximately one week prior to the initiation of the experiment, the rats were all given mock injections over a 2-day protocol during which they were introduced to both the i.p. injection procedures and HF diet (60% fat; 5.2 kcal/g D12492; Research Diets, New Brunswick, NJ, USA). After the mock injections, rats were fed chow diet ad libitum and allowed to rest 1 week before experimentation began.

On the first day of the experiment, all rats were acutely fasted for 2 hours prior to the onset of the dark cycle. Just before the dark cycle, the rats received an i.p. injection of either vehicle (7.7% DMSO, 4.6% Tween 80, 87.7% saline) or 0.83 mg/kg AM251 (dissolved in 7.7% DMSO, 4.6% Tween 80, 87.7% saline; Cayman Chemical, Ann Arbor, MI; half-life of 22 hours). Injection solutions were prepared fresh each day. At the time of the injection, either chow or HF diet was given to the rats and they were allowed ad libitum access to the food for the remainder of the week-long experiment. The rats were given the i.p. injection of vehicle or AM251 for 6 consecutive days an hour before the onset of the dark cycle. Body weight and food intake were recorded daily. At the conclusion of the injection period, serum was drawn by tail nicking, and the rats were allowed to rest for approximately 1 week. For the next phase of the experiment, a crossover design was employed such that the rats previously receiving vehicle now received 2.78 mg/kg AM251 and the rats previously receiving AM251 now

received vehicle using the same experimental design. Again, serum was collected by tail nicking at the conclusion of this portion of the experiment.



Experiment 2

Young adult (n = 15, 6 months of age) rats that were maintained on chow food were then provided both chow and HF diets, simultaneously, ad libitum. These rats experienced the immediate increase in food intake, and the rats were allowed to eat ad libitum until food intake (measured in grams) values had returned to pre-HF diet levels. At this point (Day 5), daily i.p. injections of 0.83 mg/kg AM251 were administered as described in Experiment 1, while still allowing the rats free access to both diets.

In a second paradigm, aged (n=15, 30 months of age) rats that were maintained on chow food were provided the choice of chow or HF diet and simultaneously administered daily i.p. 0.83 mg/kg AM251.

Results

Experiment 1

When first introduced to a HF diet, rats display an immediate hyperphagia accompanied by an increase in body weight (77, 132). With either age or leptin resistance, this HF diet-induced hyperphagia is further exacerbated (77). In this experiment, we tested responsiveness to two doses, 0.83 and 2.78 mg/kg/day, of

AM251 in young adult and aged rats with and without simultaneous introduction of a HF diet.

As expected, chow-fed, vehicle-treated young adult rats maintained a steady caloric intake and body weight throughout the study, whereas HF-fed, vehicle-treated rats demonstrated a transient hyperphagia with a peak caloric intake of approximately 100 kcal, a 38% increase in cumulative caloric consumption, and a steady gain in body weight (Figure 4-1, open symbols, open bars). While the low dose (0.83mg/kg/day) of AM251 in young adult rats did not significantly reduce either caloric intake (Figure 4-1, A) or body weight (Figure 4-1, B) in chow-fed rats, this dose combined with HF feeding resulted in a 22% reduction in peak caloric intake (Figure 4-1, A) and a prevention of HF diet-induced weight gain (Figure 4-1, B). Similarly, cumulative food consumption was unchanged in the AM251 chow-fed but diminished with AM251 in the HF-fed animals (Figure 4-1, A inset). Adiposity levels, lean mass, and the fat-to-lean mass ratio were not affected by diet or this lower dose of drug treatment in these young adult rats (Table 4-1). However, despite no overall increase in adiposity with this short-term HF feeding, there was a nearly 50% increase in serum leptin levels in the young adult rats, which was unchanged by AM251 treatment (Table 4-1).

The higher dose (2.78 mg/kg/day) of AM251 caused a complete inhibition of the HF diet-induced hyperphagia in young adult rats (Figure 4-1, C). Caloric intake was reduced on both diets, with the reduction in cumulative caloric intake during the 5-day treatment period being 21% in chow-fed and 27% in HF-fed young adult rats with AM251 treatment (Figure 4-1, C inset). None of the vehicle-treated, but all of the rats treated with the high dose of AM251 immediately lost body weight compared to diet-

matched controls by Day 1, regardless of dietary treatment (Figure 4-1, D). Moreover, the AM251-mediated body weight loss was greater with HF feeding beginning on day 1 with an overall reduction in body weight of approximately 10 grams in chow- and 16 grams in HF-fed young adult rats by day 5 compared with diet-matched controls (Figure 4-1, D). Body composition was unchanged in the young adult rats with this dose of AM251 (Table 4-1).

In the aged rats, the vehicle–treated, chow-fed animals maintained a stable body weight and caloric intake while the HF-fed, vehicle-treated rats demonstrated a hyperphagia of approximately 150 kcal, nearly 50% greater than that observed in the young adult rats (Figure 4-2, open symbols). Unlike treatment in the young adult rats, the low dose (0.83 mg/kg) of AM251 caused a 16% reduction in the HF-induced peak in caloric intake, and reduced the normal period of hyperphagia from 30 days (77) to 6 days (Figure 4-2, A). Similarly, the five-day cumulative caloric intake was reduced by 24% in chow-fed and 20% in HF-fed aged rats with AM251 treatment (Figure 4-2, A inset). HF feeding resulted in a considerable gain in body weight in the vehicle treated rats (Figure 4-2, B). Peripheral AM251 treatment reduced body weight beginning at day one in chow-fed and partially prevented body weight gain by day 1 in the HF-fed rats. By the end of the 5-day period, body weight differed by 17 grams in chow-fed and 22 grams in HF-fed aged rats compared to diet-matched controls (Figure 4-1, B). In addition, AM251 treatment with HF feeding improved the fat-to-lean mass ratio by 14% compared to diet-matched controls, while whole body adiposity and lean mass were unchanged (Table 4-2). Interestingly, this low dose of AM251 significantly blunted the HF-diet-induced increase in serum leptin levels in the aged rats (Table 4-2).

The high dose of AM251 in aged rats completely inhibited HF diet-induced hyperphagia. Interestingly, the absolute level of caloric intake in both chow- and HF-fed aged rats was nearly equivalent by Day 5 (Figure 4-2, C). However, when compared to their diet-matched controls, the response to AM251-treatment in the HF-fed aged rats was considerably greater than that in the young adult rats with a reduced cumulative caloric intake of 76% compared with 29%, respectively (Figure 4-2, C inset). This higher dose of AM251 caused a parallel reduction in body weight in chow- and HF-fed aged rats. However, if these rats are compared to their diet-matched controls, the AM251 responsiveness was dramatically augmented in the HF-fed aged rats, with body weight differing by approximately 27 and 58 grams in chow-fed and HF-fed aged rats, respectively (Figure 4-2, D). Body composition analysis revealed that adiposity, lean mass, and serum leptin were not significantly different with this high-dose AM251 treatment in chow-fed, whereas the fat-to-lean mass ratio was improved by 9% (Table 4-2). In contrast, drug treatment coupled with HF feeding reduced adiposity by 23%, the fat-to-lean mass ratio by 19%, and serum leptin levels by 73%, whereas lean mass was unchanged (Table 4-2). In essence, the HF diet-induced elevation in adiposity and serum leptin levels and the decrease in fat-to-lean mass ratio were reversed with high-dose AM251 treatment in aged rats (Table 4-2).

Experiment 2

Data regarding CB1 receptor-mediated actions on food palatability are limited and conflicting. To examine if CB1 receptor antagonistm is able to change the food palatability or preference of a HF diet relative to chow, we administered AM251 in young adult and aged rats while providing both HF and chow food in two different protocols.

In the young adult, HF food was first introduced along with the chow and AM251 injected after the HF-induced hyperphagic period normalized. Prior to addition of the second diet (60% HF food), the young adult rats consumed approximately 20 grams of chow food each day (Figure 4-3, A inset). Upon initiation of the HF diet (in addition to the chow), total food intake spiked at approximately 28-29 grams/day, primarily due to HF diet intake and equivalent to approximately 140 kcal/day. The hyperphagia was gradually normalized over the next few days. During this phase of the experiment, the rats ate almost exclusively of the HF diet even though they were given the free choice between the diets (Figure 4-3, A). When food consumption, measured in grams/day, returned to pre-choice level, approximately 20 grams/day, we began daily AM251 treatment.

On day 5, we began AM251 treatment and observed an immediate decrease in caloric intake and body weight similar to that seen in Experiment 1 (Figure 4-3, A & B). Interestingly, CB1 antagonist treatment did not change the preference for chow vs. HF food in the young adult rats. Throughout the experiment and even after termination of drug treatment (data not shown), the rats continued to eat exclusively HF diet (Figure 4-3, A).

In a second paradigm, the effect of daily AM251 treatment was examined on diet selection in aged rats. The aged rats were maintained on chow diet and were given the choice between the chow and HF diets simultaneous with daily i.p. AM251 treatment. Thus, we administered i.p. vehicle or AM251 simultaneously with the introduction of the diet choice on Day 0. Similar to the results in aged rats in Experiment 1, AM251 treatment significantly reduced HF-diet intake on Days 2 and 3, and caused a reduction

in body weight, which began on Day 1 and lasted throughout the treatment period (Figure 4-4, A & B). Moreover, as seen in the young adult animals, AM251 treatment did not change the preference for the aged rats to consume almost exclusively HF diet (Figure 4-4, A).

Discussion

Ad libitum access to a highly palatable diet stimulates rodents to immediately consume more calories for a period of time, the peak and duration of which is dependent upon the animal's state of leptin responsiveness (77). Aged-obese, leptin resistant rats display a heightened sensitivity to this HF-diet-induced hyperphagia and experience a prolonged elevation of caloric intake and exacerbated body weight gain (15, 37, 81). Obesity is also associated with elevated EC levels and increased CB1 receptor activity (18, 38). In fact, leptin administration significantly reduces both peripheral and central EC levels, suggesting that endogenous leptin negatively modulates EC levels and that this downregulation may be blunted or absent in the leptin resistant state (38, 162). Genetically obese rodents, including models with impaired leptin signaling, display enhanced anorectic responses to CB1 antagonist administration (5, 144), predicting that our aged-obese, leptin resistant rats will also display enhanced decreases in body weight and caloric intake during AM251 treatment. Indeed, the data described here demonstrate that CB1 receptor antagonist responsiveness is enhanced with both age-related obesity and short-term HF feeding. However, for the dose and diets tested, AM251 was unable to increase the preference of the chow diet when compared to the highly palatable HF diet.

Conflicting evidence exists regarding the ability of a CB1 receptor antagonist to reduce the intake of chow and/or highly palatable diets. Some studies have suggested

that CB1 antagonists preferentially reduce caloric intake on a highly palatable diet versus a bland chow diet (5, 144). These researchers showed that the CB1 antagonist SR141716 selectively reduced intake of a sweet diet, in both solid and liquid forms, without affecting chow or water intake. Their data suggest that the endogenous EC activity may act by increasing the preference of the available diets or drinking solutions (103, 161). This is partially consistent with the present study in that AM251-mediated CB1 antagonism, regardless of dose or age of animal tested, reduced HF-diet intake to a much greater extent than chow intake.

However, our larger dose of AM251 in Experiment 1 was also able to significantly, albeit to a lesser degree, reduce caloric intake on the chow diet. While these data appear to conflict with that mentioned above, it is consistent with McLaughlin, et al. and Verty, et al., who showed that AM251 equally suppressed feeding on chow, high-carbohydrate, and HF diets in Wistar and Sprague-Dawley rats (74). These authors suggest that data interpretation has a large impact on the conclusions reached. For example, when rats are provided a HF or high carbohydrate diet ad-libitum, they consume more of these diets than they do of chow, creating higher baseline consumptions for these highly palatable diets. If the AM251-induced reduction of intake of these diets is compared to their own baselines, the results show a more dramatic anorectic effect in the highly palatable diets when compared to normal chow, whereas analysis using grams of food consumed after AM251 treatment did not reach significant differences among groups. Another study by Jarrett, et al. showed that AM251 was able to reduce the intake of both sweet and mildly bitter solutions, suggesting that the feeding behaviors after CB1 receptor antagonist treatment may be mediated by a

change in perceived food palatability (77). Thus, possible explanations for these contradictions include dietary composition, data interpretation, and dose and type of CB1 receptor antagonist used.

The present study showed that AM251 responsiveness is enhanced in aged rats and even further increased with HF feeding. We propose that this hyper-responsiveness is due to the state of leptin resistance in the aged rats. If endogenous leptin is unable to down-regulate brain EC levels, the EC levels may be elevated and create a situation in which CB1 receptors may be hyper-responsive to antagonism. HF feeding has been shown to increase serum leptin levels (64, 153) so this may explain the enhanced responsiveness with HF feeding, rather than the palatability of the food.

Interestingly, AM251 treatment was able to reverse both age-related and diet-induced elevations in adiposity and serum leptin levels, which has also been seen in studies by other investigators (146). More experiments are required to determine if this is a cause-effect relationship or if they are merely parallel responses. Regardless of the mechanism, the lower leptin levels may at least partially restore leptin responsiveness.

Experiment 2, examining AM251-induced changes in preference of the chow vs. HF diets, showed that 0.83 mg/kg/day AM251 reduced total caloric intake by causing a reduction in only the HF-diet intake. Thus, AM251 treatment did not increase the preference of the chow diet versus the HF diet because both young adult and aged rats continued to consume only HF diet when given the choice. Very few data regarding CB1 antagonist treatment during a food choice test are available, but our results are consistent with South, et al. These investigators gave C57BL/6 mice access to high- and low-fat diets ad libitum and then administered 5 mg/kg/day AM251, i.p., for 4 days.

This dose is considerably higher than the dose tested here, but these researchers found that AM251 treatment reduced intake of only the HF-diet (5). Similarly, a study with Wistar rats showed that CB1 receptor antagonism preferentially reduced intake of palatable sucrose pellets over regular chow (8, 117, 156). Therefore, CB1 antagonism dose not appear to reduce the preference of a 60% HF diet relative to the regular chow consisting of 15% fat.

In summary, we showed that there is a dose-dependent reduction in HF diet-induced hyperphagia and caloric intake in young adult and aged rats with AM251 administration. The aged rats displayed enhanced responsiveness to AM251 treatment, and the anorectic responses were further amplified during HF feeding in both ages. In the second experiment, we showed that AM251 treatment does not change the preference for chow vs. a 60% HF diet in either young adult or aged rats. Examination of AM251 responses during the HF diet-induced hyperphagic period is a novel approach and one that may provide new insights into both age-related and diet-induced obesity and leptin resistance.

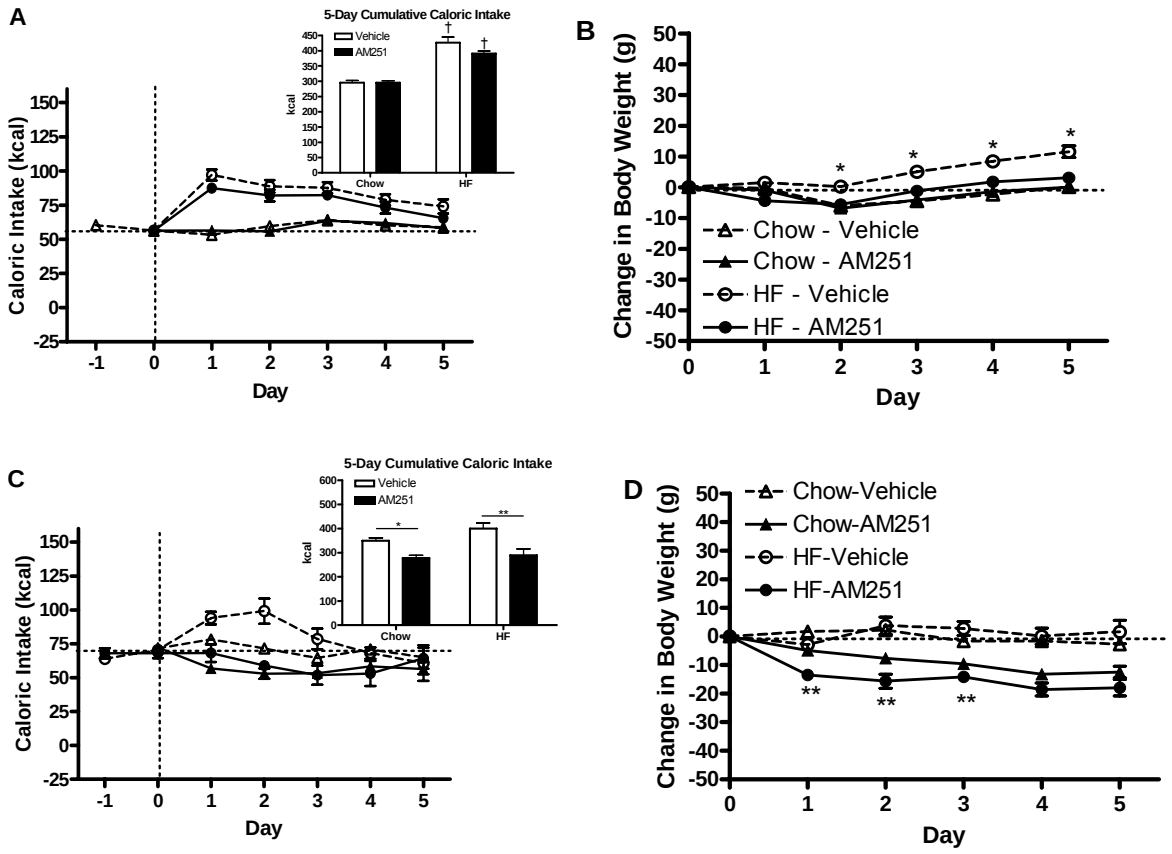


Figure 4-1. Change in caloric intake (A & C) and body weight (B & D) in young adult rats during daily i.p. 0.83 mg/kg or 2.78 mg/kg AM251 administration. Data represent mean ± s.e.m. of chow-fed with vehicle treatment (n = 6, open triangles), chow-fed with AM251 treatment (n = 6, closed triangles), HF-fed with vehicle treatment (n = 6, open circles), and HF-fed with AM251-treatment (n = 6, closed circles). A) AM251 treatment did not reduce caloric intake in young adult rats, regardless of dietary treatment. Inset: HF diet stimulated an increase in cumulative caloric intake (†P-value < 0.001) that was unchanged by AM251. B) Only the HF-fed vehicle-treated young adult rats gained weight during the experimental period. *P-value < 0.01 for the gain in body weight in HF-fed vehicle-treated compared to all other groups starting at day 2 by t-test. C) AM251 treatment inhibited the HF diet-induced hyperphagia. Inset: 5-day cumulative caloric intake was significantly reduced by AM251 treatment in chow-fed (*P < 0.05) and HF-fed (**P < 0.01) rats. D) AM251 treatment caused body weight loss in young adult rats on both diets (P < 0.05 for the difference between AM251-treated rats and their diet-matched controls, starting on Day 1). **P<0.05 for the difference between HF-fed AM251-treated and Chow-fed AM251-treaded rats on Days 1-3.

Table 4-1. Adiposity, lean mass, and serum leptin levels after respective AM251 doses in young adult rats during hyperphagia.

	Experimental Group	Adiposity (g)	Lean Mass (g)	Fat/Lean Mass Ratio	Serum Leptin (ng/mL)
Young Adult Rats, 0.83mg/kg AM251	**Chow-Vehicle**	70.0 ± 3.5	191.6 ± 9.3	0.37 ± 0.01	6.0 ± 1.1
	Chow-AM251	71.7 ± 2.7	190.6 ± 5.1	0.38 ± 0.01	6.1 ± 1.2
	HF-Vehicle	73.2 ± 4.5	200.3 ± 10.0	0.36 ± 0.01	12.5 ± 1.6 *
	HF-AM251	70.7 ± 1.7	196.2 ± 6.5	0.36 ± 0.00	11.5 ± 1.5 *
Young Adult Rats, 2.78mg/kg AM251	**Chow-Vehicle**	93.2 ± 5.6	230.0 ± 10.4	0.40 ± 0.01	No data available
	Chow-AM251	89.7 ± 2.9	222.6 ± 5.3	0.40 ± 0.01	No data available
	HF-Vehicle	79.4 ± 3.9	211.9 ± 7.2	0.37 ± 0.01 *	13.1 ± 3.1
	HF-AM251	73.7 ± 4.2 *	200.2 ± 11.9	0.37 ± 0.01 *	8.4 ± 1.5

Data represent mean ± s.e.m. of 6 rats per group analyzed by 2-way ANOVA. * indicates the difference with HF feeding from corresponding chow-fed rats.

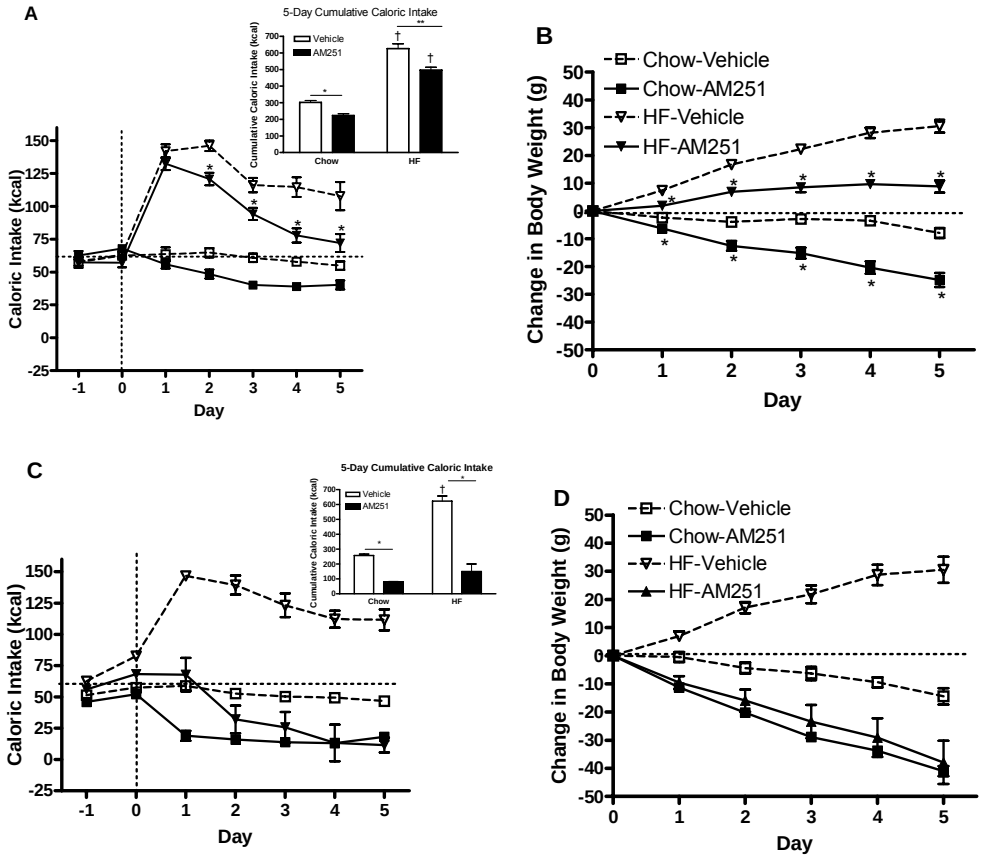


Figure 4-2. Change in caloric intake (A & C) and body weight (B & D) in aged rats during daily i.p. 0.83 mg/kg or 2.78 mg/kg AM251 administration. Data represent mean ± s.e.m. of chow-fed and vehicle-treated (n =6, open triangles), chow-fed and AM251-treated (n = 7, closed triangles), HF-fed and vehicle-treated (n = 7, open squares), and HF-fed and AM251-treated (n = 8, closed squares). A) AM251 treatment significantly reduced caloric intake in HF-fed rats beginning on Day 2. *P-value < 0.01 for the difference in caloric intake between HF-fed vehicle-treated and AM251-treated aged rats. Inset: 5-day cumulative caloric intake is reduced in all AM251-treated aged rats (*P < 0.05, **P < 0.001) †P-value < 0.001 for the increase in cumulative caloric intake with HF-feeding compared to treatment-matched chow-fed rats. B) AM251 treatment caused a reduction in body weight compared to diet-matched, vehicle-treated aged rats (*P-value < 0.01). C) AM251 completely inhibited HF diet-induced hyperphagia and inhibited chow intake in aged rats. Inset: 5-day cumulative caloric intake is reduced with AM251 treatment on both diets (*P < 0.001, †P-value < 0.001 for the increase in cumulative caloric intake with HF-feeding compared to treatment-matched chow-fed rats.) D) AM251 reduced body weight of both chow- and HF-fed aged rats beginning at Day 1 (P < 0.001 for difference between AM251-treated and diet-matched vehicle-treated rats at all days during experimental period, by t-test).

Table 4-2. Adiposity, lean mass, and serum leptin levels after respective AM251 doses in aged rats during hyperphagia

	Experimental Group	Adiposity (g)	Lean Mass (g)	Fat/Lean Mass Ratio	Serum Leptin (ng/mL)
Aged Rats, 0.83 mg/kg AM251	**Chow-Vehicle**	154.7 ± 5.5	305.7 ± 5.3	0.51 ± 0.01	24.2 ± 1.9
	Chow-AM251	139.1 ± 7.4	304.0 ± 7.7	0.46 ± 0.02	20.7 ± 2.2
	HF-Vehicle	184.4 ± 6.3	318.8 ± 4.3	0.55 ± 0.02	70.0 ± 9.8 *
	HF-AM251	149.8 ± 9.8	321.6 ± 11.6	0.47 ±0.03 †	40.8 ± 5.9 †
Aged Rats, 2.78mg/kg AM251	**Chow-Vehicle**	148.8 ± 5.6	295.7 ± 5.3	0.50 ± 0.01	18.0 ± 2.4
	Chow-AM251	136.1 ± 4.8	296.9 ± 5.8	0.46 ± 0.01 †	14.6 ± 1.8
	HF-Vehicle	163.9 ± 10.4	307.8 ± 9.0	0.54 ± 0.02	50.8 ± 10.5 *
	HF-AM251	126.3 ± 8.5 †	292.5 ± 10.0	0.43 ± 0.02 * †	13.9 ± 3.9 †

Data represent mean ± s.e.m. of 6-8 rats per group analyzed by 2-way ANOVA. * indicates the difference with HF feeding from corresponding chow-fed rats; † indicates the difference with AM251 treatment from corresponding diet-matched rats.

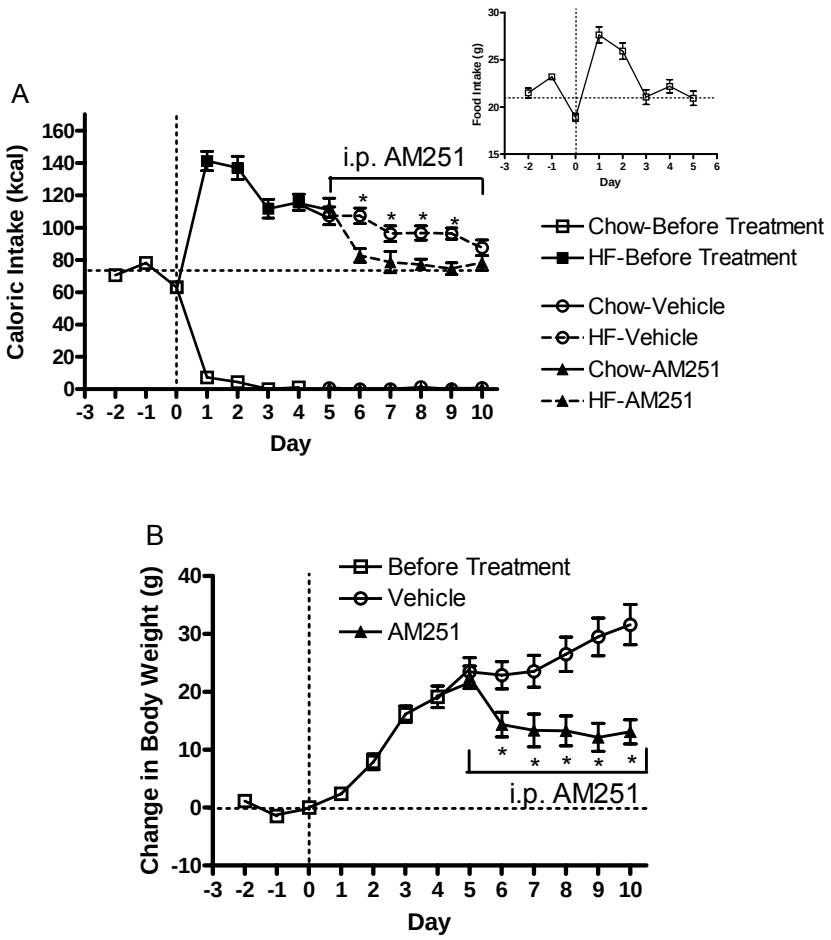


Figure 4-3. Caloric intake and change in body weight in young adult rats during food choice and i.p. vehicle or 0.83 mg/kg AM251 treatment. Data represent mean ± s.e.m. of vehicle-treated (n = 8, circles) chow (solid line) or HF (dotted line) intake and AM251-treated (n = 7, triangles) chow (solid line) or HF (dotted line) intake. A) Young adult rats exclusively consume the HF diet both before and during AM251 treatment. *P-value < 0.05 for the difference in HF-diet intake between vehicle- and AM251-treated rats by t-test. Inset: Food intake in young adult rats prior to AM251 treatment. B) Peripheral AM251 treatment immediately prevented HF-diet-induced body weight gain in young adult rats. *P-value < 0.05 for the difference between vehicle and AM251 treated rats by t-test.

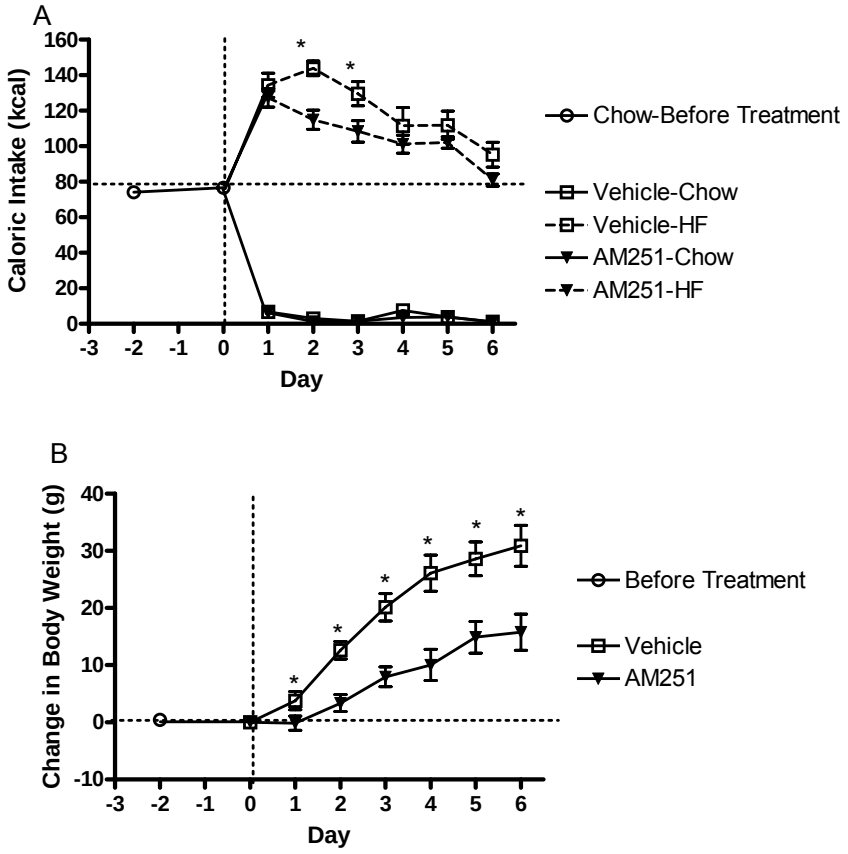


Figure 4-4. Caloric intake and change in body weight in aged rats during food choice and vehicle or 0.83 mg/kg AM251 treatment. Data represent mean ± s.e.m. of vehicle-treated (n = 7, squares) chow (solid line) or HF (dotted line) intake and AM251-treated (n = 8, triangles) chow (solid line) or HF (dotted line) intake. A) AM251 treatment reduced the HF-diet-induced hyperphagia beginning on Day 2. *P-value < 0.05 on Day 2 and 3 for the difference in HF-diet intake between vehicle- and AM251-treated rats by t-test. B) Peripheral AM251 treatment prevented the HF-diet-induced body weight gain normally seen in aged rats. *P-value < 0.05 for the difference between vehicle and AM251 treated rats by t-test. Data represent mean ± s.e.m. of 6-8 rats per group analyzed by 2-way ANOVA. * indicates the difference with HF feeding from corresponding chow-fed rats; † indicates the difference with AM251 treatment from corresponding diet-matched rats.

CHAPTER 5
RESPONSES TO THE CANNABINOID RECEPTOR-1 ANTAGONIST, AM251, ARE MORE ROBUST WITH AGE, WITH ESTABLISHED HIGH-FAT FEEDING-INDUCED OBESITY, AND WITH LEPTIN RESISTANCE

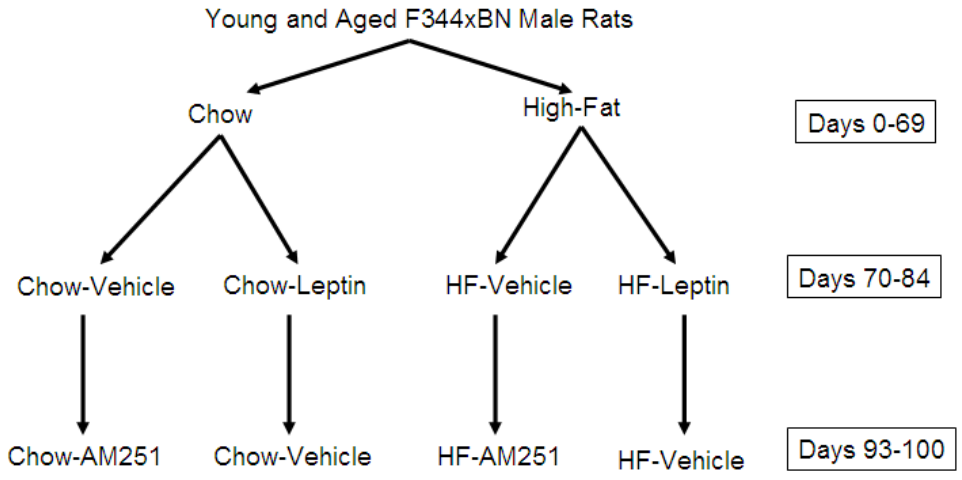
Introduction

Endocannabinoids (ECs), such as anadamide (AEA) and 2-arachidonoylglycerol (2-AG), act through G-protein-coupled cannabinoid-1 (CB1) receptors in the brain and in various peripheral organs to increase both energy intake and adipogenesis (162). CB1 receptor knockout mice, compared to wild-type littermates, consume less food after being fasted (101). These knockout mice also display reduced adiposity levels and resistance to HF diet-induced obesity, even when they consume the same amount of food as controls (38, 162).

Obese animals with established obesity and leptin resistance demonstrate enhanced responsiveness to CB1 receptor antagonists compared with lean animals (8, 60, 62, 101). Other evidence suggests that both a highly palatable diet and obesity, which can both induce leptin resistance in the long term, raise central and peripheral EC levels (100). Conversely, CB1 antagonist anorectic responses can be enhanced in lean animals after EC tone is increased, for example, when animals are exposed to a highly palatable diet or acutely fasted (90). Collectively, this suggests that there is higher EC tone in the obese state. Similarly, this predicts that the proposed leptin negative modulation of ECs may be absent or blunted in the obese, leptin resistant state. If so, then CB1 receptor blockade in obese rodents will result in greater physiological responses, particularly in long-term HF fed young adult and more so in long-term HF fed aged-obese rats. To this end, we tested the AM251 responsiveness in young adult and aged rats with established HF diet-induced obesity.

Experimental Design

Young adult (n=29, 4 months of age) and aged (n=32, 29 months of age) rats initially weighing 311.5 ± 5.4 g and 559.5 ± 6.3 g, respectively, were provided a chow (15% fat, 3.3 kcal/g diet 2018; Harlan Teklad, Madison, WI, USA) or HF (60% fat, 5.2 kcal/g D12492; Research Diets, New Brunswick, NJ, USA) diet ad libitum for 60 days. Body weight and food intake were recorded daily. On Day 60, a 14-day osmotic minipump (model 2002, Durect, Cupertino, CA), administering either saline or 0.1 mg/day leptin, i.p. was implanted in a subcutaneous pocket on the dorsal surface of the rat. After the infusion period, the rats were allowed to recover for approximately 2 weeks before AM251 injections began. A crossover design was employed, in that rats previously receiving saline now received 0.45 mg/day AM251 (dissolved in 7.7% DMSO, 4.6% Tween 80, 87.7% saline; Cayman Chemical, Ann Arbor, MI), and those rats previously receiving leptin now received vehicle (7.7% DMSO, 4.6% Tween 80, 87.7% saline) with all groups maintained on their respective diets, ad libitum. Rats were administered either vehicle or AM251, daily, by i.p. injection for 6 consecutive days, and afterwards, the rats were killed for tissue analysis. Blood was collected by cardiac puncture for serum leptin measurements.



Results

The responsiveness to AM251 was examined in young adult and aged rats with established obesity as a result of 60 days of HF feeding as compared with chow-fed animals. Prior to examining AM251 responsiveness, but after 60 days of chow or HF feeding, leptin responsiveness was examined in both age groups by peripheral leptin infusion. The HF-fed young adult rats demonstrated a partial leptin resistance and all the aged rats displayed a complete leptin resistance (Figure 5-1). The absolute reduction in body weight on Day 10, on which the rats displayed the greatest response to the leptin treatment, was reduced by both HF feeding and age (young adult chow-fed: 15.5 ± 3.1g, young adult HF-fed: 11.8 ± 1.2g, aged chow-fed: 9.7 ± 2.3g, and aged HF-fed: 4.3 ± 1.5g).

After a two week recovery period, responsiveness to AM251 was examined. The young adult, chow-fed rats didn't show a decrease in cumulative caloric intake during the 7-day AM251 treatment (0.45 mg/day), although they did lose body weight (Figure 5-2, A & B), suggesting that an increase in energy expenditure accounts for the decrease in body weight. The anorectic responses to AM251 were more marked with HF feeding compared to chow feeding. Cumulative caloric intake was reduced by 23% in HF-fed AM251-treated rats, but there was no effect on cumulative caloric intake in chow-fed rats (Figure 5-2, A). Initially, the young adult rats on both the chow and HF diets responded to the 7-day AM251 treatment (1.2 mg/kg/day or 0.45 mg/rat/day) with similar decreases in body weight. Beginning at day 5, the chow-fed animals started to regain the lost weight, such that by the end of the treatment period, the decrease in body weight was nearly double in the HF-fed compared with the chow-fed rats. This is indicated by the positive slope of the body weight change in chow-fed compared with

the negative slope in the HF-fed rats (Figure 5-2, B dotted boxes). The reduction in body weight in AM251-treated versus vehicle-treated on Day 7 was 60% greater in HF-fed compared with chow-fed young adult rats (Figure 5-2, B). In addition, there was a decrease in adiposity level in AM251-treated young adult rats on both chow- and HF-diets compared with an increase in adiposity in the vehicle-treated young adult rats (Table 5-1).

Similar to the young adult rats, the responses of AM251 were more pronounced in aged rats with HF feeding compared with chow feeding. In contrast to the young adult, the aged rats also displayed a robust responses to daily AM251 (0.8 mg/kg/day or 0.45 mg/rat/day) treatment in the chow-fed rats. The cumulative caloric intake was significantly reduced by AM251 treatment by 30% in chow-fed young adult rats, with an even greater 45% reduction in HF-fed aged rats of (Figure 5-3, A). Similarly, AM251 treatment reduced body weight by Day 7 by 21 and 34 grams in chow- and HF-fed aged rats, respectively, when compared to vehicle-treated, diet-matched controls (Figure 5-3, B). Both adiposity and lean body mass levels were also reduced in aged rats treated with AM251 compared to their diet-matched, vehicle-treated controls (Table 5-2).

The degree of AM251 responsiveness is directly correlated to the degree of peripheral leptin responsiveness in these young adult and aged rats (Figure 5-4). The young adult, chow-fed rats, which display the greatest body weight loss in response to peripheral leptin treatment, display the least anorectic response to daily AM251 treatment. Conversely, the aged, HF-fed rats, which are completely leptin resistant, display the greatest anorectic responses to AM251. The responses in young adult, HF-

fed and aged, chow-fed fall between these two extremes such that the correlation is significant, with an R squared value of 0.9649.

Biochemical Indicators

In rats with established obesity, several indicators of energy homeostasis were assessed. Several neuropeptides, including leptin, signal through phosphorylation of Signal Transducer and Activator of Transcription-3 (STAT3) (131, 170). At death, hypothalamic pSTAT-3 levels were elevated with age and with HF feeding in both young adult and aged rats (Table 5-1 & 5-2). This is consistent with previous reports where HF feeding increased hypothalamic P-STAT3 levels compared to chow-fed controls (1). More interestingly, whereas AM251 treatment raised pSTAT-3 levels in both young adult and aged chow-fed rats, it only reached significance in aged rats (Table 5-1 & 5-2).

Phosphorylation of Acetyl Co-A Carboxylase leads to fatty acid oxidation and the breakdown of adipose tissue to increase available substrate (77). Both HF feeding and age decreased pACC levels in PWAT (Table 5-1 & 5-2), consistent with the positive energy balance under both of these conditions. Surprisingly, AM251 treatment also caused a significant reduction in pACC levels in aged rats, despite the state of negative energy balance (Table 5-2).

The elevation of UCP1 protein levels in BAT is often used as a marker of energy expenditure (77, 170, 174). In accordance with previous reports, in the present study HF feeding increases UCP-1 levels in both young adult and aged rats (5, 64, 144). In addition, the current study found that AM251 tended to increase UCP-1 levels in BAT in chow-fed young adult and aged rats, although these increases did not reach significance (Table 5-1 & 5-2).

Comparisons of Young Adult and Aged Rats with Chow and HF Feeding

In addition to the comparisons with diet, the responsiveness of AM251 with age was compared using the data from Chapters 4 and 5. Figure 5-5 summarizes the loss in body weight in AM251-treated compared to vehicle-treated rats for each diet and dose of AM251 across ages. Based on the data presented in Figures 5-2 and 5-3, it appears that the 0.83 mg/kg/day dose is submaximal whereas the 2.78 mg/kg/day of AM251 is maximal. With chow feeding, only the aged rats responded to the low dose of AM251 suggesting increased sensitivity to AM251 with age (Figure 5-5, A). Second, the responsiveness to this dose within HF-fed rats of both ages is increased, indicating increased sensitivity with HF feeding (Figure 5-5, A). Comparison of the maximal dose (Figure 5-5, B), indicates that maximum efficacy is increased with HF feeding and further augmented with age.

Comparisons with age were made in rats with established obesity, as described in Chapter 5. In this case, comparisons across age were complicated by the dosing regime. In this experiment, the same dose of AM251 per animal (0.45 mg/rat/day) was administered to rats of both ages. Because of the considerable differences in body weights between young adult and aged rats, the dose if recalculated based on rodent body weight yields 1.20 mg/kg/day and 0.80 mg/kg/day, respectively for young adult and aged rats. Thus, direct comparisons cannot be made between the young adult and aged rats. However, the more robust body weight reduction was observed in the aged rats, which received the smaller dose per kilogram of body weight. With AM251 treatment, the HF fed aged rats with established obesity lost 65% greater body weight compared with the corresponding chow-fed rats (Figure 5-5, C). Lastly comparison among aged rats between those first introduced to the HF diet (Chapter 4) and those

HF fed rats with established obesity (Chapter 5) indicates that low-dose AM251 treatment evoked more robust body weight reduction in latter (Figures 5-5, A and C, right pairs of bars).

Discussion

When the data from this chapter are evaluated with the data from Chapter 4, they demonstrates that the responsiveness of the CB1 receptor antagonist was enhanced with both HF diet-induced obesity and age, with both increased sensitivity and maximum efficacy. These enhanced responses were observed with respect to both body weight reduction and the anorectic response. The latter includes the time course of food consumption as well as the overall reduction in cumulative caloric intake during HF feeding compared to chow feeding.

In addition, the HF diet-induced increase in AM251 sensitivity appears to be additive with age, with the greatest degree of responsiveness observed in aged, HF fed rats. In HF fed young adult rats, enhancement in AM251 antagonism is modest. These data are consistent with previous reports that CB1 receptor antagonists preferentially block consumption of a more palatable diet as compared with standard chow diet (38). With age, the AM251 antagonist response is greatly magnified, with this antagonistic response further exacerbated in HF-fed aged rats. Because both age and HF diet-induced obesity are associated with leptin resistance, these data are consistent with a positive relationship between CB1 receptor antagonist efficacy and leptin resistance. Leptin is believed to down regulate EC levels in the brain. Thus, in leptin resistant animals, the EC system may be free from leptin down regulation, leading to hyperactive CB1 receptors (38, 44, 96). Under these circumstances, leptin resistance would expect to be associated with enhanced AM251 responsiveness. Moreover, the present study

provides a direct relationship between leptin resistance and blockade of CB1 receptor activity.

The increase in AM251 efficacy with HF feeding appears to be influenced by the duration of the diet and resulting established obesity as well as the presence of leptin resistance. Young adult rats with long-term HF feeding displayed both reduced perhipheral leptin and enhanced AM251 responsiveness when compared to chow-fed young adult rats. However, in aged rats the efficacy of AM251 was enhanced to an even greater degree with long-term HF feeding and established obesity. Moreover, there is a strong correlation between enhanced AM251 responsiveness and leptin resistance in young adult and aged chow- and HF-fed rats. Similarly, these responses in long-term chow- or HF-fed rats were enhanced when compared to rats given short-term exposure to the respective diets. This suggests that the duration of dietary exposure and resulting obesity as well as the development of diet-induced obesity significantly contribute to the enhanced AM251 responsiveness observed in long-term fed rats.

The present data directly link the presence of leptin resistance to the increase in AM251 responses. Recent data indicate that some obese states lacking normal leptin function are associated with elevated EC levels (38). In fact, hypothalamic EC levels are significantly elevated in Zucker rats which have defective leptin receptors, db/db mice which lack leptin receptors; and ob/ob mice which lack leptin (38). In ob/ob mice, exogenous administration of leptin is effective in reducing EC levels back to those matching lean littermates (38, 112). Other evidence also suggests that EC levels are elevated in animals with both diet-induced and genetic obesity, but status of the CB1

receptor activity with obesity is unclear (4). In some cases, receptor levels are reciprocally related to EC levels, thus diminished with HF feeding. These data do not support our findings of enhanced AM251 efficacy in the presence of a HF diet. While the mechanism underlying the increased efficacy with a HF diet or with age remains speculative, leptin resistance appears to play a significant role.

One interesting observation is that the HF fed rats displayed elevated UCP1 protein levels in BAT, even in the aged, HF fed rats. Perhaps there is a component of BAT thermogenesis that is contributing to energy homeostasis in the aged HF-fed rats that is absent in aged chow-fed rats, and blockade of this component by AM251 contributes to the increased efficacy with age, though additional experiments are necessary to solidify these speculations.

In summary, both age and a HF diet are associated with enhanced CB1 receptor antagonist efficacy, with the greater response in the aged rats, and the greatest enhancement in HF fed aged rats. The enhanced efficacy with age and a HF diet appears to be more related to the diet, than the presence of leptin resistance, and the degree of obesity may play a role. In addition, CB1 receptor activity appears to contribute to the hyperphagia observed with the introduction of a HF diet.

However, the complete underlying mechanism of the enhanced CB1 receptor antagonist responsiveness remains speculative.

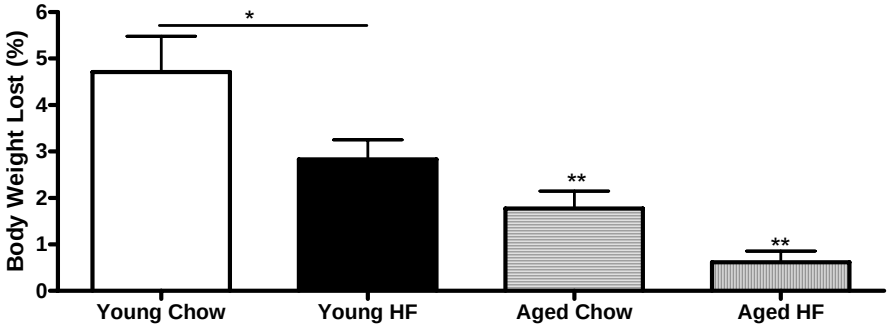


Figure 5-1. Decrease in body weight at day 10 following peripheral vehicle or leptin infusion in chow or HF fed rats. Data represent the difference in body weight from pretreatment values and is the mean ± s.e.m. of chow-fed young adult (n = 8), HF-fed young adult (n = 8), chow-fed aged (n = 6), and HF-fed aged (n = 8). HF feeding and to a greater extent, age caused a reduced responsiveness to peripheral leptin infusion (*$P < 0.05$ for the difference with HF feeding in young adult rats; **$P < 0.01$ for the difference with age, regardless of dietary treatment. Delta body weight in all groups were significantly different from pretreatment (Day 0) by paired t-test).

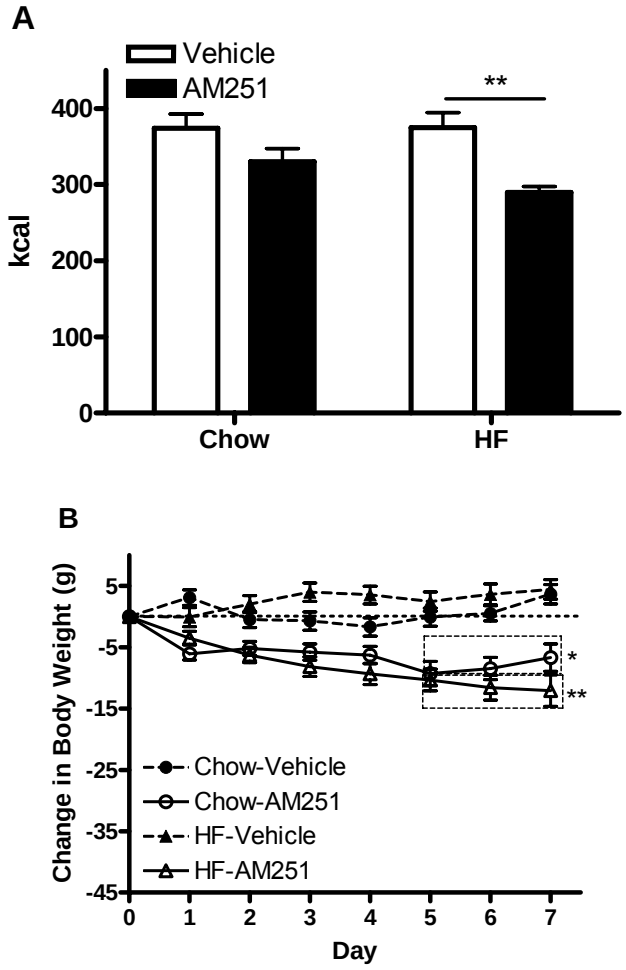


Figure 5-2. Change in cumulative caloric intake and body weight in young adult rats during 7-day daily i.p. administration of AM251 (0.45 mg/rat/day; 1.2mg/kg/day). Data represent mean ± s.e.m. of chow- or HF-fed and vehicle-treated (n = 8, closed symbols), chow- or HF-fed and AM251-treated (n = 7, open symbols). A) The combination of HF feeding and AM251 treatment caused a reduction in cumulative caloric intake. **P-value < 0.01 for the difference with AM251 treatment in HF-fed rats. B) AM251-induced body weight reduction was similar in chow- and HF-fed young adult rats.

Table 5-1. Change in body composition and biochemical markers of young adult rats during daily i.p. vehicle or AM251 injections.

	Chow		HF	
	Vehicle	**AM251**	**Vehicle**	**AM251**
Change in Adiposity (g)	3.8 + 1.2	-2.1 + 1.8 †	3.5 + 0.9	-3.6 + 1.6 †
Change in Lean Mass (g)	-0.4 + 1.6	-4.6 + 2.2	0.7 + 1.3	-2.2 + 3.4
Hypo P-STAT3	1.00 ± 0.08	1.32 ± 0.07	1.79 ± 0.11*	1.72 ± 0.09*
PWAT pACC	1.00 ± 0.19	1.31 ± 0.29	0.47 ± 0.16	0.29 ± 0.13*
BAT UCP-1	1.00 ± 0.08	1.19 ± 0.07	1.42 ± 0.05*	1.53 ± 0.10*
NPT mRNA	1.00 ± 0.06	1.00 ± 0.02	0.96 ± 0.04	0.96 ±0.04
PTP1B mRNA	1.00 ± 0.11	1.30 ± 0.11	1.00 ± 0.05	1.20 ± 0.26
POMC mRNA	1.00 ± 0.08	1.05 0.09	0.97 ± 0.07	0.98 ± 0.13

Data represent mean ± s.e.m. of 4-8 rats per group analyzed by 2-way ANOVA. Adiposity and lean mass were determined prior to and after 7-day vehicle or AM251 daily injections; biomolecular marker protein levels, measured by Western analysis, and mRNA levels, measured by RT-PCR, of all young adult, chow-fed & vehicle-treated groups are set to 1.00 and s.e.m. adjusted accordingly. † $P < 0.05$, indicates the difference with AM251 treatment from corresponding vehicle-treated and diet-matched rats. *$P < 0.05$, indicates the difference with HF feeding from corresponding chow-fed rats.

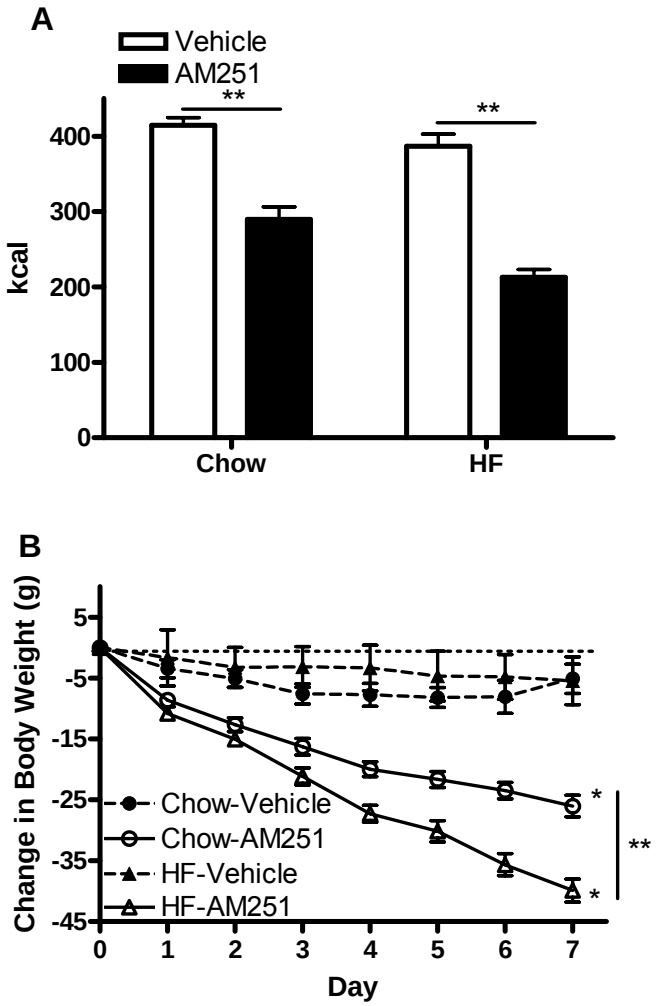


Figure 5-3. Change in cumulative caloric intake and body weight in aged rats during 7-day daily i.p. administration of AM251 (0.45 mg/rat/day; 0.8 mg/kg/day). Data represent mean ± s.e.m. of chow- or HF-fed and vehicle-treated (n = 8, closed symbols), chow- or HF-fed and AM251-treated (n = 7, open symbols). A) Cumulative caloric intake was reduced by AM251 administration, regardless of dietary treatment (**P-value < 0.001). B) AM251 treatment induced a loss of body weight in aged rats that was enhanced with prolonged HF feeding. *P-value < 0.001 for the difference with diet and AM251 treatment in aged rats. **P-value < 0,001 for the difference in change in body weight in both dietary groups.

Table 5-2. Change in body composition and biochemical markers of aged rats during daily i.p. vehicle or AM251 injections.

	Chow		HF	
	Vehicle	**AM251**	**Vehicle**	**AM251**
Change in Adiposity (g)	-4.9 + 1.6	-18.1 + 2.2 †	-7.2 + 1.7	-25.0 + 1.9 †
Change in Lean Mass (g)	-0.7 + 1.3	-7.6 + 1.3 †	-0.4 + 1.8	-10.3 + 1.8 †
Hypo P-STAT3	1.26 ± 0.10	1.87 ± 0.25†	2.35 ± 0.24 *	2.08 ± 0.10
PWAT pACC	0.42 ± 0.06	0.20 ± 0.03 †	0.22 ± 0.04 *	0.11 ± 0.02
BAT UCP-1	0.91 ± 0.04	1.29 ± 0.12	1.79 ±0.15 *	1.78 ± 0.09 *
NPY mRNA	0.74 ± 0.03†	0.91 ± 0.03	0.85 ± 0.03	0.82 ±0.05
POMC mRNA	0.76 ± 0.11†	1.27 ± 0.12	1.10 ± 0.09	0.95 ± 0.14
PTP1B mRNA	0.78 ± 0.04	0.81 ± 0.01	0.90 ± 0.10	0.70 ± 0.05

Data represent mean ± s.e.m. of 4-8 rats per group analyzed by 2-way ANOVA. Adiposity and lean mass were determined prior to and after 7-day vehicle or AM251 daily injections; biomolecular marker protein levels, measured by Western analysis, and mRNA levels, measured by RT-PCR, of all young adult, chow-fed & vehicle-treated groups are set to 1.00 and s.e.m. adjusted accordingly. † $P < 0.05$, indicates the difference with AM251 treatment from corresponding vehicle-treated and diet-matched rats. *$P < 0.05$, indicates the difference with HF feeding from corresponding chow-fed rats.

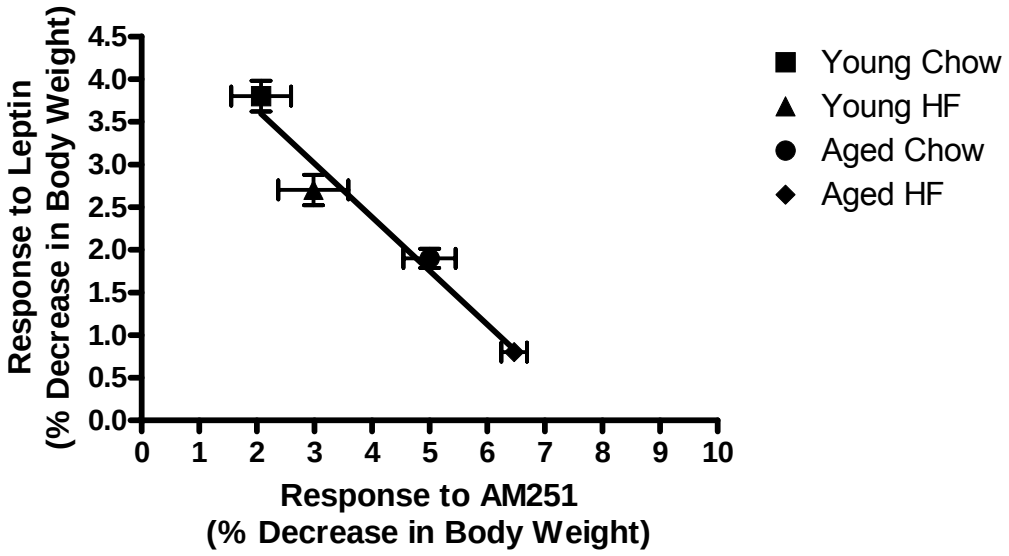


Figure 5-4. Percent change in body weight in response to peripheral leptin infusion versus peripheral AM251 injections in young adult and aged rats with and without HF feeding. Data represent mean ± s.e.m. of young adult chow-fed (n = 5, squares), young adult HF-fed (n = 8, triangles), aged chow-fed (n = 7, circles) and aged HF-fed (n = 7, diamonds). P-value = 0.0177 and R^2 = 0.9649.

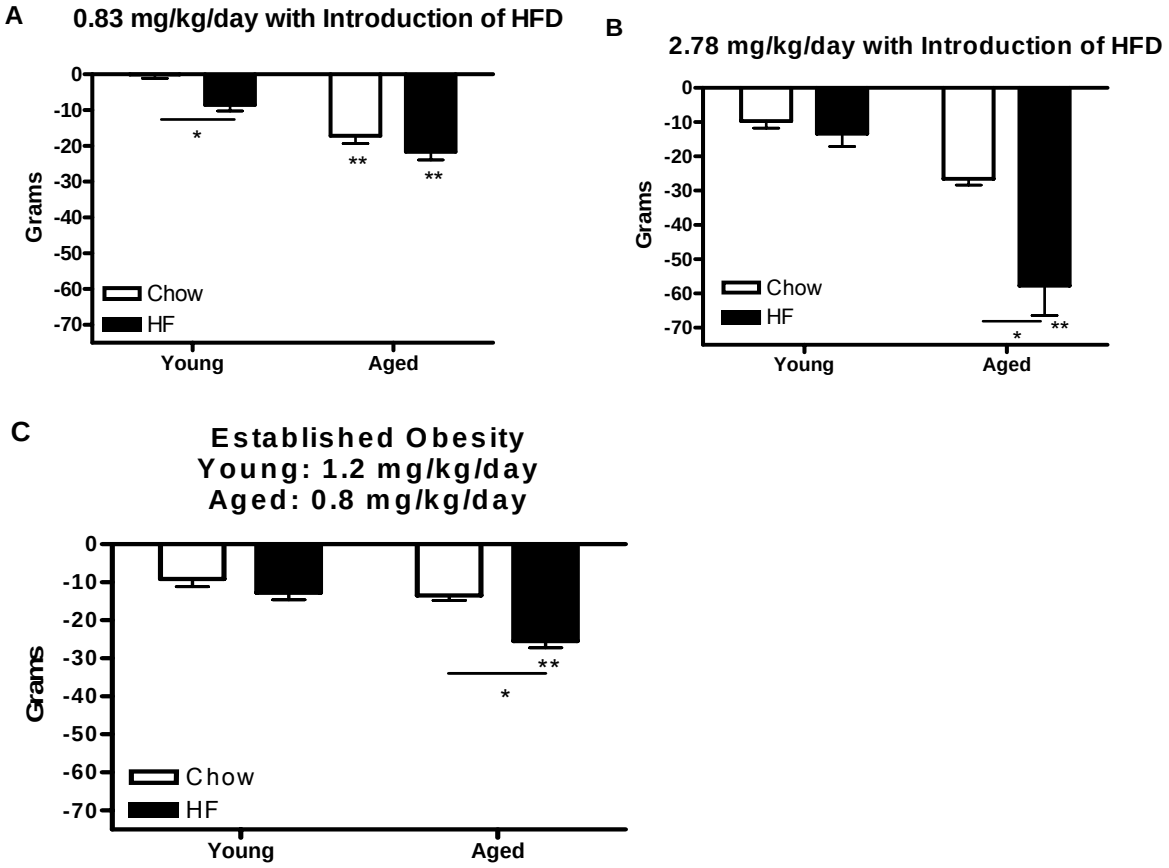


Figure 5-5. Change in body weight in AM251-treated rats compared to vehicle-treated rats on Day 5 of the respective experiments. A) 0.83 mg/kg AM251 treatment during hyperphagia reduced body weight in young adult HF-fed and aged chow- and HF-fed rats. *P-value < 0.05 for the difference in HF-fed compared to chow-fed young adult rats. **P-value < 0.001 for the in aged rats versus diet-matched young adult rats. B) 2.78 mg/kg AM251 treatment during hyperphagia caused an increase in sensitivity with HF feeding in aged rats. *P-value < 0.001 for the difference with HF feeding in aged rats. **P-value < 0.001 for the difference with age during HF-feeding. C) 0.45mg/rat/day during prolonged chow or HF feeding caused a significant reduction in body weight in aged rats, which was enhanced with HF feeding. *P-value < 0.001 for the difference with HF feeding in aged rats. **P-value < 0.001 for the difference in age on a HF diet.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Obesity is a major health issue, and the rates of obesity are continually on the rise in both adults and children around the world. Obese individuals often experience a reduced quality of life and are at higher risk for many other diseases, including type 2 diabetes, heart disease, stroke, and certain types of cancer (2). There are many hormones and signaling molecules involved in the modulation of energy homeostasis, but one of the key regulators is leptin. Leptin is produced in adipose tissue and acts in the central nervous system to suppress food intake while increasing energy expenditure (2, 47, 105, 148). Upon its discovery, leptin was believed to be the magic pill for curing obesity, but it was soon apparent that the situation was far more complex than researchers first thought. While leptin was effective at treating patients with congenital leptin deficiencies, it had little impact in obese rodents and humans with normal genetic profiles (109). Circulating leptin levels rise in proportion to adiposity, but this elevated leptin over time becomes unable to properly regulate energy homeostasis in obese individuals (18, 75, 97). This phenomenon is termed leptin resistance, and is common in many obese humans and rodents.

Leptin signaling opposes the activity of the ECS, primarily through downregulating ECs and/or inhibiting their biosynthesis (101, 102, 112, 147). Activation of the CB1 receptor stimulates energy intake and storage. This signaling system is hyperactivated during HF feeding, and data suggest it is chronically overactivated in obese individuals (38). Studies suggest that in states of leptin resistance, leptin is unable to downregulate EC levels, thus allowing the ECS to become overactivated (77).

The increasing obesity rate in humans is often attributed to the consumption of a HF diet. These diets are readily available, energy dense, and highly palatable. In fact, when rodents are provided access to a HF diet ad libitum, they respond with immediate hyperphagia, which normalizes to pre-HF diet levels over time (174). This normalization is dependent upon leptin receptor activity (139, 174).

In this dissertation, the major objective was to further understand the role of leptin and the ECS in energy regulation and the changes associated with both aging and HF feeding. We put forth three major hypotheses during our studies. First, the leptin resistance associated with age-related obesity results in a prolonged hyperphagia during HF feeding, contributing to exacerbated weight gain. Second, the overactive ECS associated with obesity and likely with age-related obesity contributes to the exacerbated hyperphagia and weight gain observed in aged-obese rats during HF feeding. Third, long-term HF feeding or aging leads to leptin resistance, therefore it is likely that the leptin negative modulation of CB1 receptor activity is blunted or absent in obese rodent models. Thus, we predicted that CB1 receptor antagonism would be enhanced in young adult rats with diet-induced obesity due to long-term HF feeding and that this exacerbated CB1 receptor antagonism would be further enhanced in long-term HF fed aged rats. The goals of this dissertation were to, first, characterize physiological responses to a HF diet in rats of varying ages; second, to examine the effects of a CB1 receptor antagonist on body weight and caloric intake with or without HF feeding – both short- and long-term.

Major Finding and Conclusions

In Chapter 3, we demonstrated that as male F344xBN rats age, they are more susceptible to the detrimental effects of a HF diet. When rodents are provided a highly

palatable caloric dense diet ad libitum, they immediately consume an elevated level of calories that gradually normalizes to control levels. However, leptin resistant animals display impaired normalization of this hyperphagia (57, 135, 175). Because aged obese rats are leptin resistant, I hypothesized that they would fail to rapidly normalize the elevated caloric intake after HF diet exposure. Indeed, the 30-month-old rats, the oldest tested, displayed the most exaggerated hyperphagia, reaching the highest peak intake level and requiring the longest amount of time to normalize. Investigation of this phenomenon at multiple ages proved that both the peak of caloric intake up on initiation of HF feeding and the time to normalization increased with age. Moreover, this hyperphagia was a result specific to HF feeding because all of the ages consumed the same amount on the chow diet prior to initiation of the HF diet, confirming earlier studies (152).

This failure to normalize caloric intake after initiation of HF feeding appears to result in greater rate of body weight gain in the aged rats during the first few days of HF feeding. In addition, the rats continued to gain weight even after caloric intake normalization. Together, this suggests that responses to HF feeding are dependent upon the degree of leptin resistance, and the aged rats might have an accompanying decrease in energy expenditure. However, both the young adult and aged HF fed rats responded equally with an increase in UCP1 protein level in BAT. Thus, any changes in energy expenditure do not appear to be related to the thermic effect of food. Research has shown that body weight and physical activity are inversely related so we tested voluntary wheel running, one measure of volitional activity that involves motivational, exploratory, muscular, age, and body size components (152). Because locomotor

activity tends to decline with both age and obesity (22, 30, 41), I predicted that both age and HF feeding would negatively impact voluntary wheel running. In Chapter 3, I showed that the aged rats ran significantly less than the young adult rats on a chow diet, and HF feeding also suppressed the voluntary wheel running activity in the young adult rats. This is consistent with previous reports that aged rats or rats on a HF diet run significantly less than controls (70, 71). Interestingly, the aging and HF feeding suppressive effect on wheel running do not appear to be independent. For example, the oldest group tested ran the least, but HF feeding had little additional suppressive effect. Collectively, these data suggest that the tendency for inactivity with age may be one contributing factor in age-related obesity. In fact, the inactivity with HF feeding may accelerate the rate of diet-induced obesity in all ages.

Using body composition measurements, I showed that aging also increases the susceptibility for fat storage with HF feeding. Studies have shown that rats that are naturally growing to maturity have the ability to store energy as both proteins and lipids. However, as the rats age, protein deposition becomes almost nonexistent and, eventually, all energy consumed is stored as fat (137). Supporting this, the data in Chapter 3 demonstrated that only some young adult rats are susceptible to diet-induced weight gain, whereas all aged rats are susceptible to this negative effect upon initiation of the HF diet. Body composition analysis proved that the aged rats gained a disproportionate amount of body fat compared with young adult counterparts during HF feeding. These data indicate that all aged rats, as opposed to only some young adult rats, are prone to develop diet-induced obesity on a HF diet.

We then verified that 3-month-old male F344xBN rats are leptin responsive while 30-month-olds are completely leptin resistant. The young adult rats displayed dose-dependent anorectic responses, measured by reductions in both food intake and body weight, to a peripheral leptin infusion, but the aged rats were completely resistant to leptin's anorectic effects. Aged rats have reduced numbers of leptin receptors and a parallel decrease in leptin signaling (5, 144). In Chapter 3, I also found increases with age in SOCS-3, a negative regulator of leptin signaling, and PTP1B, a phosphatase that dephosphorylates activated components in the leptin signaling cascade. These changes, along with evidence of leptin resistance in the aged animals are likely to impair the native responses to the endogenous leptin elevation triggered by HF feeding. However, it should be noted that leptin responsiveness was only examined in the 3- and 30-month-old rats. While they demonstrated leptin resistance by 30 months of age, we cannot dismiss the possibility that this resistance may be fully manifested prior to this age. If this is the case, the leptin resistance may only be one factor in the progressive susceptibility to the adipogenic effects of a HF diet with age.

In Chapter 4, I set out to prove that dysregulation of the ECS is at least one contributor in the exacerbated hyperphagia observed in HF fed aged rats. Because these aged rats are leptin resistant, the endogenous leptin may be unable to downregulate ECs. This predicts that CB1 receptors are overactive and that CB1 antagonist administration will have increased efficacy in these leptin resistant rats.

I first demonstrated that daily i.p. AM251 administration in both young adult and aged rats was able to reduce HF-diet intake to a greater extent than chow intake. This is consistent with other studies that showed CB1 antagonist administration preferentially

reduced intake of a sweet diet, in both solid and liquid forms, without affecting chow or water intake (103, 161). However, the larger dose of AM251 tested in Chapter 4 was also able to significantly reduce caloric intake on the chow diet, albeit to a lesser degree than the HF diet. While other researchers have demonstrated that AM251 suppresses feeding equally on chow, high-carbohydrate, and HF diets in rats, they suggest that data interpretation has a large impact on the conclusions reached (5, 144). For example, when rats are provided a highly palatable diet ad libitum, they consume more of these diets than of chow, creating a higher baseline consumption. Thus, if the AM251-induced reduction in caloric intake is compared to the baseline of the respective diet, the results show a more dramatic anorectic effect in the highly palatable diets compared to normal chow.

This aforementioned method of data analysis was employed in Chapter 4, confirming that CB1 receptor antagonism produced greater reductions in the consumption of the highly palatable HF diet when compared to the chow diet. Taken together, these data suggest that the endogenous EC activity may act to increase the preference of the palatable food (77). One possible explanation for the results in Chapter 4 is that the largest dose of AM251 tested (2.78 mg/kg/day) was able to override the majority of the endogenous orexigenic signals, causing the rodents to consume fewer calories overall. In contrast, the lower dose may only override some of endogenous orexigenic signals, explaining why the rats experienced only a minor decrease in caloric intake during low-dose AM251 treatment.

Second, the data in Chapter 4 verified that AM251 responsiveness is enhanced in aged rats and further increased with HF feeding. One possibility, although it is untested

in this dissertation, is that this hyper-responsiveness is due to the state of leptin resistance in the aged rats. When the endogenous leptin is unable to downregulate brain EC levels, the ECs may become elevated and allow the CB1 receptors to become hypersensitive to antagonism. In addition, HF feeding stimulates serum leptin levels. This elevation of leptin levels may induce or exacerbate leptin resistance. If so, the state of leptin resistance may explain the enhanced AM251 responsiveness with HF feeding (64, 153), rather than the palability of the food.

Third, AM251 administration was able to reverse both age-related and diet-induced elevations in adiposity and serum leptin levels. This is consistent with other studies (5, 146), but more experiments are needed to determine if this reversal is a direct effect of AM251 treatment or if it is merely a parallel response. Either way, the lower leptin levels may help to restore leptin responsiveness in these aged rats.

Lastly, I showed that, at the dose tested (0.83 mg/kg/day), AM251 treatment did not alter the preference of the chow and HF diets. When given the choice between the chow and HF diet during daily AM251 administration, the rats – both young adult and aged – continued to consume the HF diet exclusively. Very few other researchers have investigated this phenomenon, but our data are consistent with South, et al. and Arnone, et al. who demonstrated that CB1 receptor antagonist treatment reduced the intake of only the highly palatable HF diet (38, 162).

After examining AM251 responsiveness during the HF diet-induced hyperphagic period in Chapter 4, I sought to investigate AM251 responsiveness after long-term chow or HF feeding in both young adult and aged rats in Chapter 5. Obese animals display enhanced responses to CB1 receptor antagonists compared with lean controls (8, 60,

62, 101). In addition, obesity and consumption of a highly palatable diet raise both central and peripheral EC levels (100). Moreover, anorectic responses to CB1 antagonists are enhanced in lean animals after EC tone is increased, which can be done by exposure to a highly palatable diet or acute fasting (38). These data suggest that there is higher EC tone in obese rodents, and predicts that long-term HF feeding will further enhance AM251 responsiveness in both young adult and aged rats.

Indeed, the data in Chapter 5 verify that the AM251 responsive was enhanced with long-term HF feeding in young adult and aged rats, as measured by an increase in sensitivity and maximum efficacy. These responses were observed with respect to both body weight reduction and the anorectic response, which includes the time course of food consumption as well as the overall reduction in cumulative caloric intake. Moreover, the HF diet-induced increase in AM251 sensitivity is additive with age, with the greatest degree of responsiveness in the aged HF-fed rats. When compared with the aged rats, the HF diet-induced enhancement of AM251 antagonism is modest.

The enhanced efficacy with age and HF feeding appears to be both related to the duration of the diet and the associated established obesity as well as the presence of leptin resistance. In Chapters 4 and 5, the young adult rats displayed similar responsiveness to AM251 regardless of the duration of feeding. However, the AM251 efficacy in the aged rats was enhanced to a greater degree with long-term HF feeding and established obesity (Chapter 5) when compared to the introduction of the HF diet (Chapter 4). In fact, the aged, HF-fed rats were less responsive to peripheral leptin infusion and more responsive to AM251 administration when compared with chow-fed aged rats, although the difference in leptin responses did not reach statistical

significance. This suggests that the duration of dietary exposure and resultant obesity as well as the development of diet-induced obesity significantly contribute to the enhanced AM251 responsiveness observed in long-term HF-fed rats. Because both age and HF diet-induced obesity are associated with leptin resistance, and there is a strong correlation between enhanced AM251 responsiveness and leptin resistance in young adult and aged chow- and HF-fed rats, these data are consistent with the concept that leptin resistance is one contributor to enhanced CB1 receptor antagonist efficacy with HF feeding and age (38, 44, 96).

Obese rodents lacking normal leptin function have higher EC levels than normal controls (38). For example, hypothalamic EC levels are elevated in Zucker rats with defective leptin receptors, db/db mice with no leptin receptors, and ob/ob mice with no leptin (38, 112). However, exogenous administration of leptin in ob/ob mice and normal rodents reduces EC levels back to those of lean control animals. In addition, it is well-known that EC levels are elevated in animals with diet-induced and genetic obesity, but the status of the CB1 receptor activity is still unclear (65). In fact, some data suggest that receptor levels are downregulated during HF feeding. These data do not support my findings of enhanced AM251 efficacy with HF feeding. Thus, the mechanism behind the increased efficacy with HF feeding and age remains speculative.

Interestingly, all the HF-fed rats displayed elevated UCP1 protein levels in BAT, suggesting that there is a component of BAT thermogenesis that is contributing to energy homeostasis in the aged HF-fed rats that is absent in the aged chow-fed rats. Perhaps blockade of this component by the CB1 receptor antagonist contributes to the increased efficacy with age.

In summary, this dissertation compared young adult and aged for to their responses to chow or HF feeding, peripheral leptin infusion, and daily i.p. CB1 antagonist treatment. When male F344xBN rats are provided access to a HF diet ad libitum, they immediately consume more calories than chow-fed controls. In fact, as the rats age, the peak and days required to normalize the elevated caloric intake increases. This hyperphagia in the aged rats is accompanied by a disproportionate gain in fat tissue, and all the aged rats are susceptible to the adipogenic effects of the HF diet while some of the young adult rats appear obesity resistant. After both short- and long-term exposure to the HF diet, AM251 treatment in these young adult and aged rats preferentially reduced consumption of the highly palatable HF diet, but was apparently unable to alter the palatability or preference of the diets when the rats were given a choice. We do not believe that the anorectic effects of AM251 are due to aversion to the drug. However, we did not test this aversion by reintroducing the diets subsequent to the AM251 treatment but in absence of the drug administration. Moreover, the anorectic effects, measured by body weight and caloric intake reduction, of the CB1 antagonist were enhanced with both age and HF feeding. However, this enhanced responsiveness cannot be clearly attributed to the presence of leptin resistance so the underlying mechanism remains speculative.

Future Directions and Potential Improvements

This dissertation was founded on 3 major hypotheses. First, the leptin resistance associated with age-related obesity results in a prolonged hyperphagia during HF feeding, contributing to exacerbated weight gain. Second, the overactive ECS associated with obesity and likely with age-related obesity contributes to the exacerbated hyperphagia and body weight gain observed in aged-obese rats during HF

feeding. Third, long-term HF feeding or aging leads to leptin resistance, and it is likely that the leptin negative modulation of CB1 receptor activity is blunted or absent in obese rodent models. This predicts that CB1 receptor antagonism will be enhanced in young adult rats with diet-induced obesity due to long-term HF feeding and this exacerbated CB1 receptor antagonism will be further enhanced in long-term HF fed aged rats.

In Chapter 3, I confirmed the first hypothesis by demonstrating that as the rats age, both the peak and normalization period of the HF diet-induced hyperphagia increases. This prolonged hyperphagia contributes to an exaggerated body weight gain and, moreover, a disproportionate gain in fat versus lean mass in the aged, leptin resistant rats. In Chapters 4 & 5, I partially confirmed the second and third hypotheses by demonstrating that administration of a CB1 receptor antagonist produces anorectic effects in young adult, leptin responsive and, to a greater extent, in aged, leptin resistant rats. However, more experiments are needed to prove whether the HF diet-induced hyperphagia is dependent on both leptin and CB1 receptor activity and whether the enhanced responsiveness observed in aged, leptin resistant rats is dependent upon the state of leptin resistance.

In Chapter 3, we observed that only some young adult rats, but all aged rats, are susceptible to the adipogenic effects of a HF diet. However, we were unable to provide a clear underlying mechanism for this phenomenon. Future studies could compare measures of energy expenditure during rest and activity, locomotor activity levels, and muscular strength in rats during HF feeding with increasing age. In Chapter 3, we demonstrated that HF feeding and, moreover, aging decreased voluntary wheel running, but it is unclear if the gain in body weight associated with HF feeding and aging

is a contributor to this decrease in activity. Because wheel running may be dependent on muscle strength, we subsequently subjected the aged rats to a grip strength and inclined plane test. There was no statistical significance between dietary groups so limited conclusions could be drawn. Perhaps muscle strength and voluntary activity was already maximally diminished in these rats. Thus, measures of energy expenditure, locomotor activity levels, and muscular strength with HF feeding at various ages throughout the rat's life may provide important information regarding the exacerbated hyperphagia and body weight gain observed during HF feeding. If energy expenditure decreased with age, it would suggest that the exacerbated body weight gain during HF feeding with age, especially that which occurs after the hyperphagia normalizes, may be at least partially due to the decrease in energy expenditure.

In Chapter 4, we speculate that dysregulation of the ECS is at least partially responsible for the exacerbated hyperphagia observed in aged rats on a HF diet. Our data demonstrate that daily administration of a CB1 receptor antagonist reduces both the peak and the days required to normalize the HF diet-induced hyperphagia. At both the low and high doses tested, the CB1 receptor antagonist reduced HF intake to a greater extent than chow intake and prevented HF diet-induced body weight gain in both young adult and aged rats. However, we were unable to prove beyond a doubt that central CB1 receptors are overactivated during this hyperphagia. This could be tested by administration of a CB1 receptor agonist in young adult rats, which would hypothetically increase both the peak and the days required to normalize the caloric intake. In the aged rats, CB1 agonist treatment may not further exacerbate the HF diet-

induced hyperphagia, suggesting that the CB1 receptors are maximally stimulated in these completely leptin resistant rats.

Thus, we administered the CB1 agonist WIN 55, 212-2 by daily i.p. injection using the same experimental protocol as described in Chapter 4 (data not shown). In contrast to our predictions, WIN 55,212-2 treatment reduced caloric intake in the young adult rats. We chose to use WIN 55,212-2 because, at the time, it was one of the most extensively studied commercially available CB1 receptor agonists, it produces all the pharmacological effects of tetrahydrocannabinol (THC), and it has been successfully substituted for all other cannabinoids in discriminative stimulus tests (54, 81). However, because it has high affinity for both CB1 and CB2 receptors and has a moderate selectivity in favor of the CB2 receptor, we feared that AM251 was activating peripheral CB2 receptors, which are highly associated with the immune system. If so, activation of the immune system could cause a discomfort prompting the rats to consume less food. To avoid activating peripheral CB2 receptors, we next administered WIN 55,212-2 directly into the hypothalamus (data not shown).

A brain infusion cannula directed at the ventral medial hypothalamus (VMH) or ventral tegmental area (VTA) and infusion pump were surgically implanted in young adult rats. The rats were allowed to rest for one week before WIN 55,212-2 was loaded into the infusion pumps. However, even central infusion of WIN 55,212-2 caused a reduction in caloric intake and body weight that was not seen in control rats. There is no agreement in the literature regarding the effect of CB1 agonist administration on food intake. While some investigators report CB1 agonist-mediated decreases in food intake or no change at all (73, 104), others report significant increases in food intake after

central or peripheral administration of various CB1 agonists (174). In fact, it appears that many factors contribute to apparent controversial results in the literature, including the hunger status of the rodent; the location, time, and method of agonist delivery; and the dose of the agonist. However, future studies could examine CB1-mediated feeding behavior using another agonist. An ideal agonist is one that preferentially binds to the CB1 receptor, is stable at room temperature, is more water soluble, and produces the full range of pharmacological effects observed with THC.

Another way to investigate the interactions of the leptin and ECS during HF diet-induced hyperphagia is to examine the relationship between maximal leptin signaling and the hyperphagia. Studies in the Scarpace lab have demonstrated that maximal leptin signaling capacity is blunted in young adult rats after only 2 days of HF feeding, suggesting that leptin receptor desensitization occurs rapidly (56). We propose that this desensitization may play an important role in the duration of caloric normalization during HF feeding. Future studies could compare the maximal leptin signaling level at different points along the caloric normalization curve in both young adult and aged rats. Increased desensitization may increase both the peak and the days required to normalize the hyperphagia, whereas maximal leptin signaling may be restored by the time caloric intake is fully restored. In addition, this may shed some light on the interactions of the leptin and CB1 signaling systems during this HF diet-induced hyperphagia. If leptin receptors are desensitized to a greater extent in the aged rats, the leptin negative modulation of the ECS will be absent or blunted for a longer period of time. Because of this, the CB1 receptors may be allowed to become even further overactivated, causing the rats to consume more food for a longer duration.

In order to directly implicate the dysregulation of the ECS during the HF diet-induced hyperphagic period, future studies could also measure CB1 receptor protein level by Western and/or message level by RT-PCR. However, to date, commercially and personally produced CB1 receptor antibodies have been variable, unreliable and nonspecific for the CB1 receptor (18). If specific, reliable antibodies were available, an increase in hypothalamic CB1 receptor number with age and HF feeding may indicate hyperactivation of the ECS. Alternatively, a decrease in hypothalamic CB1 receptors with age and HF feeding would support our data that the same dose of AM251 was able to produce greater anorectic effects in the aged obese rats. In addition, measurements of EC levels in white adipose tissue would provide an important piece of the puzzle (64). AM251 administration produces body weight reductions long after the food intake response has normalized (81). This suggests that a peripheral activation of CB1 receptors, particularly on adipose tissue, may be involved.

Similarly, future studies could include measurements of brain EC levels with age and HF feeding. EC levels have been shown to vary in different brain regions, but the changes most related to feeding behaviors are those in regions of the brain dealing with feeding, satiety, and reward (38, 67). Of particular interest are those regions that contain both leptin and CB1 receptors, such as the hypothalamus and the VTA (140). If EC levels in these regions are elevated with both age and HF feeding, both of which are associated with leptin resistance, this would support the hypothesis that leptin in a leptin resistant state is unable to downregulate EC levels. Thus, these EC levels may become elevated, creating hyperactive CB1 receptor activity, and further inducing food intake in these leptin resistant animals.

In Chapter 4, we demonstrated that daily i.p. administration of AM251 in young adult and aged rats was unable to alter the preference of the chow diet when compared to the HF diet. However, future studies may examine the effect of AM251 on food intake of two diets closer in palatability. For example, if the rats were given a choice between 60% and 32% HF diets, there may be a decrease and increase in the respective intake of diets with AM251 treatment. In addition, studies suggest that experience with the diets before the choice is important. Unpublished studies in the Scarpace lab indicate that when rats are conditioned on one diet and then introduced to another, they will initially consume elevated amounts of the second diet. Thus, our choice experiments in Chapter 4 barely scratch the surface in this area of research because there are many other diet combinations and experimental designs that can be studied in the future.

In Chapter 5, we suggested that the leptin negative modulation of CB1 receptor activity is impaired with age. We confirmed that CB1 antagonist administration produced greater anorectic effects, measured by a decrease in caloric intake and an inhibition in HF diet-induced body weight gain, in aged rats compared with young adult rats. In fact, this enhanced efficacy was further emphasized with HF feeding, producing the greatest anorectic effects in the aged, HF-fed rats. Moreover, we were able to verify that these results were directly correlated to the leptin resistant state in these rats. One possible future study to provide further confirmation is to create leptin resistant young adult rats using leptin gene delivery to induce central leptin overexpression. A previous study in the Scarpace lab showed that central leptin gene delivery in young adult rats induced a 40% increase in cerebrospinal fluid leptin levels nearly 50 days after the third

ventricle injection (130). Moreover, the leptin-treated young adult rats did not become obese as is typically associated with increased leptin levels. After the development of leptin-induced leptin resistance, these animals display exacerbated hyperphagia and body weight gain, similar to what has been described in this dissertation in aged rats, when exposed to a HF diet . I predict that daily i.p. CB1 antagonist treatment in these rodents will be highly efficacious and produce dramatic anorectic effects, like those described here in aged, leptin resistant rats. Therefore, this animal model may prove to be an ideal situation for studying CB1 antagonist responsiveness in leptin resistant, but not obese, animals. If hypothalamic EC and CB1 receptor levels are elevated in these rats, it would provide a direct connection between leptin resistance and the overactive ECS.

Conclusions

Obesity is a serious, chronic disease that increases the risk of developing various secondary diseases including type 2 diabetes, uterine cancer, gallbladder disease, osteoarthritis, stroke, hypertension, coronary heart disease, breast cancer, and colon cancer. Caloric dense, highly palatable HF diets are often implicated as a primary contributor to the prevalence of obesity today. The leptin and endocannabinoid signaling systems induce opposing effects on feeding behavior and fat storage, and the dysregulation of these systems may be important in age-related and diet-induced obesity.

In the research described in this dissertation, we demonstrated that all aged rats, while only some young adult rats, are susceptible to the adipogenic effects of a HF diet. These aged, leptin resistant rats, display increased and prolonged caloric consumption during HF feeding when compared to young adult controls. We revealed that the CB1

antagonist-mediated anorectic effects are greater in these aged rats and further enhanced with both short- and long-term HF feeding. These results contributed to the understanding of the interactions between the leptin and endocannabinoid signaling systems as well as how these interactions change with both age and HF-feeding. I hope these findings will contribute to the prevention and treatment of obesity.

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BIOGRAPHICAL SKETCH

Melanie Kae Judge was born in Sarasota, Florida in 1983 to Robert & Susette Mitchell. Four years later, the Mitchells were expecting again. They were crazy enough to give her the opportunity to name her new baby sister, already knowing that Melanie had a female doll which she called “Tim.” Thankfully, one of Melanie’s best friends at the time had a unisex name so Robin Lynn Mitchell soon became a shining star in the Mitchell family. Robin, being blessed with many talents, dabbled in horseback riding, playing the violin, gymnastics, dance, oil painting, and other various forms of art. On the other hand, Melanie excelled in science and math from an early age. Throughout elementary and middle school, she won first place in many school science fairs and even won first place in the Martin County Science Fair in second grade. Melanie used sports (soccer, basketball, softball, track & field, swimming) as a means of staying in shape, meeting new people, and keeping a competitive edge. She attended the prestigious Florida State University and was awarded many leadership and community service honors, including induction into the Seminole Torchbearers, Omicron Delta Kappa, and the National Society of Collegiate Scholars. Melanie received a Bachelor of Science degree in biochemistry in 2005 with the hopes of joining a Federal Bureau of Investigation forensic science team. When these plans changed rather abruptly, she enrolled in the Interdisciplinary Program in Biomedical Research at the University of Florida in 2005. She has spent the last several years working with Dr. Philip J. Scarpace studying the molecular basis of obesity. In the future, Melanie plans to continue her career in obesity research while raising at least 3 children and continuing to do a lot of baking.