IMMUNOLOGICAL AND BEHAVIORAL CONSEQUENCES OF HIGH-FAT DIET FEEDING IN MICE

BY

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THESIS

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ABSTRACT

Obesity is a harmful and costly condition which continues to increase in prevalence. Co-morbidities accompanying this disease include brain-based disorders that impact cognition and mood. Anhedonia is a biobehavior associated with depression that manifests in mice as a loss of interest in consuming a highly palatable sucrose or saccharin solution. Here we show that obese mice fed a high-fat diet (HFD), fail to exhibit an interest in saccharin (27% vs 74%) compared to mice fed a low-fat diet (LFD) ($p < 0.007$). When HFD mice undergo a fasting period of 24 hours, saccharin preferences equilibrate to water consumption (51% saccharin vs 49% water). Other behaviors such as immobility during the forced swim test (FST) and burrowing behavior are improved by fasting in HFD mice. We have previously shown and demonstrate here that basal serum leptin and IL-1RA concentrations are increased in mice fed HFD for 12 weeks compared to mice fed LFD. Leptin has been associated with modulation of motivation to obtain reward and anti-depressant activity, and IL-1RA has been implicated in the mediation of central leptin resistance. To examine if these cytokines contributed to the increased saccharin consumption and other behaviors observed in fasted HFD mice, we measured serum concentrations during the fed and fasted states. Leptin decreased, although not significantly, in mice fed a HFD (43,844 vs 42,791 pg/mL) and LFD (8,118 vs 3,104 pg/mL) after a 24-hour fast. IL-1RA did not significantly change in either group fed LFD or HFD when fasted, and IL-1β was not detectable in any group. We further investigated differences in leptin, leptin receptor (LepR), IL-1RA, IL-1R1, IL-1R2, IL1α and IL-1β brain expression in fed and fasted HFD and LFD mice utilizing real-time PCR (RT PCR) gene expression analysis. A 2-fold increase in leptin expression was seen in both LFD (1 vs 2) and HFD (2 vs 4) after a 24 hour fast ($p < 0.05$). IL-1RA expression was increased 2.3-fold in HFD compared to LFD in the fed state (1 ± 0.31 vs 2.26 ± 0.66) ($p < 0.05$) and trended toward increasing in the HFD fed vs fasted (1.89 vs 4.16) but this was not significant. IL-1R1 expression was decreased in fed (0.79 ± 0.09 vs 1 ± 0.08) and fasted (0.68 ± 0.14 vs 0.88 ± 0.07; $p < 0.05$) HFD mice compared to LFD mice. IL-1R2 expression was decreased during fasting in mice fed HFD compared to mice fed LFD (1.013 ± 0.12 vs. 1.40 ± 0.08; $p < 0.005$). IL-1α expression was increased 1.5-fold after fasting in mice fed HFD compared to mice fed LFD (0.923 ± 0.14 vs. 0.59 ± 0.15; $p < 0.05$) and decreased by 1.6-fold during fasting within the LFD group compared to its fed state (0.6 ± 0.15 vs 1 ± 0.19; $p < 0.05$). Taken together, these data show that changes in brain gene expression of leptin, IL-1α, IL-1R2, IL-1R1 and IL-1RA occur during fasting, and that these changes may be affected by nutritional status and contribute to differences in saccharin preference in mice fed a HFD. In order to determine if IL-1RA played a role in saccharin consumption, IL-1RA knockout mice were given a saccharin preference test. Similar to mice fed HFD, IL-1RA KO mice had an aversion to saccharin compared to control WT mice. IL-1RA KO mice consumed 12% saccharin solution compared to control WT mice that consumed 65% of their total daily fluid intake from saccharin solution ($p < 0.005$). Taken together, these data indicate that a deviation in normal levels of brain IL-1RA may contribute to saccharin aversion, and that fasting has a behavioral benefit in mice fed HFD that may be reliant on increased leptin expression in the brain.

To my husband

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LIST OF ABBREVIATIONS

Blood brain barrier (BBB), central nervous system (CNS), dopamine (DA), diet-induced obesity (DIO), forced swim test (FST), hypo-pituitary adrenal (HPA), indoleamine 2,3–dioxygenase (IDO), insulin-like growth factor (IGF-1), interferon (IFN), interleukin-1 (IL-1), interleukin -1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-1 beta converting enzyme (ICE), interleukin-1 receptor 1 (IL-1R1), interleukin-1 receptor antagonist (IL-1RA), interleukin-6 (IL-6), interleukins (IL), intracerebroventricular (ICV), leptin receptor (Ob-R), leptin receptor a (Ob-Ra), leptin receptor b (Ob-Rb), lipoprotein lipase (LPL), major depressive disorder (MDD), mitogen activated protein kinase (MAPK), pathogen associated molecular patterns (PAMPS), selective serotonin reuptake inhibitor (SSRI), serotonin (5-HT), tail suspension test (TST), toll-like receptors (TLRs), tumor necrosis factor alpha (TNF-α), type 2 diabetes (T2D), white adipose tissue (WAT), wild-type (WT)

CHAPTER ONE

INTRODUCTION

This section provides a review of the literature on obesity, the innate immune system, and related behavioral consequences of obesity. Obesity is a state of increased lipid accumulation in white adipose tissue (WAT) that is linked not only to weight gain, but also to chronic, low-grade inflammation (Lago et al., 2007). Over the past two decades, inflammation has been recognized as a major contributor to the pathogenesis of several common diseases, including atherosclerosis, diabetes, asthma, cancer, and obesity. Inflammation of adipose tissue accompanies obesity and contributes to its sequelae (Nathan, 2008). Recognition of the connection between obesity and inflammation dates back to 1993, when the inflammatory cytokine tumor necrosis factor (TNF-α) was shown to be secreted from adipose tissue in obese rodents and contribute to insulin resistance (Hotamisligil et al., 1993). Ten years later, it was determined that adipose tissue is infiltrated by macrophages in obese children and adults (Weisburg et al., 2003) and mice (Xu et al., 2003), in proportion to how far they exceed normal body weight. These macrophages produce inflammatory substances that can act in the periphery and in the central nervous system. Recently, inflammatory changes including increased leakiness of vessels and adhesion of leukocytes and platelets were observed in the visceral, adipose tissue in obese but not lean mice (Nishimura et al., 2008). Obesity leads to inflammation, and having excess adipose tissue can exacerbate obesity by dysregulating adipocyte-derived regulators of feeding (Nathan, 2008).

Cytokines and Obesity

IL-1

Cytokines that are produced by leukocytes and exert their effects preferentially on other white cells are called interleukins (IL). These mediators are also produced by and act on many other cells other than leukocytes (Litwack, 2006). IL-1 is produced from two distinct genes that encode proteins, IL-1α and IL-1β, that share only 26% homology (Dinarello et al., 1994). IL-1β is synthesized in an inactive form as a precursor that is cleaved by IL-1β converting enzyme (ICE) to its bioactive form (Schmidt, 1984). A third member of the IL-1 family is a naturally occurring inhibitor, IL-1 receptor antagonist (IL-1RA), which blocks the binding and biological activity of both IL-1α and IL-1β (Cominelli et al., 1990). The IL-1RA molecule binds specifically to interleukin-1 receptors (IL-1Rs), but fails to trigger internalization of the ligand-receptor complex inhibiting initiation of intracellular responses (Arend et al., 1993).

Two receptor isoforms have been identified and exist in both membrane-bound and soluble forms (Slack et al., 1993). These include an 80 kDa type 1 IL-1 receptor (IL-1R1) (Resch et al., 1986; Horuk et al., 1987) and a 68 kDa type II IL-1 receptor (IL-1R2) (Re et al., 1994). Soluble forms of the receptors are generated by proteolysis of the extracellular domains and soluble IL-1R2 seems to bind to IL-1β (Symons et al., 1991), while soluble IL-1R1 seems to bind to IL-1ra (Svenson et al., 1993) and IL-1α (Dinarello et al., 1994). Of the two membrane-bound receptors, only IL-1R1 is physiologically functional, activating intercellular IL-1 signaling cascades. IL-R2 acts as a decoy receptor and negative regulator of IL-1β and IL-1α because binding does not initiate intercellular signaling (Re et al., 1994; Labriola-Tompkins et al., 1991). These observations indicate that the relative proinflammatory activities of IL-1 in a diseased

tissue will depend on the concentrations of IL-1α, IL-1β, IL-1RA, membrane bound forms of IL-1R1 and IL-1R2, and soluble forms of the two IL-1Rs.

IL-1β stimulates many physiological responses including activation of the hypothalamic-pituitary-adrenal axis (HPA), febrile response, sickness behaviors and anorexia (Sternberg et al., 1989; MacPherson et al., 2005). IL-β has been shown to be increased in the serum of obese diabetics and is related to progression of associated complications (Pickup, 2004).

IL-1α polymorphisms have been suggested to be associated with rheumatoid arthritis and Alzheimer's disease (Rainero et al., 2004). Additionally, IL-1α is essential for maintenance of skin barrier function, especially with increasing age (Barland et al., 2010).

IL-1β, IL-1RA, and Obesity

In the absence of a chronic disease state such as obesity and type II diabetes (T2D), IL-1 plays an important role in energy homeostasis (Matsuki et al., 2003). It has been suggested that IL-1β can modulate lipid metabolism by suppressing the activity of lipoprotein lipase (LPL) (Doerrler et al., 1994) and may regulate adipocyte function by inhibiting maturation (Gregoire et al., 1992).

IL-1 receptor antagonist levels are elevated in the circulation of patients with a variety of inflammatory, infectious, and post-surgical conditions (Arend, 2002), with obese patients reported to have a 7-fold increase in serum IL-1RA relative to controls (Meier et al., 2002). It is likely that IL-1RA in the circulation diffuses into the tissues and influences the local ratio of IL-1RA to IL-1β. However, because of the spare receptor effect, 100-fold or greater levels of IL-1RA over IL-1β are necessary to functionally inhibit the biologic effects of IL-1β on target cells (Arend, 1990). It has been shown that IL-1RA is a pro-adipogenic factor that inhibits IL-1β

induced energy expenditure leading to weight gain and obesity (Perrier et al., 2006). IL-1RA knockout mice are much leaner than wild type mice due to a significant decrease in fat mass, which is attributed to impaired lipid accumulation (Matsuki et al., 2003). Therefore, the IL-1β/IL-1RA system may exhibit some control on lipid metabolism through direct control of adipose tissue.

The balance between IL-1RA and IL-1β levels in local tissues influences the physiologic or pathophysiologic effects of IL-1β. An imbalance between IL-1β and IL-1RA is one of the factors influencing the course, the susceptibility to and the severity of many diseases (Arend et al., 2002). In morbid obesity, leptin may exert its effects through the IL-1 pathway, and IL-1RA may contribute to central leptin resistance because it crosses the BBB (Guitierrez et al., 1994). Leptin induces IL-1RA expression in the brain including the hypothalamus (Hosoi et al., 2002), and injection of IL-1RA into the cerebral ventricles inhibits leptin-induced reduction in food intake (Luheshi et al., 1999). IL-1RA knockout mice have an increased food intake relative to their body weight, but have a lean phenotype (Somm et al., 2005). This could be a consequence of increased energy expenditure or a significant reduction in blood leptin levels (6-fold lower compared to wild type). Circulating levels of proinflammatory cytokines including IL-1 are not significantly different from those in wild type mice (Somm et al., 2005)

Long-term feeding on a HFD modulates the expression of more than 10% of genes expressed in the hypothalamus of rodents (DeSouza et al., 2008). The majority of genes undergoing differential expression belong to families of genes with immunological function. Among these, a few cytokines were identified by real-time PCR, ELISA and immunoblot. These cytokines included TNF-α, IL-1β, and IL-6, which were all significantly increased in the hypothalamus of rats fed a HFD.

Leptin

Given that its absence accounts for the obese phenotype of ob/ob mice, this 16-kDa protein was named leptin, derived from the Greek, *leptos*, meaning *thin* (Halaas et al. 1995). Leptin was originally identified in 1994 as the gene responsible for the obesity syndrome in *ob/ob* mice by Freidman and colleagues (Zhang et al., 1994). Leptin is highly conserved among species, and its structure is cytokine-like, particularly resembling interleukin-6 (IL-6) (Madej et al., 1995). Therefore, the structure of leptin suggests that it might be classified as a cytokine. It is produced by adipose tissue in proportion to body mass (Juge-Aubry et al., 2002) and is best known for its role in regulating satiety and energy storage in the form of fat.

In addition to playing a key role in energy regulation, leptin regulates endocrine and immune functions (Faggioni et al., 2001). Leptin levels increase acutely during infection and inflammation, and may represent a protective component of the host response to inflammation (Faggioni et al., 2001). Leptin acts in response to challenge of the innate immune system, leading to increased phagocytic function in monocytes/macrophages and augmented LPS-induced proinflammatory cytokines such as TNF-α, IL-6, and IL-12 (Matarese et al., 2005) as well as the anti-inflammatory cytokine IL-1RA (Dreyer et al., 2003). Leptin deficiency increases susceptibility to infections and inflammatory stimuli and is associated with the dysregulation of cytokine production. It is clear that leptin has many roles in mammalian physiology by the complex syndrome exhibited by leptin-deficient *ob/ob* and receptor deficient *db/db* mice. These mice are not only obese, but have abnormalities in reproductive function (Chehab et al., 1996), hormone levels (Flier et al., 1998), wound repair (Frank et al., 2000), bone structure (Ducy et al., 2000) and immune function (Howard et al., 1999).

Leptin receptors are richly expressed in the centers of the brain responsible for maintaining energy homeostasis (Friedman et al., 1998), and leptin exerts most of its classical regulatory roles through this system. Leptin receptors are also expressed in peripheral tissues including those of the immune system such as the spleen (Gainsford et al., 1996), thymus (Fei et al., 1997), lung (Tsuchiya et al., 1999), and leukocytes (Cioffi et al., 1996). Six leptin receptor isoforms have been identified to date, all of which have identical or nearly identical extracellular domains, but differ in their transmembrane and intracellular domains (Tartaglia, 1997). Leptin receptor b (Ob-Rb) is the only isoform with complete transmembrane and intracellular domains and as such signals primarily via the JAK2-STAT3 pathway (White et al., 1997) as well as mitogen-activated protein kinase (MAPK) signal transduction pathways (Vaisse et al., 1996). The Ob-Rb is also expressed on all cells of the innate immune system (Matarese et al., 2005) and exerts action on the immune system via T-cells, lymphocytes, monocytes, and macrophages (Juge-Aubry et al., 2002).

Ob-Rb has even been shown to be expressed in a limited population of taste cells of lean mice (Kawai et al., 2000). This study, demonstrated that the taste organ is a peripheral target for leptin and that leptin inhibits sweet responses through activation of outward K^+ currents in lean mice. They concluded that defects in the leptin suppression system in *db/db* mice may lead to their enhanced neural and behavioral sensitivities to sweet stimuli. When energy levels are low, leptin secretion decreases and various orexigenic neuropeptides are generated contributing to feelings of hunger. Leptin acts through its full-length isoform functional receptor (Ob-R) (Matarese et al., 2005) and has been shown to cross the BBB (Banks et al., 1996) via a specific, saturable transport mechanism that is believed to involve leptin receptor a (Ob-Ra) (Banks et al., 1996). It can also cross by way of the median eminence, where its main action is in the central

nervous system (CNS), especially the arcuate, ventromedial, and dorsomedial hypothalamus (Elmquist et al., 1998).

Leptin and Obesity

The adipocyte-derived hormone, leptin, plays a central role in energy homeostasis and resistance to its actions is associated with obesity (Friedman et al., 1998). Since leptin can also modulate inflammation (Faggioni et al., 2001), it may integrate energy balance and systemic inflammation. Leptin is involved in the pathogenesis of insulin resistance, and induces pancreatic β cell apoptosis and impairs β cell function via IL-1β signaling in human islets (Maedler et al., 2004). The concentration of leptin in blood plasma is maintained between 1 and 20 ng/mL in lean animal and human subjects, but it may be as high as 100 ng/mL in obese subjects (Maffei et al., 1995). The circulating level of leptin reflects its production by white adipocytes and exhibits both long-term (days) and short-term (hours) changes. Mechanisms that drive changes in fat storage regulate long-term changes in leptin production (Frederich et al., 1995). Not only are increased adipocytes expected to produce more leptin, but also adipocytes containing more fat synthesize more leptin (Hamilton et al., 1995). Short-term changes occur within a few hours, independent of a change in body mass and appear to involve insulin, as evidenced by a positive correlation between plasma levels of leptin and insulin (Ahren et al., 1997) and by the ability of insulin treatment to increase leptin production (Mueller et al., 1998).

Rodents with diet-induced obesity (DIO) exhibit high plasma leptin and resistance to the hypermetabolic and anorexic effects of leptin (Lin et al., 2000). Resistance to the weight-reducing effects of leptin can contribute to the progression of obesity and unresponsiveness to leptin appears to involve decreased leptin transport across the BBB and impairment of Ob-Rb

signaling (Munzberg et al., 2005). Behavioral depression is a manifestation of systemic inflammation that is augmented in the absence of leptin signaling and aimed at energy conservation (Romanovsky et al., 1996). Therefore, it is possible that suppression of leptin production under conditions of negative energy balance augments behavioral depression as well, though this possibility has not been previously tested in a direct experiment. In diet-induced obese animals that are unresponsive to leptin, this malfunction can result in increased behavioral depression of systemic inflammation (Steiner et al., 2006).

Leptin and Starvation

During fasting or starvation, leptin levels drop disproportionately to the decrease in adipose tissue mass and the correlation between leptin levels and fat stores is lost (Ahima et al., 1996). During starvation, leptin is not only an indication of energy reserve, but a neuroendocrine signal that is critical for the brain to initiate adaptive responses to starvation including metabolic, endocrine, and immunological changes. Suppression of immune, reproductive, and thyroid function and stimulation of the HPA axis are changes that are induced by starvation (Schwartz et al., 1997). A consequence of prolonged fasting is lymphoid atrophy, suggesting that leptin may be required to maintain normal lymphoid tissue. This is substantiated in that spleen and thymus weight, as well as thymocyte number were normalized in starved ob/ob mice treated with leptin (Howard et al., 1999). In a study by Mito et al (2004), control mice and mice fed a HFD were fasted for 48 hours and weights of immune organs, cytokine production and proliferation of cultured splenocytes were measured. Although starvation of the control mice dramatically reduced all three parameters, these levels returned to those of free-feeding groups with

exogenous leptin administration. However, these effects of leptin were not observed in obese mice.

Leptin and Reward

Food restriction of experimental animals has been historically imposed to increase the occurrence of the behaviors the experimenters sought to study. Ferster and Skinner (1957) observed that response rates on different schedules of reinforcement in pigeons were significantly increased by restriction of their body weights. They described an inverse relationship between the birds' body weight and their response for food pellets. Obesity may be partly related to reward and hedonic mechanisms and a failure of regulation of the reward systems. Food deprivation or negative energy balance, promotes responding for foods and other reinforcements. Our current knowledge is that negative energy balance leads to lower levels of circulating leptin, pointing to the hypothesis that low levels of this hormone might be associated with an increased response to obtain rewards (Figlewicz et al., 2009). The corollary hypothesis is that increased levels of leptin might be sufficient to attenuate response to a reward.

The major neurotransmitter pathways associated with motivation and hedonics include mesolimbic dopamine (DA) and certain CNS opioid pathways (Figlewicz et al., 2009). Activation of midbrain DA neurons has been implicated in the motivating, rewarding, reinforcing and incentive salience properties of natural stimuli such as food and water (Berridge et al., 1998), as well as drugs of abuse (Smith, 1995). In human (Wang et al., 2001) and animal studies (Bina et al., 2000), changes in central DA have been suggested to contribute to the development of obesity. Therefore, it is possible that dysregulation of these circuits may adversely affect body weight regulation and that obesity is capable of altering processes within

the endogenous reward system of the brain. Sipols and colleagues demonstrated suppression of acute sucrose licking and sucrose self-administration with intraventricular leptin administration (Figelwicz et al., 2006) in rats fed chow *ad libitum*. Taken together, these results indicate that leptin, across a concentration range from fasting to free-feeding to elevated can modify behaviors that reflect acute and learned reward evaluation independent of their regulatory actions on body mass and adiposity.

There is direct evidence that leptin decreases DA release and the electrical activity of DA neurons, resulting in decreased locomotor activity and decreased intake of palatable substances (Hommel et al., 2006). In a study that used rats fed HFD for an extended period of time, sucrose self-administration decreased. Plasma leptin levels were increased after 3 months (Davis et al., 2008). It could be inferred from this study that a "contrast effect" for food occurred and that the difference in response to sucrose was due to the already present palatable HFD. Consistent with this, DA turnover in the nucleus accumbens (NAc) was substantially decreased in the HFD relative to LFD. Other studies suggest that leptin may have trophic effects on DA neurons; therefore, models of extreme starvation, leptin resistance, or obesity may result in impaired DA neuronal function and signaling (Weng et al., 2007). Leptin effects on reward circuitry may be bimodal, such that low concentrations of leptin sustain dopaminergic neuronal viability and intermediate physiological levels suppress reward function but elevated levels, linked to resistance and metabolic pathophysiology, lead to impaired dopaminergic function. This theory may provide a link between obesity and common psychiatric disorders such as depression and drug addiction (Figlewicz et al., 2009).

Cytokine-Induced Sickness Behavior

Sickness behavior refers to a coordinated set of behavioral changes that develop during the course of an infection. At the molecular level, these changes are caused by the effects of pro-inflammatory cytokines, such as IL-1β and TNF-α in the brain (Dantzer, 2001). Symptoms of infection and inflammation include fever and other physiological and behavioral changes. A sick individual may experience weakness, malaise and inability to concentrate. Depressed mood, manifesting as decreased interest in physical surroundings and normally pleasurable food and drink, may occur as well. The individual becomes preoccupied with their own body and the suffering that they are experiencing. This constellation of nonspecific symptoms is referred to as "sickness behavior" (Dantzer, 2001). In mice, injection of IL-1β or TNF-α causes a behavioral inclination to stay in the corner of their home cage in a hunched posture and show little or no interest in their physical and social environment. These animals also show decreased motor activity, social withdrawal, reduced food and water intake, increased slow-wave sleep and altered cognition (Dantzer et al., 2007). Anti-inflammatory cytokines such as IL-1RA and IL-10 regulate the intensity and duration of sickness behavior, possibly by inhibiting proinflammatory cytokine production and attenuating pro-inflammatory cytokine signaling (Heyen et al., 2000; Arakawa et al., 2009). In particular, intracerebroventricular (ICV) administration of IL-10 or insulin-like growth factor (IGF-1) attenuates behavioral signs of sickness induced by centrally injected lipopolysaccharide (LPS) (Bluthe et al., 1999). These data indicate that in the brain, the natural balance between pro-and anti-inflammatory cytokines regulate the intensity and duration of the response to immune stimuli.

In the brain

Soluble mediators released from macrophages during inflammation, such as IL-1β, IL-6, TNF-α, and the interferons (IFN) can act on the brain in several ways. Primary afferent neurons innervating the body site where the inflammation is transducing their signal to the brain. Additionally, in the humoral pathway, Toll-Like Receptors (TLRs) on macrophage-like dendritic cells located in the circumventricular organs and the choroid plexus outside the BBB, respond to circulating pathogen-associated molecular patterns (PAMPs) by producing pro-inflammatory cytokines which can pass the BBB and enter the brain by volume diffusion (Quan et al., 1998). A third pathway comprises cytokine transporters at the BBB. Pro-inflammatory cytokines overflowing in the systemic circulation can gain access to the brain through saturable transport systems (Banks et al., 2006). Finally, a fourth pathway involves IL-1 receptors that are located on perivascular macrophages and endothelial cells of brain venules (Schiltz et al., 2002; Konsman et al., 2004).

Molecular biology studies have demonstrated that peripheral cytokines induce the synthesis and release of cytokines in the brain by microglial cells (Gatti & Bartfai, 1993; Laye et al., 1994). For instance, administration of IL-1RA into the lateral ventricle of the brain to block brain IL-1 receptors abrogated the depressive effect of peripherally administered IL-1 on social exploration in rats exposed to a juvenile (Kent et al., 1992). The brain circuitry that mediates various behavioral actions of cytokines is not completely known. Different behaviors may be mediated in different brain areas, but ultimately the site of action of the cytokine message depends on the localization of cytokine receptors or receptors for intermediates such as prostaglandin E2 (Dantzer et al., 2007). IL-1 receptors were first localized in the granule cell layer of the dentate gyrus, the pyramidal cell layer of the hippocampus and the anterior pituitary

gland (Parnet et al., 2002). More recently, they were identified in endothelial cells of brain venules throughout the brain, at a high density in the preoptic and supraoptic areas of the hypothalamus and the subfornical organ, and a lower density in the paraventricular hypothalamus, cortex, nucleus of the solitary tract and ventrolateral medulla (Konsman et al., 2004).

Role for cytokines in depression and relation to obesity

Depression is the most prevalent and life-threatening mental disorder with an overall lifetime prevalence rate of 20% of the population worldwide (Lu, 2007). Obesity has been found to be associated with negative health outcomes (Must et al., 1999; Pi-Sunyer, 1993), functional impairment (Fontaine & Barofsky, 2001) and increased mortality (Allison et al., 1999). Obesity and depression may perpetuate one another; in that obesity may increase the risk for depression and depression may promote obesity. Understanding and managing the mechanisms that link obesity and depression is crucial to the treatment of individuals who are afflicted with both conditions.

The HPA axis has been suggested as a possible neuroendocrine link between depression and the development of obesity. It has been suggested that antidepressants may exert their primary effect through regulation and normalization of the HPA response (Holsboer & Barden, 1996) and that failure to regulate the HPA response during treatment elevates the risk for depressive relapse (Holsboer, 2000).

The contribution of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IFN-γ to the development and pathogenesis of depression has been examined (Kenis & Maes, 2002). Increased inflammatory responses have been found in depressed individuals (Carney et al., 2002)

and this may be exaggerated if obesity is also present (Miller et al., 2002). IL-6 levels decrease with selective serotonin reuptake inhibitor (SSRI) treatment in patients with major depressive disorder (MDD) (Basterzi et al., 2005).

It has also been suggested that the enzyme indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan to kynurenine, plays a key role in inflammation induced depression. When tryptophan is shuttled down the kynurenine pathway, there is less tryptophan available to make serotonin (5-HT). This resembles tryptophan depletion, which is used as an experimental paradigm to induce depressive symptoms in susceptible individuals (Neumeister et al., 2003). In microglia and macrophages, IDO expression is induced by Th1 cytokines, IFN-γ, IL-1β, and TNF-α, and inhibited by Th2 cytokines, IL-4 and IL-10 (Muller & Schwartz, 2007). Therefore, excess inflammation may reduce 5-HT levels through activation of this pathway in central and peripheral macrophages, contributing to depression.

Current treatment of depression is largely prescription of antidepressants that exert their therapeutic effects by promoting monoaminergic neurotransmission (Berton et al., 2006). However, this treatment does not fulfill expectations in terms of efficacy, onset of action and tolerability. Antidepressants have also been shown to inhibit IFN-γ induced microglial production of IL-6 and nitric oxide (Hashioka et al., 2007) suggesting that inhibition of brain inflammation may be one mechanism of action of antidepressants.

When activation of the peripheral immune system continues unabated, immune signaling to the brain can lead to an exacerbation of sickness and the development of symptoms of depression in vulnerable individuals. This may account for the increased prevalence of clinical depression in physically ill people (Dantzer et al., 2007). The similarity between the symptoms of cytokine-induced sickness behavior and depression are abundant. In both cases, there is

withdrawal from the physical and social environment that is accompanied by pain, malaise and decreased reactivity to reward (anhedonia) (Dantzer et al., 2007). Importantly, components of sickness behavior, such as decreased preference for sweet solutions and reduced social exploration, are improved by anti-depressant treatment (Yirmiya et al., 1999). Major depressive disorders are more prevalent in patients afflicted with conditions that lead to chronic inflammation, including cardiovascular disease, T2D, rheumatoid arthritis and obesity compared to the general population (Markowitz et al., 2008).

In the absence of any knowledge of the causal factors of depression, most animal models of depression are based on behavioral and pharmacological analogies (Dantzer, 2001). At the behavioral level, the two main symptoms that are usually considered include the deficit in escape/avoidance learning and anhedonia which is the diminished ability to experience normally pleasurable stimuli. Attenuated response to a saccharin solution in response to LPS indicating anhedonia was observed and reconciled by chronic but not acute treatment with the antidepressant drug Imipramine (Yirmiya, 1996). Depression may represent a maladaptive version of cytokine-induced sickness occurring when the activation of the innate immune response is increased in intensity and/or duration or takes place in the context of an increased vulnerability to depression (Nemeroff & Vale, 2005).

An example of how inflammation can modulate depression is a study that indicated that aged mice were more sensitive than young adults to not only LPS-induced sickness behavior but also LPS-induced depressive-like behavior, and this is associated with an exaggerated inflammatory response in the brain. However, in the absence of an acute immune stimulation, aged mice do not differ from young adults in their behavior in animal models of depression

(Godbout et al., 2008). In summary, in this study, their increased inflammatory status remains behaviorally silent until it is challenged.

Chronic stress acts as a predisposing factor to the onset of depression in humans (McEwen, 2004). Rats or mice exposed to chronic unpredictable stress or chronic social defeat stress develop behavioral deficits and endocrine abnormalities, mimicking symptoms of human depression and showing decreases in leptin levels in the plasma (Lu et al., 2006). The observation that low circulating leptin levels in animal models of depression indicates that leptin insufficiency may underlie behavioral deficits. Systematic administration of leptin in normal mice can reverse the chronic stress-induced decrease in sucrose preference (Lu et al., 2006).

Another symptom of clinical depression that can be modeled in rodents is behavioral despair, which can be measured and assessed with a forced swim test (FST) and tail suspension test (TST). These tests have high predictive validity for antidepressant activity and have been used for screening of antidepressant drugs (Cryan et al., 2005a,b). Systemic leptin administration was found to produce a dose-dependent reduction of the duration of immobility in both tests (Lu et al., 2006; Kim et al., 2006). These data support the view that leptin may have antidepressant-like efficacy when functioning adequately in homeostatic proportions. Genetic deletion of the leptin receptor or pharmacological inhibition of leptin signaling in specific brain areas may help to determine whether a reduction in leptin signaling can directly lead to depressive behaviors (Lu et al., 2006).

Motivational Aspects of Sickness Behavior

The motivational interpretation of sickness behavior is that it is not the consequence of physical debilitation caused by the disease process, but the expression of a highly organized

strategy enabling the organism to fight infections (Dantzer, 2001). Therefore, sick individuals should be able to reorganize their behavior depending on the consequences and internal constraints to which they are exposed. The flexibility to do this is characteristic of what psychologists call motivation (Dantzer, 2001). The first evidence that sickness behavior is the expression of motivational state rather than the consequence of weakness was provided in a series of experiments designed to search for motivational signal for thirst (Miller, 1964). In these experiments, rats injected with bacterial endotoxin stopped pressing a bar for water but, when given water, ingested substantial amounts but to a lesser extent than normally. An important characteristic of motivational state is that it competes with other motivational states for behavioral output. The normal expression of behavior requires a hierarchical organization of motivational states that is continuously updated according to fluctuations in the internal state and occurrence of external events (Dantzer, 2001). Fatigue symptoms such as lack of energy and loss of interest occur very frequently in depressed patients. These symptoms are incorporated in the basic description of depressive episodes, and in fact, in the 10th revision of the *International Classification of Disease,* the entry for "Depression" begins with the statement that "the subject suffers from a lowering of mood, reduction of energy, and decrease in activity. Capacity for enjoyment, interest and concentration are impaired, and marked tiredness after even minimum effort is common."

CHAPTER TWO

METHODOLOGY

Materials

All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except IL-1RA (MRA00) IL-1β (MLB00B), and leptin (MOB00) ELISA, R&D Systems (Minneapolis, MN). High-Capacity cDNA Reverse Transcription Kit (PN 4368813), Applied Biosystems (Foster City, CA).

Animals

Animal use was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as we have described (Johnson et al., 2007) and also approved by the University of Illinois IACUC. C57BL/6J and B6.129S-Il1rntm1Dih/J (IL-1RA knockout) male mice were bred in house. All knockout mice were bred on a C57BL/6 background. Mice on diets were either bred in house or purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in standard shoebox cages and allowed water and food *ad libitum*. Housing temperature (22.2°C) and humidity (45-55%) were controlled as was a 12/12-h reversed dark-light cycle (2000-0800). Where required, mice were sacrificed by CO_2 asphyxiation.

Diets

Chow was closed source NIH-31 7013 from Teklad (Indianapolis, IN). Experimental diets were open source uniform-base diets. These diets contained either 10% fat (D12450B,

Research Diets) or 60% fat (D12492, Research Diets) (New Brunswick, NJ). Diets were fed to mice for 12 weeks beginning at the age of 4 weeks.

Blood Glucose

After a 24 hour fast, blood was collected from the lateral saphenous vein and blood glucose measured using a FreeStyle Freedom® glucometer (Abbott) as per manufacturer's instructions as we have described (O'Connor et al., 2005)

Behavior

Saccharin preference test

The saccharin preference test was used to measure anhedonia. To examine this behavior, mice were housed singly in standard cages fitted with adapted wire tops to allow access to two fluids: water or a 0.4% sodium saccharin solution (Sigma-Aldrich, CN 4-7839). Prior to experimental treatment, mice were exposed to two bottles of water in order to acclimate them to the presence of two fluid sources in the cage. Following a three day acclimation period, one of the two bottles in each mouse's wire top cage was replaced with a 0.4% sodium saccharin solution, place preference was accounted for by alternating the position of saccharin solution and water per cage, and fluid levels were monitored and recorded twice daily to measure total daily saccharin solution and water consumption for a total of 5 days during the fed state or 1 day after a 24 hour fast. Two independent studies were performed to measure saccharin preference in fed and fasted mice. Results are expressed as a percentage of total daily fluid consumption over the course of 5 days or 1 day and shown as means ± SEM. Food consumption and body weight were

also measured during these two independent studies, and results are expressed as average food intake in grams per day and average body weight change per day.

Forced swim test

The forced swim test (FST) was conducted as described by Porsolt (2000). Briefly, each mouse was placed individually in a cylinder (diameter: 16cm; height: 31cm) containing 15cm of water maintained at 25 ± 1 C. The water was changed and the cylinders were cleaned thoroughly between testing sessions. Mice were tested for 6 min and then returned to their home cage. The duration of immobility was evaluated during the last 5 min of the test.

Burrowing

Lab-made cylindrical burrows were placed in the home cage 24 hours prior to testing. On the day of the test, mice were single housed and burrows were filled with 200 g of food pellets and placed along the wall of a clean cage with a thin layer of bedding, with the closed end of the burrow against the back wall of the cage. Food was removed from the hopper, but water was still provided *ad libitum.* The test was started 3 hours before the dark cycle and total amount burrowed was measured by weighing the amount displaced from the burrow and subtracting that from the total amount given at the start of the test, at 2 and 24 hours.

Cytokine measurements

All cytokine measurements were performed using commercially available ELISA (R&D Systems) on serum from the inferior vena cava from expired mice. Blood was allowed to clot at

room temperature for 2 hours, and then centrifuged at 2,000 x g at 4°C for 20 min. Remaining serum was extracted from the sample and used for analysis.

Real-time RT PCR

RNA isolation and real-time RT PCR was performed as we have described (Sherry et al., 2007). In brief, TaqMan Gene expression primer for IL-1β (Mm99999061_mH), IL-1RA (Mm01337566_m1), leptin (Mm00434759_m1), leptin receptor (Mm00440181_m1), IL-1R1 (Mm00434237_m1), IL-1R2 (Mm00439622_m1), and IL-1α (MM99999060_m1) were used in real-time RT PCR performed on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix. To normalize gene expression, a parallel amplification of endogenous β-actin (Mm00607939_s1) was performed with TaqMan Gene Expression primer. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene levels was performed by comparing threshold cycle (ΔC_t).

Statistical analysis

The experimental design for behavioral experiments was a completely randomized design, with a 2 x 2 factorial arrangement of treatments (2 levels of pretreatment and 2 levels of treatment). All data were analyzed using the two way (with or without repeated measures) ANOVA procedure of Sigma Plot (San Jose, CA). The statistical model for saccharin preference included the effects of diet/phenotype x day. *Post hoc* comparisons of individual group means were performed with the Tukey's test. Statistical significance was denoted at $p < 0.05$.

CHAPTER THREE

RESULTS

Saccharin preference is decreased in mice fed a high-fat diet (HFD)

The saccharin preference test is a measure of anhedonia in rodents, and the preference for sweet solutions has been shown to be decreased during inflammation or infection (Dantzer, 2001). Mice fed HFD display an aversion to saccharin solution compared to control mice fed a LFD, which show a significant preference for the saccharin solution (**Fig.1**). After 5 days of free access to both saccharin and water, mice fed HFD consumed less saccharin solution as a percentage of total fluid intake (27% vs. 74%) [$F= 20.761$, $p < 0.0001$] compared to LFD fed mice. Two-way ANOVA with one factor repetition (diet x mouse x day) of saccharin preference revealed significant diet interactions [$F = 9.587$, $p = 0.007$].

The body weights of LFD and HFD mice during the 5 day saccharin preference test are presented in **Fig. 2**. Two-way repeated measures ANOVA (one factor repetition) revealed significant interactions between diet and day [$F = 4.590$, $p = 0.007$] on day 5 alone. Mice fed HFD had a significantly increased weight gain compared to mice fed a LFD [$F = 3.113$, $p = 0.034$] on day 5 of the test. However, food intake does not significantly change over the 5 day measurement period in either LFD or HFD fed mice. Based on this finding, we can conclude that saccharin preference does not influence food intake in either group.

The total food consumption measurements indicate that there is no main effect of diet on food consumption in HFD or LFD fed mice during the 5 day saccharin preference, although there is a statistical significance in food consumption between LFD and HFD fed mice on day 1 of the

saccharin preference test [$p = 0.006$] (Fig. 3). Taken together these findings indicate that mice fed HFD exhibit a saccharin aversion compared to mice fed LFD without influencing overall food intake.

Burrowing is decreased in mice fed HFD

Burrowing behavior is a sensitive method for detecting behavioral dysfunction because all rodents display burrowing behavior. The test is sensitive to prion disease in mice, mouse strain differences, lesions of the hippocampus and prefrontal cortex in mice, and effects of LPS and IL-1β in rats (Deacon, 2006). HFD fed mice burrow significantly less than LFD fed mice as shown in **Fig. 4**. Two hours after introduction of the burrowing material, HFD fed mice burrowed only 25.4 g of material compared to LFD fed mice which burrowed 46.5 g [$F = 11.149$, $p < 0.02$]. By 24 hours, HFD fed mice still exhibited decreased burrowing compared to LFD [59.9 g vs 89.8 g; $p < 0.02$].

Saccharin aversion is decreased in HFD mice after 24 hour fast.

HFD and LFD fed mice were fasted for 24 hours, then saccharin preference was measured. In mice fed a HFD, saccharin preference increased to 51% of the total daily fluid intake, while mice fed a LFD only increased to 84% of the total daily intake after fasting (**Fig. 5**). There is a statistically significant difference in saccharin consumption [$F = 10^{-40}$, $p < 0.001$] and water consumption [$F = 10^{-40}$, $p < 0.001$] in the HFD vs LFD fed mice. However, there was no significant difference between water and saccharin consumption within the HFD fed group. Taken together, these findings indicate that fasting HFD fed mice for 24 hours increases

saccharin preference, although not significantly different from water consumption. This indicates that there is no longer a saccharin aversion in HFD fed mice when fasted.

Burrowing is increased in HFD mice after 24 hour fast

In mice fed a HFD, burrowing increases after a 24 hour fast at both 2 [25.43 ± 5.04 vs. 73.26 ± 11.9g, $p < 0.005$] and 24 hours [59.98 ± 10.4 vs. 93.86 ± 4.5g, $p < 0.001$] (**Fig. 6**). Mice fed a HFD burrow similar amounts to mice fed a LFD after a 24 hour fast. These results indicate that fasting HFD mice for 24 hours completely restores burrowing behavior to that of control mice.

Immobility in the forced swim test (FST) decreases after fasting in LFD and HFD fed mice

FST is a sensitive test stimuli that provokes depressive-like behavior, measuring a behavioral dimension that is relevant to depression (Cryan et al., 2005). In the fed state, HFD mice were immobile for an average of 4:06 minutes, which is not significantly different from mice fed a LFD who were immobile for an average of 4:16 minutes (**Fig. 7**). However, once fasted, both groups had a significant decrease in immobility [3:02 vs 3:31; $F = 29.295$, $p < 0.001$], compared to their respective fed groups.

HFD increases serum leptin and IL-1RA, which are not affected by fasting.

Mice fed a HFD for 12 weeks have a 5-fold increase in serum leptin concentration [$F = 323.004$, $p < 0.001$] and a 1.5-fold increase in serum IL-1RA [$F = 6.546$, $p < 0.016$] compared to mice fed a LFD (**Table 1**). After a 24 hour fast, leptin and IL-1RA in the serum are not significantly changed in HFD mice. IL-1β is not detectable in any group. These findings

indicate that a change in peripheral leptin, IL-1RA, and IL-1β may not contribute to the behavioral changes observed in fasted HFD fed mice.

Body weights decrease after a 24 hour fast in both LFD and HFD fed mice.

Mice fed LFD lose a significant amount of body weight during a 24 hour fast, decreasing from 30.8 ± 0.6g to 25.2 ± 1.31g [$F = 27.914$, $p < 0.001$]. Mice fed HFD also lose a significant amount of body weight during a 24 hour fast, decreasing from 49.7 ± 0.96g to 47.2 ± 6.7g [$p<0.05$] (**Table 2**). The extent of fasting induced weight loss in HFD fed mice is less than that observed in mice fed a LFD. As expected, HFD fed mice weigh more in both the fed and fasted states compared to mice fed a LFD [$p < 0.001$].

Blood Glucose decreases after a 24 hour fast in both LFD and HFD fed mice.

When LFD fed mice are fasted for 24 hours, their blood glucose decreases significantly from 147.7 ± 5.44 to 70 ± 0.33 dg/mL [$F = 127.518$, $p < 0.001$]. Similarly, when mice fed a HFD are fasted for 24 hours, their blood glucose decreases from 176 ± 6.66 to 121 ± 0.81 dg/mL [$p < 0.05$] (**Table 3**). Mice fed a HFD have a significantly higher blood glucose in both the fed and fasted states compared to mice fed a LFD [$F = 45.98$, $p < 0.001$].

Brain based cytokine expression changes after a 24 hour fast.

Brain based gene expression was analyzed in mice fed HFD or LFD during a fed period (*ad libitum*) and a fasted period (24 hours). The results of RT PCR gene expression analysis of several genes including leptin, leptin receptor (Lep R), IL-1RA, IL-1R1, IL-1R2, IL-1α and IL-1β are shown in **Fig. 8-12**. As shown in **Fig. 8**, mice fed LFD, had a 2-fold increase in leptin

after fasting [1 ± 0.15 vs 2.18 ± 0.34 Δ mRNA, $p < 0.05$] and mice fed a HFD had a 2 –fold increase [1.94 ± 0.51 vs 4.08 ± 0.34 Δ mRNA $p < 0.05$] as well. IL-1α expression was increased 1.5-fold during fasting in mice fed HFD compared to mice fed LFD [0.923 ± 0.14 vs. 0.59 ± 0.15 Δ mRNA, $p < 0.05$] and decreased by 1.6-fold during fasting within the LFD group compared to its fed state [0.6 ± 0.15 vs. 1 ± 0.19 Δ mRNA, $p < 0.05$) as shown in **Fig. 9**. IL-1R2 was decreased during fasting in mice fed HFD compared to mice fed LFD (1.013 ± 0.12 vs. 1.40 ± 0.08 Δ mRNA, $p < 0.005$) as shown in **Fig. 10**. IL-1R1 was decreased in the fed [0.79 ± 0.09 vs. 1 ± 0.08 Δ mRNA, $p < 0.05$] and fasted [0.68 ± 0.14 vs 0.88 ± 0.07 Δ mRNA, $p < 0.05$] in HFD mice compared to LFD mice as shown in **Fig. 11**. IL-1RA was increased 2.3-fold in HFD compared to LFD in fed [1 ± 0.31 vs 2.26 ± 0.66 Δ mRNA, $p < 0.05$] and 2.43-fold in the fasted state [1.89 ± 0.16 vs 4.16 ± 0.67 Δ mRNA] as shown in **Fig. 12**, although the latter was not significant. There were no differences in Lep R expression between HFD and LFD and no differences due to fed/fasted state (data not shown). For IL-1β, no differences in gene expression between HFD and LFD or fed and fasting states were observed (data not shown). Leptin expression in mice fed a HFD was the only gene to significantly increase expression from fed to fasting. Taken together, these data show that changes in brain gene expression of leptin, IL-1α, IL-1R2, IL-1R1 and IL-1RA occur during fasting, and these changes may be affected by nutritional status and contribute to differences in saccharin preference in mice fed HFD.

Saccharin preference is decreased in IL-1RA KO mice..

In order to determine if IL-1RA plays a role in saccharin preference, IL-1RA KO mice were given a saccharin preference test over the course of 5 days. Similar to mice fed a HFD, IL-1RA KO mice had an aversion to saccharin compared to control wild-type (WT) mice (**Fig. 13**).

IL-1RA KO mice consumed 12% saccharin solution compared to control WT mice that consumed 65% of their total daily intake from saccharin solution. The mean values of saccharin consumption and water consumption in IL-1RA KO are significantly decreased [$F = 729.97$, $p < 0.001$; $F = 29.52$, $p < 0.006$] compared to WT mice. Taken together, these data indicate that depletion of IL-1RA may contribute to saccharin aversion.

CHAPTER FOUR

TABLES AND FIGURES

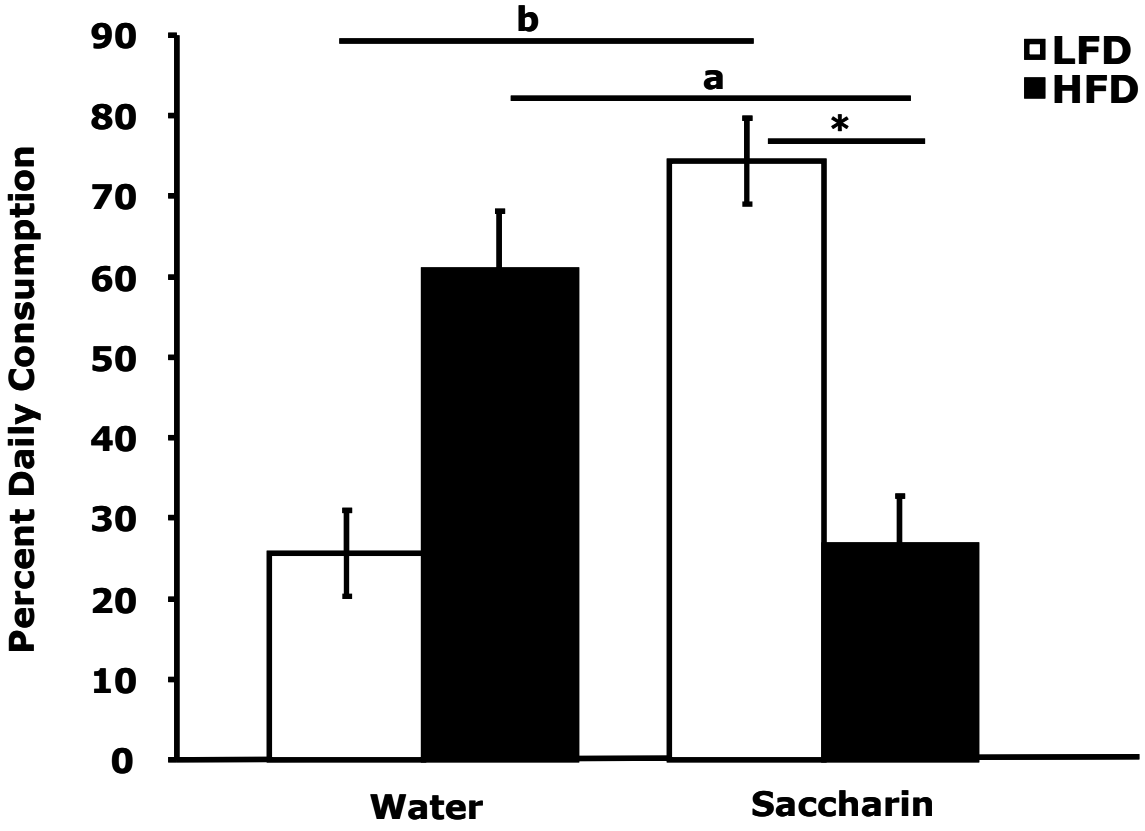

Fig. 1. Saccharin preference is decreased in mice fed a high-fat diet. Mice were fed a 60% fat diet (HFD) or a 10% fat diet (LFD) and saccharin preference was measured utilizing a 2-bottle test for 5 days. Results are expressed as a percentage of daily consumption over 5 days, means ± SEM; n=10; *$p < 0.007$, LFD saccharin vs HFD saccharin, $^{a}p < 0.05$, HFD water vs saccharin, $^{b}p < 0.001$, LFD water vs. saccharin.

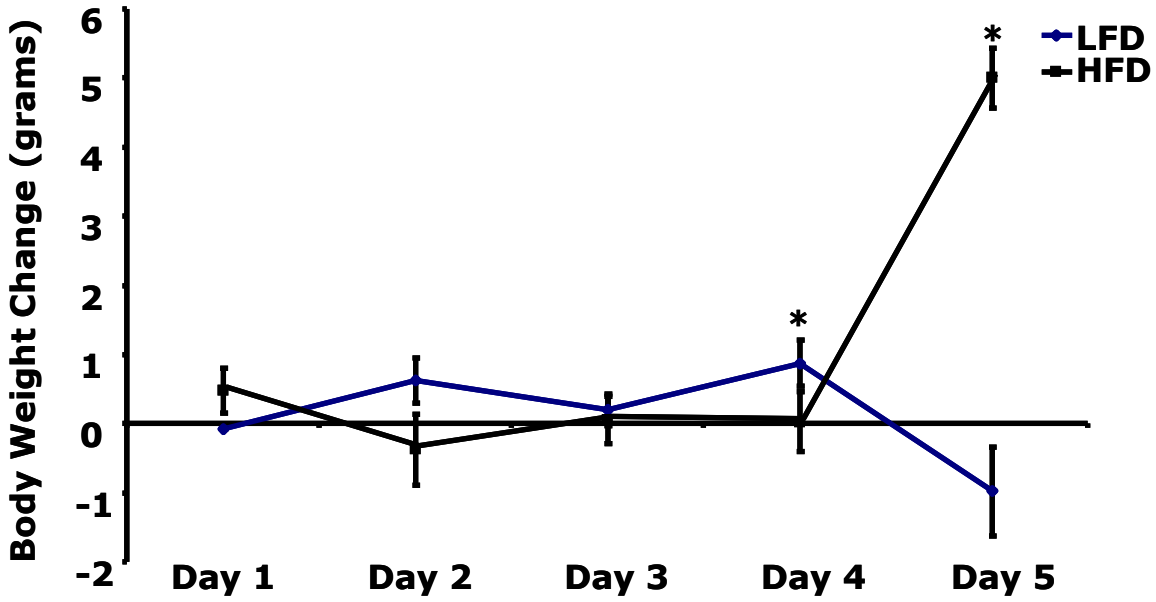

Fig. 2. Saccharin preference test does not induce weight changes in HFD or LFD fed mice. Mice were fed HFD or LFD and saccharin preference was measured utilizing a 2-bottle test for 5 days. During this test, body weights were taken daily. Results are expressed as an average of body weight change in grams per day, means ± SEM; n = 4, *$p < 0.05$ factor effect of day.

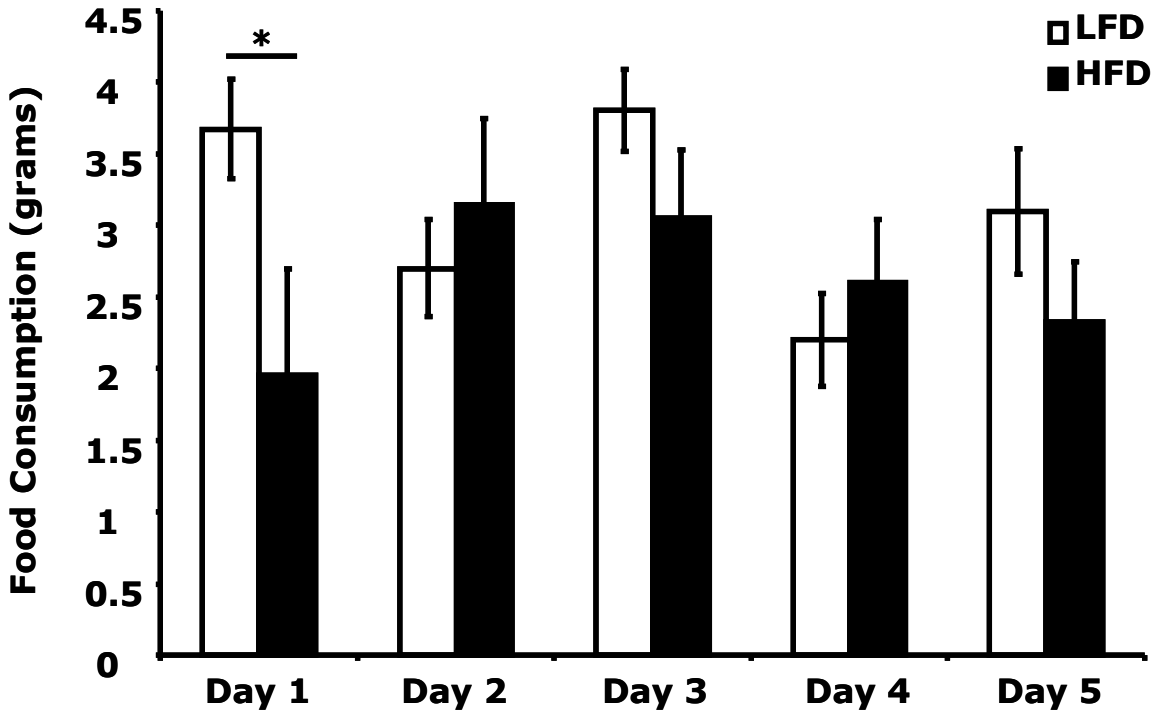

Fig.3. Saccharin preference test does not affect food intake in HFD and LFD mice. Mice were fed HFD or LFD and saccharin preference was measured utilizing a 2-bottle test for 5 days. During this test, food weights were taken daily. Results are expressed as an average of food intake in grams per day, means ± SEM; n = 4,*$p < 0.05$, LFD vs HFD on day 1.

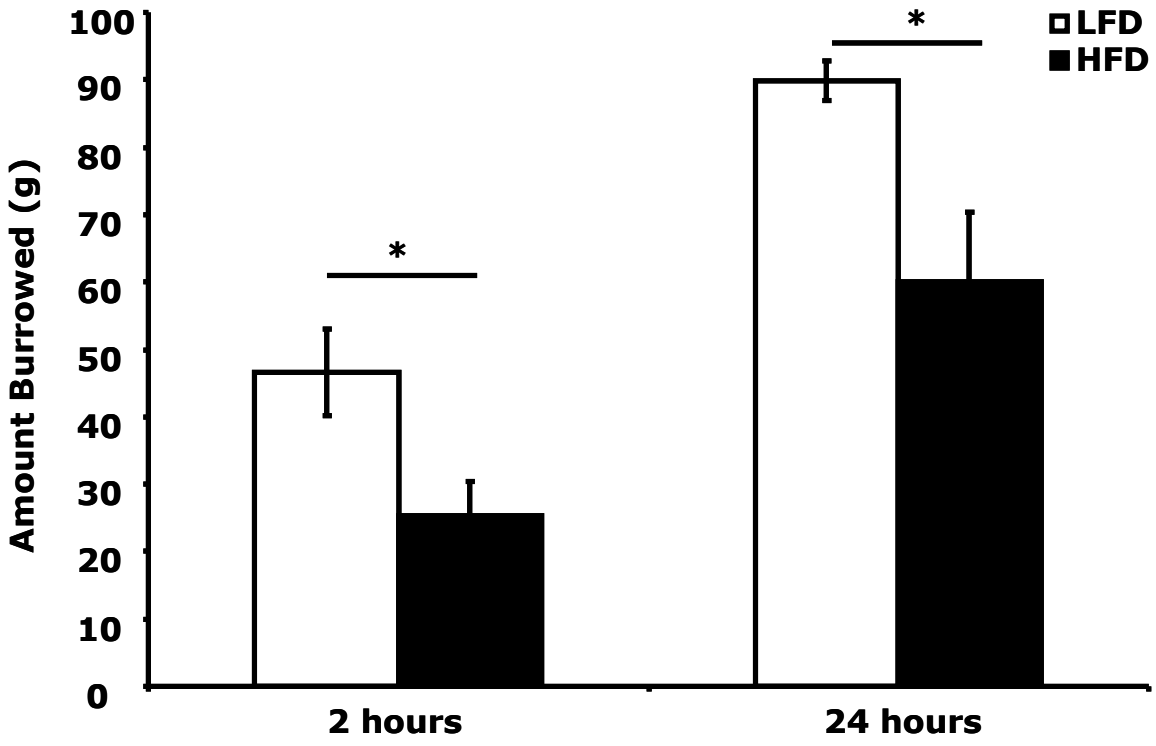

Fig.4. Mice fed LFD exhibit increased burrowing compared to mice fed HFD at both 2 and 24 hours. Mice were fed HFD for at least 12 weeks or LFD for 12 weeks and burrowing behavior was measured at 2 and 24 hours after introduction of burrowing tubes and material. Results are expressed as material burrowed in grams, means ± SEM; n = 4, *$p < 0.02$ LFD vs HFD at 2 and 24 hours.

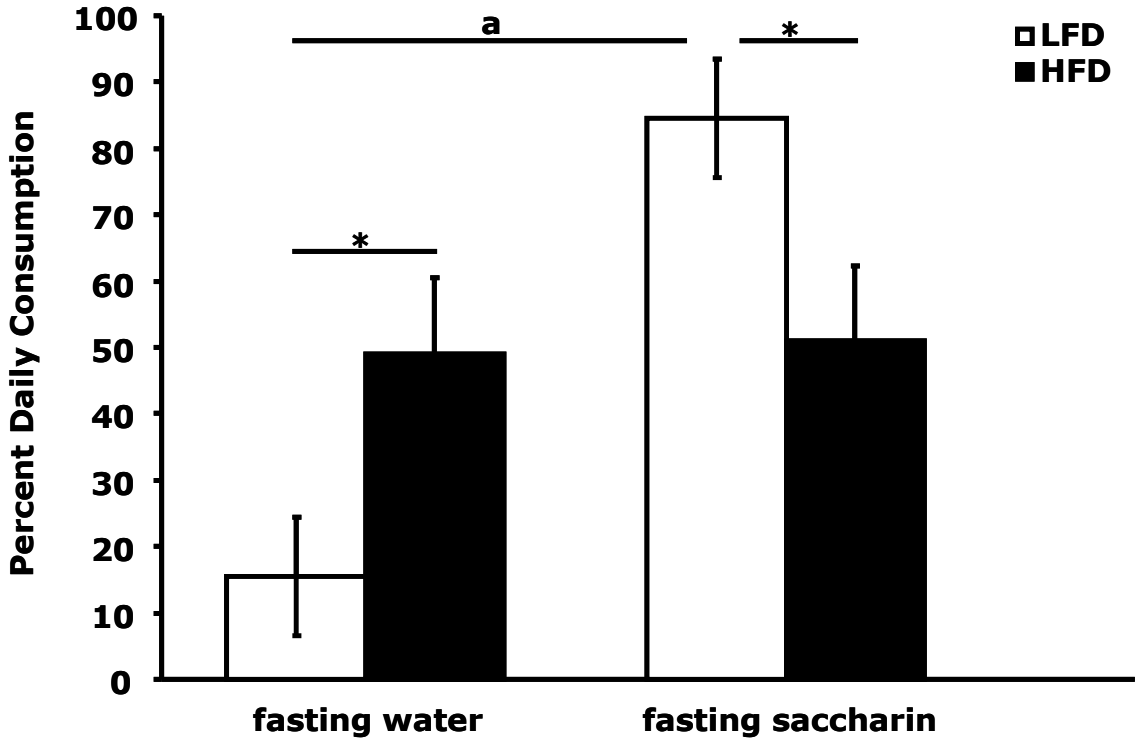

Fig. 5. Saccharin preference is increased in HFD mice after 24 hour fast. LFD and HFD fed mice were fasted for 24 hours and then saccharin preference was measured utilizing a 2-bottle test over 1 day. Results are expressed as a percentage of daily consumption over 1 day, means ± SEM; n = 8 per group, *$p < 0.001$, LFD water vs HFD water; LFD saccharin vs HFD saccharin, [a]$p < 0.001$, LFD saccharin vs water.

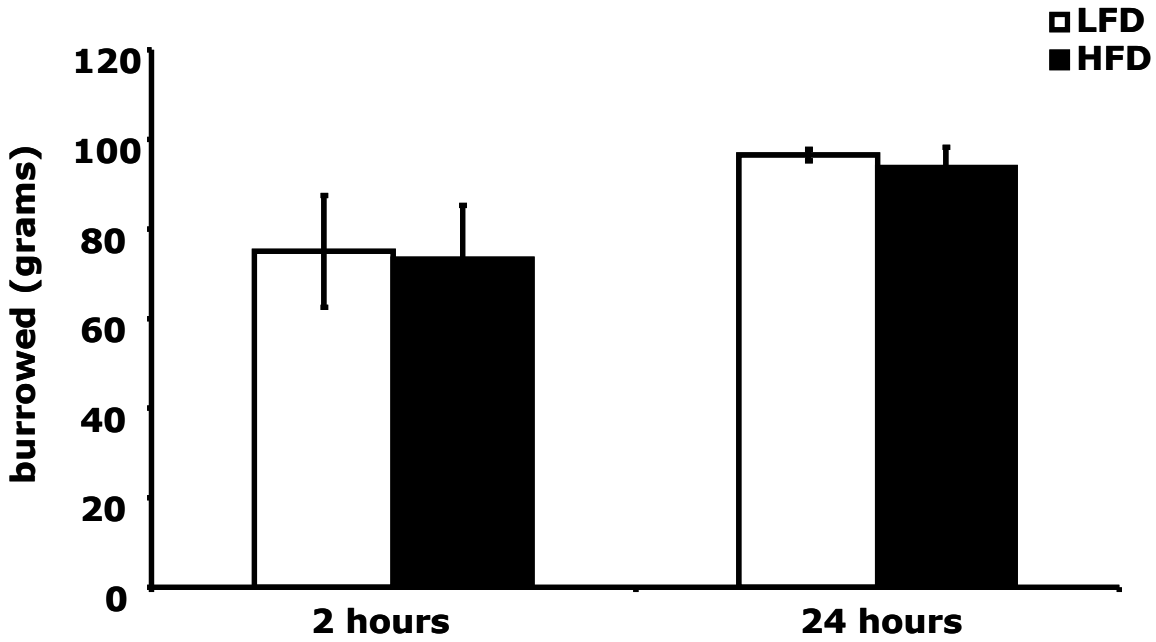

Fig. 6. Burrowing activity in HFD increases after fasting at 2 and 24 hours. Mice were fed HFD or LFD for 12 weeks, and then fasted for 24 hours. Burrowing behavior was measured at 2 and 24 hours after introduction of burrowing tubes and material. Results are expressed as material burrowed in grams, means ± SEM; n = 8.

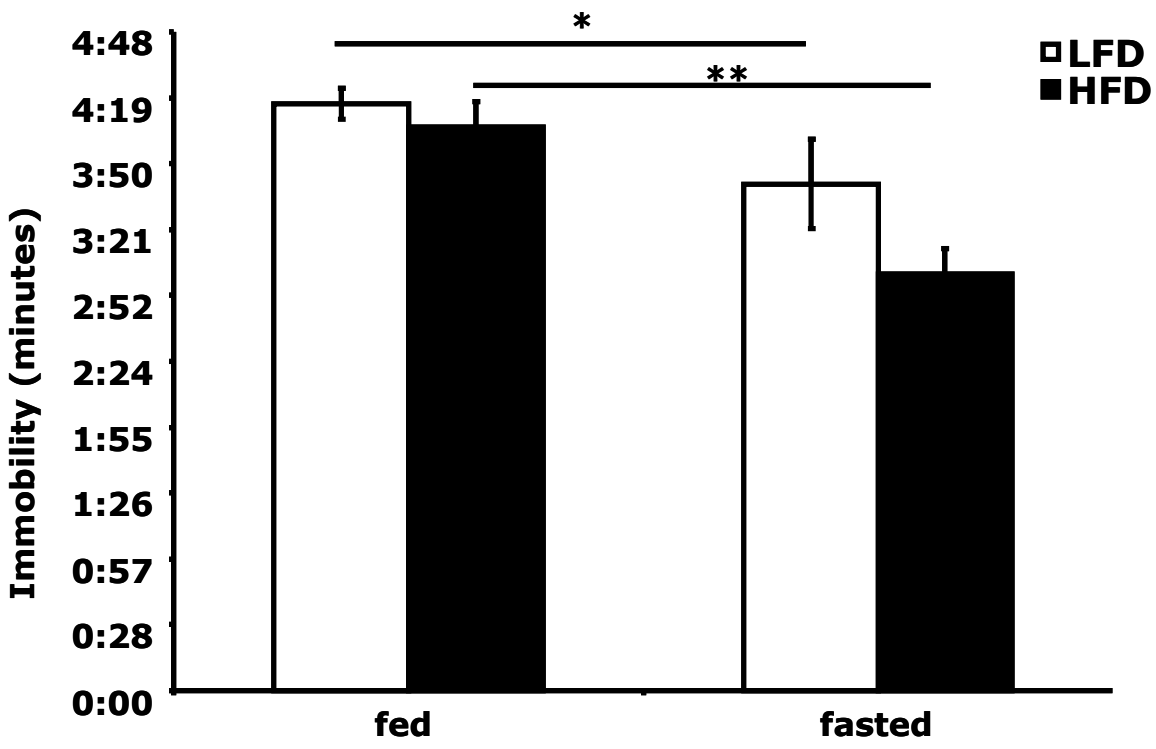

Fig.7. Immobility in the forced swim test decreases in HFD and LFD fed mice when fasted.

LFD and HFD fed mice were fasted for 24 hours and then behavioral despair was measured utilizing the forced swim test (FST). Results are expressed as total seconds of immobility during a 6 minute test period, means ± SEM; n = 8;*$p < 0.05$, LFD fed vs LFD fasted, **$p < 0.001$, HFD fed vs HFD fasted.

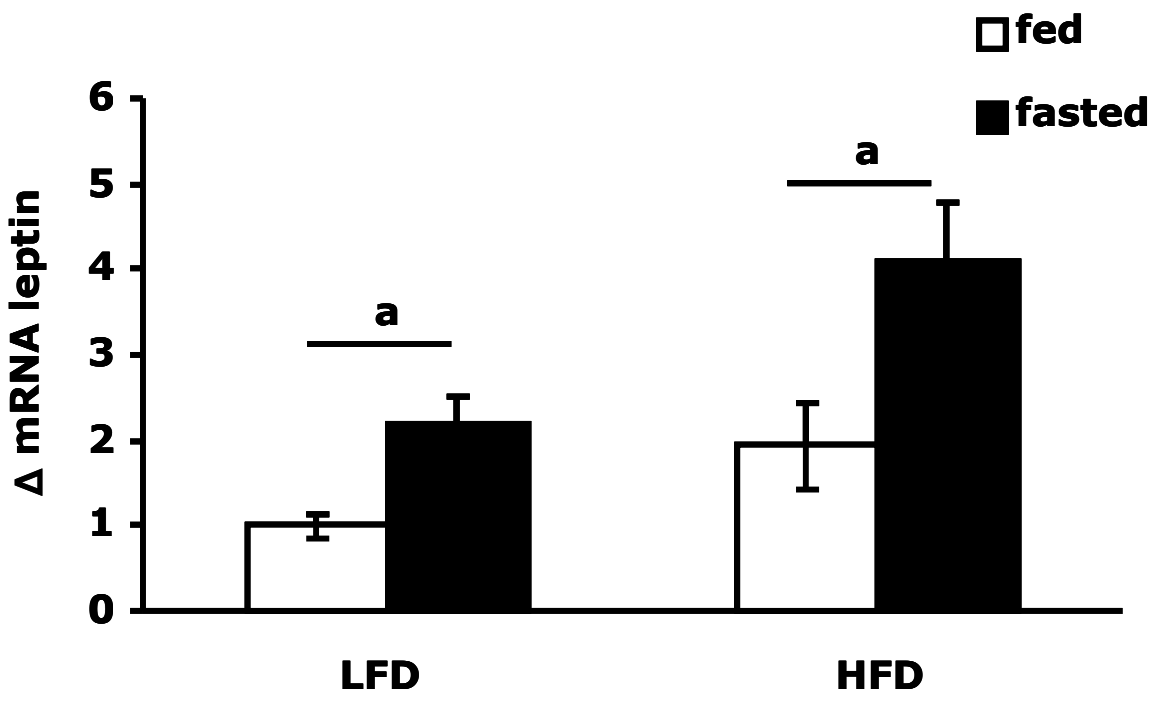

Fig. 8. ***Leptin expression in brain.*** Real-time RT-PCR was used to quantify expression in brains of mice fed LFD or HFD for 12 weeks and in mice fasted for 24 hours. Results are expressed as relative change in mRNA expression, means ± SEM; n = 8 per group; $^{a}p < 0.05$, LFD fed vs LFD fasted; HFD fed vs HFD fasted.

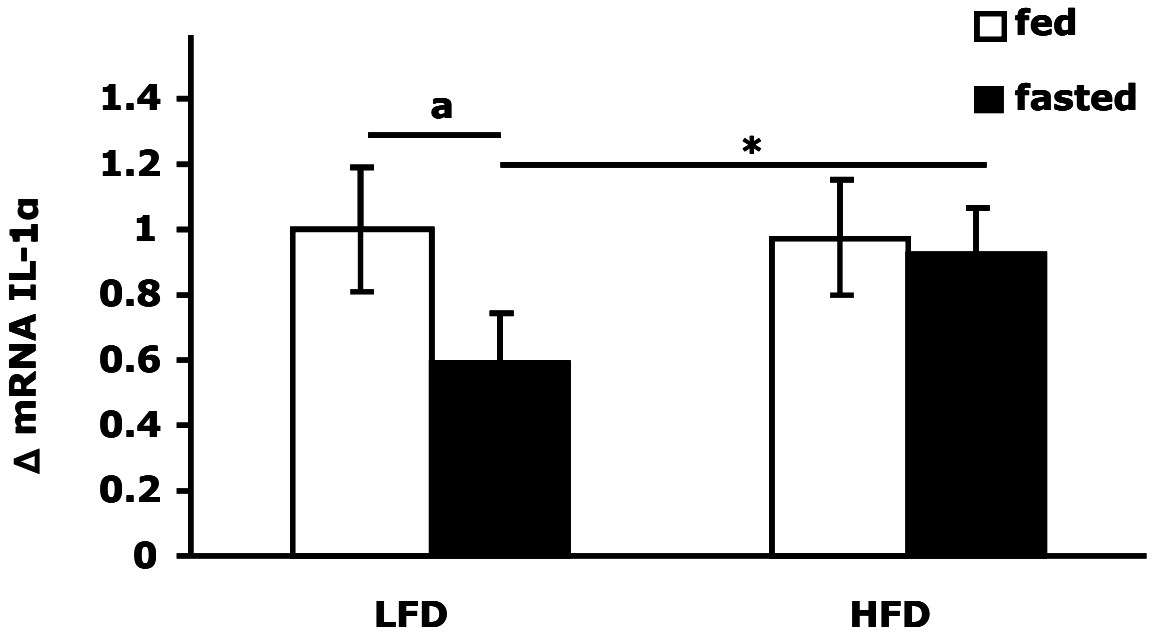

Fig. 9. ***IL-1α expression in brain.*** Real-time RT-PCR was used to quantify expression in brains of mice fed LFD or HFD for 12 weeks and in mice fasted for 24 hours. Results are expressed as relative change in mRNA expression, means ± SEM; n = 8 per group; *$p < 0.05$, LFD fasted vs HFD fasted; $^{a}p < 0.05$, LFD fed vs LFD fasted.

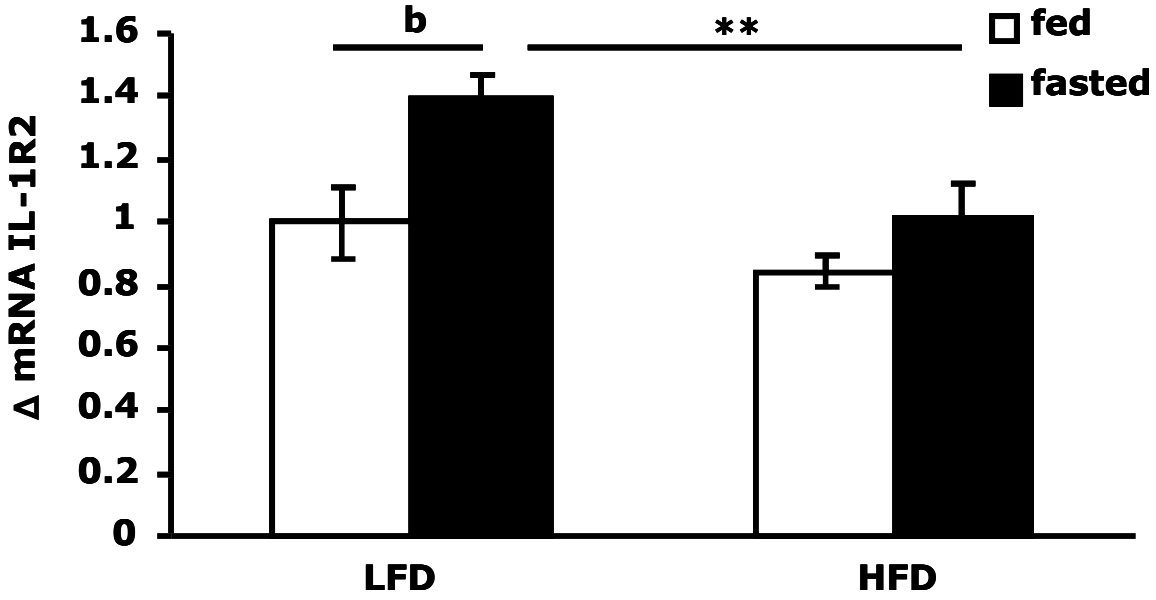

Fig. 10. ***IL-1R2 expression in brain.*** Real-time RT-PCR was used to quantify expression in brains of mice fed LFD or HFD for 12 weeks and in mice fasted for 24 hours. Results are expressed as relative change in mRNA expression, means ± SEM; n = 8 per group; **$p < 0.005$ LFD fasted vs HFD fasted; $^{b}p < 0.005$, LFD fed vs LFD fasted.

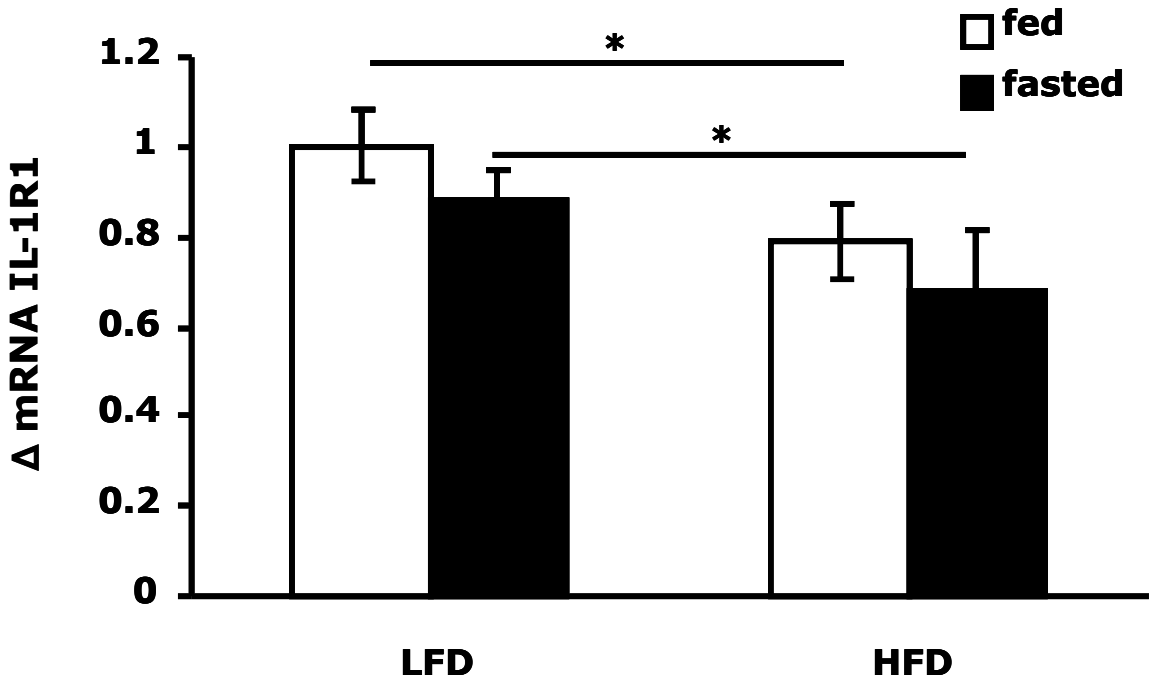

Fig. 11. ***IL-1R1 expression in brain*** Real-time RT-PCR was used to quantify expression in brains of mice fed LFD or HFD for 12 weeks and in mice fasted for 24 hours**.** Results are expressed as relative change in mRNA expression, means ± SEM; n = 8 per group; *$p < 0.05$, LFD fed vs HFD fed; LFD fasted vs HFD fasted.

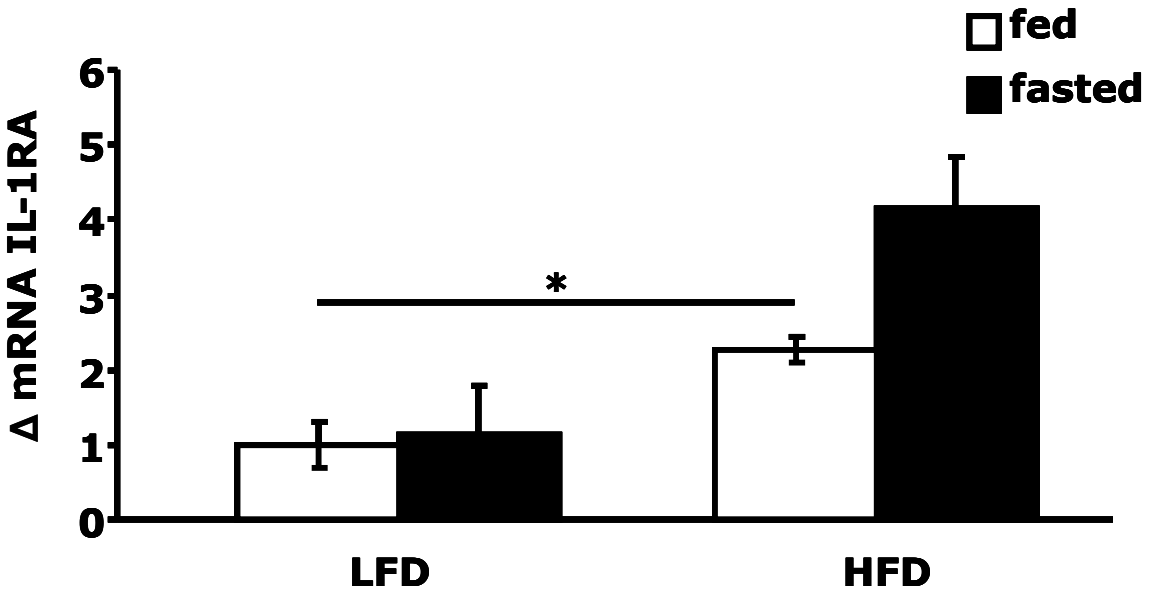

Fig. 12. ***IL-1RA expression in brain.*** Real-time RT-PCR was used to quantify expression in brains of mice fed LFD or HFD for 12 weeks and in mice fasted for 24 hours**.** Results are expressed as relative change in mRNA expression, means ± SEM; n = 8 per group; *$p < 0.05$, LFD fed vs HFD fed.

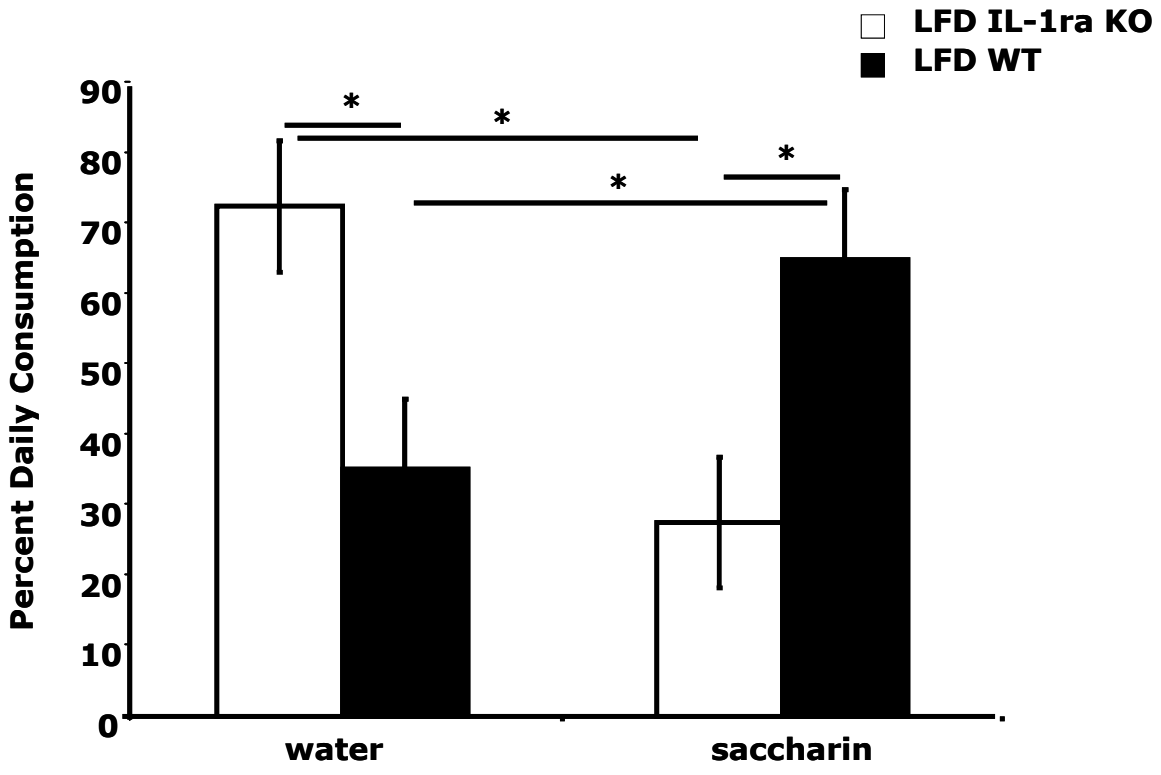

Fig. 13. Saccharin preference is decreased in IL-1RA KO mice. IL-1RA KO and WT mice were fed LFD and saccharin preference was measured utilizing a 2-bottle test for 5 days. Results are expressed as a percentage of daily consumption over 5 days, means ± SEM; n = 4, *$p < 0.005$, IL-1RA KO water vs WT water; IL-1RA KO saccharin vs WT saccharin; WT water vs saccharin; IL-1RA KO water vs saccharin.

Table 1
Leptin (pg/mL), IL-1RA (ng/mL), and IL-1β concentrations for LFD and HFD fed/fasted mice

	LFD		HFD	
	Fed	Fasted	Fed	Fasted
Leptin	8,118.8±680.3[a]	3,104.5 ±292.8[a]	43,844.52 ± 1954.7[b]	42,791.6 ± 4719[b]
IL-1RA	567.12±17.39[a]	701.33 ±54.07[a]	800.79 ± 81.51[b]	796.42 ±92.07[a]
IL-β	nd	nd	nd	nd

Table 1. Serum leptin and IL-1RA is increased in HFD fed mice. Mice were fed 10% fat (LFD) for 6 weeks or 60% fat (HFD) for 12 weeks and serum leptin, IL-1RA, and IL-1β was measured by ELISA. Nd indicate "not determined." Results are expressed as mean ±SEM, n=8. Within a row, different letter superscripts indicate significant differences, $p<0.05$.

Table 2

Body weights for fed/fasted mice fed HFD or LFD

	Fed			Fasted		
LFD	30.8	±	0.6[a]	25.2	±	0.3[b]
HFD	49.7	±	0.9[c]	47.2	±	0.8[d]

Table 2. Body weights for fed and fasted mice on a HFD or LFD. Body weights were taken from mice fed a LFD or HFD during the fed state and after a 24 hour fast. Results are expressed as a mean ± SEM, n=8 per group, different letter superscripts indicate significant differences, $p<0.05$.

Table 3

Blood glucose for fed/fasted mice fed HFD or LFD

	Fed			Fasted		
LFD	147.7	±	5.4[a]	70	±	1.3[b]
HFD	176.0	±	6.7[c]	121	±	6.7[d]

Table 3. Blood glucose for fed/fasted mice on a HFD or LFD. Blood glucose was measured in mice fed a LFD or HFD during the fed state and after a 24 hour fast. Results are expressed as a mean ± SEM, n=8 per group, different letter superscripts indicate significant differences, $p<0.001$.

CHAPTER FIVE

CONCLUSIONS

Anhedonia is defined as an inability to experience pleasure from normally pleasurable events. It can manifest in rodent behavior as a decreased preference for highly palatable solutions, and can be easily measured (Dantzer, 2001). It is a symptom of sickness behavior and depression, which is a normal response of the host to pathogens that are recognized by the innate immune system (Dantzer, 2001). Typically, systemic or central infusion of cytokines or endotoxins such as LPS, induce symptoms of sickness behavior in experimental animals and human beings, including anhedonia. In the absence of an exogenous immune stimulus, as is the case in **Fig. 1**, anhedonia is present in mice that are fed HFD containing 60% of total calories from fat for 12 weeks. The question that arises is whether this behavioral deficit is due to the low-grade inflammation that has been reported in rodent obesity (Hotamisligil et al., 1993; Xu et al., 2003), a dysregulation of specific cytokines in obesity, particularly IL-1β/IL-1RA (Matsuki et al., 2003), or a direct effect of increased levels of leptin or leptin resistance.

Other behavioral measures of depressed mood such as a decreased interest in physical surroundings may occur in a typical host response to infection/inflammation as well (Dantzer, 2001). A typical measurable behavior in rodents is burrowing. Rodents are noted for their burrowing ability which is probably an extremely ancient behavior (Deacon, 2006). Observation of this behavior shows that mice dig the burrowing material out of their designated burrows with coordinated hind- and forelimb movements and kick them outside the container, rather than carry the pellets carefully to a selected spot which could be interpreted as hoarding. It is possible that burrowing is related to tunnel maintenance, as the filled burrow may represent a blocked tunnel,

or it could reflect elements of defensive behavior in that a rodent would typically attempt to prevent predators from entering their burrows by digging and kicking debris toward an intruder. Defensive burrowing is a well-known paradigm for measuring anxiety (Pinel et al., 1978). Rat and mouse burrowing is decreased after administration of LPS and IL-1β –expressing replication-deficient adenovirus (Deacon, 2006). It has also been shown as a way of assessing recovery from surgery, in that an animal that is even slightly ill will show reduced burrowing. I have shown that burrowing behavior is significantly decreased in mice fed a HFD compared to mice fed a LFD at both 2 and 24 hours as seen in **Fig. 4**. This suggests that there is some behavioral dysfunction due to HFD feeding. It is possible that these animals are experiencing sickness syndrome or depressed mood. Interestingly, when fasted for 24 hours, as seen in **Fig. 6**, burrowing increases in HFD fed mice at 2 and 24 hours respectively, and is comparable to burrowing observed in mice fed LFD. Taken together, these results suggest a behavioral benefit of fasting in HFD fed mice.

A third behavioral test was employed as seen in **Fig. 7**. The FST is based upon the observation that "rodents eventually develop immobility when they are placed in a cylinder of water after they cease active escape behaviors (Cryan et al., 2005b)." Unlike the previous behavioral tests, there is no significant difference in time spent immobile in mice fed HFD compared to mice fed LFD. Interestingly though, when both groups of mice are fasted (**Fig. 7**), immobility is decreased, to a somewhat greater extent in mice fed a HFD. This would suggest that physiological changes that occur during fasting may contribute antidepressant action to both groups of mice regardless of diet, but to a slightly increased extent in mice fed HFD.

Leptin modulates inflammation (Faggioni et al., 2001) and has been shown to be increased in rodents fed HFD (Lin et al., 2000). It has also been associated with the modulation

of motivation to obtain reward (Figlewicz et al., 2008) and anti-depressant-like activity (Lu, 2007). Serum leptin was measured in mice fed HFD and LFD. As expected, leptin was significantly higher in mice fed a HFD. The hypothesis that elevated levels of leptin may attenuate response to a reward was tested by promoting negative energy balance by fasting mice for 24 hours. Negative energy balance has previously been shown to promote response to foods and other reinforcements (Figlezwicz et al., 2008); therefore, serum leptin was also measured after a 24 hour fast to delineate whether or not a significant decrease in serum leptin contributed to the increased response to saccharin consumption, burrowing activity, and FST. Results indicate that leptin was not significantly decreased in fasted HFD fed mice (**Table 1**), so it is likely that serum leptin did not contribute to an increase in response to stimuli in this experiment.

Given that IL-1RA levels are typically elevated in obese rodents as our lab has previously shown, as well as in the circulation of patients with inflammatory conditions (Arend, 2002) and because the balance between IL-1RA and IL-1β influences the course, susceptibility and severity to many diseases, we measured IL-1RA and IL-1β in the serum (**Table 1**). Leptin has been shown to increase peripheral IL-1RA and also induces its expression in the hypothalamus. We hypothesized that IL-1RA (or more likely IL-1 balance) may be contributing to the behavioral consequences of HFD feeding. However, while IL-1RA was increased in mice fed a HFD compared to mice fed a LFD, a 24 hour fast did not significantly change the serum concentration. IL-1β was not detected in the serum of any group. The lowest concentration of detection of the assay used was 7.8 pg/mL. This indicates that peripheral IL-1RA/IL-1β balance likely did not have an effect on the consequential behaviors observed.

Both leptin and IL-1RA can cross the BBB (Guitierrez et al., 1994) and leptin induces IL-1RA expression in the brain, including the hypothalamus (Hosoi et al., 2002). Therefore, we

measured expression of these two cytokines and their receptors, as well as IL-1α and IL-1β in the brain. As shown in **Fig. 12** and **11** respectively, in mice fed a HFD, IL-1RA was increased in the fed state, and IL-1R1 decreased compared to LFD fed mice. The only significant change due to fasting within the HFD group was an increase in leptin expression. This may indicate an increased central leptin sensitization in HFD mice that are fasted, which is important because the brain is a target site for leptin to exert its mood promoting actions (Figelwicz et al., 2008). These results indicate differences in fed state cytokine expression between HFD and LFD mice, and that leptin expression in the brain is influenced by 24 hour fasting, possibly contributing to the behavioral benefit of a 24 hour fast on HFD fed mice.

Since IL-1RA expression is increased in mice fed HFD, who exhibit saccharin aversion and decreased burrowing compared to mice fed LFD, saccharin preference was tested in IL-1RA knockout mice (**Fig. 13**). We have hypothesized that physiologically balanced IL-1/IL-1RA concentrations may be important in normal mouse behaviors. IL-1RA knockout mice display a decreased preference for saccharin solution **(Fig. 13**), similar to HFD mice, indicating that a deviation from normal physiological levels of IL-1RA may contribute to anhedonic behaviors. However, IL-1RA knockouts also have low levels of leptin, (about 6-fold decreased) compared to control mice (Somm et al., 2005), which could be the cause for anhedonic behavior.

To further complicate things, when fasted, IL-1RA expression in the brain of HFD mice trends toward increasing contrary to our expectations, and we observe increased saccharin preference, increased burrowing, and decreased immobility in the FST. Therefore, it is probably not the case that fluctuations in IL-1RA or IL-1 balance contributes to the behavioral changes observed in fasted mice fed HFD, but more likely a contribution of increased brain based leptin. Taken together, these findings indicate that changes in brain expression of specific genes,

particularly leptin occur simultaneously with the behavioral benefits observed in fasted mice fed HFD.

To further probe the mechanism of why these behavioral consequences exist in mice fed a HFD, ICV leptin could be infused continuously over the course of a 5 day saccharin preference test, to establish if central leptin infusion increases preference for saccharin. Both burrowing and FST should also be examined with leptin infusion to see if behavior changes resemble those in fasted HFD fed mice. Another set of experiments could include measuring burrowing and FST in IL-1RA knockout mice and then injecting leptin ICV and repeating the procedures to see if exogenous brain leptin improves behavioral dysfunction. The corollary experiment of the HFD study would be to examine the neuropeptides that are increased during fasting. Here we have specifically chosen to examine leptin's role, but other neuropeptides may be increased during fasting including orexin, ghrelin, melanin-concentrating hormone and neuropeptide-y. Interestingly, ghrelin has been shown to play a significant role in neurotrophy, particularly in the hippocampus, and is essential for cognitive adaptation to changing environments and the process of learning (Atcha et al., 2009). These could be candidates for further study.

CHAPTER SIX

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