Mind What You Eat:
Implications and Mechanisms of High-Fat Diet-Induced Neuroinflammatory Priming.

By

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline

Abstract

Sobesky, Julia Lillian (Ph.D., Psychology and Neuroscience, Center for Neuroscience)

Mind What You Eat: Implications and Mechanisms of High-Fat Diet-Induced Neuroinflammatory Priming.

Thesis directed by Distinguished Professor Dr. Steven F. Maier

The purpose of this dissertation is to establish a link between high-fat diet (HFD) and neuroinflammation, and to evaluate both the nature and mechanisms by which HFD influences neuroinflammatory processes. HFD-induced obese adipose tissue fosters a peripheral inflammatory environment, which contributes to the development of metabolic disease associated with obesity. In addition, HFD consumption is associated with disruptions in cognition, and hippocampal function appears to be particularly vulnerable to HFD. It is likely that HFD induces alterations in neuroinflammatory processes, which negatively influence hippocampal function and mediate cognitive decline. However, specific mechanisms by which HFD mediates neuroinflammatory processing are poorly understood.

Consumption of HFD (60% fat) was evaluated in wistar rats. With prolonged (2-5 months) HFD, rats developed obesity and hippocampal disruption, as measured by a contextual pre-exposure fear-conditioning (CPE-FC) paradigm. HFD-induced memory impairments were mediated by increased interleukin-1 beta (IL-1β) protein in hippocampus that occurred in response to a footshock during CPE-FC, as central IL-1 receptor antagonism with hIL-1RA prior to the footshock prevented the HFD-induced memory impairment. A 4-week dietary reversal (DR) in HFD rats

eliminated hippocampal IL-1β increase to footshock and restored memory function, yet DR animals were still obese. Therefore, HFD, not obesity, mediated the alterations in neuroinflammation.

To assess the impact of HFD, independent of obesity, we evaluated the neuroinflammatory phenotype following 3 days of HFD and observed a primed neuroinflammatory environment and potentiated neuroinflammatory response to a subsequent inflammatory challenge. The influence of HFD was observed most prominently in hippocampus. HFD-induced elevations of corticosterone (CORT) and HMGB1 are implicated in mediating the effects of HFD on neuroinflammatory processes through observations of CORT and HMGB1 blockade with RU486 and BoxA, respectively.

The evidence presented herein demonstrates: prolonged HFD mediates hippocampal memory by altering neuroinflammatory responding, evidence of a primed neuroinflammatory environment is observed following 3 days HFD, and HFD mediates primed and potentiated neuroinflammation through induction of hippocampal CORT and HMGB1.

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Abbreviations

11βHSD1: -----11 beta hydroxysteroid ----------dehydrogenase
ω3: ----------omega 3
ω6: ----------omega 6
ACTH: --adrenocorticotropic hormone
Ac-YVAD-CMK: ----caspase-1 inhibitor
AGE: -advanced glycation end-product
Arc: --activity-dependent cytoskeletal- ----------associated protein
BBB: ----------blood-brain barrier
BDNF: ------brain derived neurotropic ----------factor
BMI: ----------Body mass index
BoxA: ----------HMGB1 inhibitor
CD11b:-------- cluster of differentiation
CNS: ----------central nervous system
CORT: ----------corticosterone/cortisol
CPE-FC: -----context pre-exposure fear ----------conditioning
CRF: -----corticotropin-releasing factor
CSF: ----------cerebrospinal fluid
DAMP: -----danger/damage associated ----------molecular pattern
DIO: ----(high-fat) diet induced obesity
DR: ----------dietary reversal
FA: ----------fatty acid
FFA: ----------free fatty acid
GC: ----------glucocorticoid
GILZ: --glucocorticoid-induced leucine ----------zipper
GR: ----------glucocorticoid receptor
GRE: glucocorticoid-response element
HFD: ----------High-fat diet
hIL-1RA: ----------human interleukin 1 ----------receptor antagonist
HMGB1: -----high mobility group box 1
HPA: -hypothalamic-pituitary-adrenal
ICM: ----------intracisterna magna
IFNγ: ----------interferon gamma
IκBα: ---------nuclear factor kappa light ----------chain enhancer of activated ----------B cells inhibitor alpha
IL: ----------interleukin: 1β, 4, 6, 10, 18
kDa: ----------kilodalton
LCPUFA: ----------long-chain PUFA
LPS: ----------lipopolysaccharide
LTP: ----------long-term potentiation
Med: ----------medium-fat diet
MHCII: -------major histocompatibility ----------complex
MR: ----------mineralcorticoid recpeotr
MYD88: myeloid differentiation factor
NAc: ----------nucleus accumbens
NFκB: ------nuclear factor kappa-light- -----chain-enhancer of activated B cells
NLRP3: ---NOD-like receptor protein 3
NOD: ----------nucleotide-binding ----------oligomerization
PA: ----------palmitic acid
PAMP: ----------pathogen associated ----------molecular pattern
PCR: --------polymerase chain reaction
POCD: ---------post-operative cognitive ----------decline
PRR: ------pattern recognition receptor
PUFA: ------poly-unsaturated fatty acid
RAGE: ----------receptor for advanced ----------glycation end products
Reg: ----------regular chow
ROS: ----------reactive oxygen species
RU486: ----------GR antagonist
SFA: ----------saturated fatty acid
T2D: ----------type-II diabetes
TLR: ----------toll-like receptor
TNFα: ----------tumor necrosis ----------factor alpha
TrKB: ----------tropomyosin kinase B
Veh: ----------vehicle control
VTA: ----------ventral tegmental area

Chapter 1

Introduction

1.1. The Problem with Obesity

Obesity is one of the fastest growing health concerns of our time. It is a preventable condition marked by severe physiological and psychological consequences and impacts a significant portion of people in the developed world. In humans, obesity is typically described in terms of the Body Mass Index (BMI) scale, which is calculated based on the ratio of an individual's weight to height. Using this scale, an individual is considered overweight with a BMI of > 25, obese > 30 and morbidly obese > 40. It is estimated that a third of America's population qualifies as obese, which is double the rate from 40 years ago (O'Brien and Dixon, 2002).

An obese state is associated with a host of serious co-morbid metabolic disturbances, as well as shortens life-span, reduces quality of life and increases health-care costs (O'Brien and Dixon, 2002). Metabolic syndrome is a collection of serious health concerns of which obesity is a hallmark and initiating factor, and includes type-2 diabetes (T2D), insulin resistance, cardiovascular disease, glucose intolerance, hypertension, peripheral inflammation and an increased risk for dementia. T2D and insulin resistance are so highly prevalent with obesity that it has led to use of the term 'diabesity' to describe the linked conditions. Obesity is also associated with increased pain (Guneli et al., 2010, Ray et al., 2011, Somers et al.) and asthma (Wood and Gibson, 2009). Unfortunately, as rates of obesity grow, the condition predominately impacts members of low socioeconomic standing who are less able to afford and access medical health care (Monteiro et al., 2004) to treat the

associating health conditions, which further exacerbates the mortality rates of the condition.

1.1.1. Obesity and Cognition

In addition to the widespread physiological metabolic health conditions, obesity is also associated with a number of neurological, psychiatric and cognitive alterations mediated by the central nervous system (CNS). Obese patients present at higher than normal levels for depression and mood disorders (Dantzer et al., 2008, Capuron et al., Soczynska et al., Song and Wang), stroke (Drake et al., 2011), and a number of psychiatric conditions (Lopresti and Drummond, 2013). Furthermore, obesity is linked to a decline in a number of facets of cognition. BMI levels are linked with deficits in semantic memory, attention and alertness (Nilsson and Nilsson, 2009), executive function (Sellbom and Gunstad, 2012) and learning and memory (Gunstad et al., 2010). A number of studies have *indirectly* linked obesity and cognitive decline by examining obesity-related disorders. For instance, the Canadian First Nations Population Study found obesity and related metabolic syndrome were associated with decreased performance on a number of cognitive measures, most notably for tasks involving executive function (Fergenbaum et al., 2009). Another study found that metabolic syndrome and T2D are associated with significantly decreased scores of overall intellectual function and recall, and nearly significant declines on tests of learning and executive function (Hassenstab et al., 2010). In addition, a longitudinal study measuring cognitive fluency and memory decline paralleled the geographic distribution of incidence of stroke mortality (Wadley et al., 2011). While a number of cognitive functional deficits are implicated,

it appears that aspects of learning, memory and executive control are most likely disturbed in obesity (Cohen, 2010, Francis and Stevenson, 2013).

As mentioned previously, human obesity is a condition that rarely presents in the absence of concomitant disorders, thus the examination of links between obesity and cognition are confounded. Therefore, animal models have become particularly important to clarify the specific nature of the cognitive decline that occurs with obesity, and there are a number of different rodent models commonly used (Buettner et al., 2006, Buettner et al., 2007, Young and Kirkland, 2007, Chida et al., 2008). Researchers utilize two primary methods to achieve obese animal subjects for study: genetic manipulation (which tend to focus on altering leptin signaling) and high-fat or high-sugar diets. Diet-induced obesity (DIO) is particularly useful as it is believed that the quickly escalating nature of obesity in western societies is most likely attributed to alterations in diet (Archer and Mercer, 2007) and activity levels (Dwyer-Lindgren et al., 2013) rather than genetic factors. Diet-induced obesity is the result of chronic nutritional excess, which stimulates the body to convert and store extra energy as fat, expanding adipose stores. The DIO animal literature is unfortunately inconsistent, likely because species, animal strain, the nature of the dietary manipulation and the duration of feeding vary across studies. Subtle variations of any of these factors can alter the cognitive effects observed once obesity develops (Greenwood and Winocur, 1996, Dziedzic et al., 2007).

Typically, high-fat or high-sugar diets are used in studies of DIO, as they tend to induce obesity rapidly and reliably, but there is still variability in the amount and type of fat and sugar in such diets. ‘High-fat’ diets may refer to any diet within the

range of 20-60% fat, and most have high-sugar contents as well. Therefore, a review of the current literature examining DIO and related cognitive decline must be done accepting that the nature of DIO is not consistent across studies. DIO researchers primarily use rats or mice, and typically administer diet for 8-16 weeks prior to cognitive testing. This prolonged consumption allows a state of obesity, and often, other health conditions to develop, which makes distinguishing the effects of 'diet' versus the effects of 'obesity' on cognition difficult. It is for this reason that we utilized a very short-term HFD consumption protocol (3 days), data presented in Chapters 3 and 4, in an attempt to distinguish the effect of diet from obesity.

In general, the animal literature suggests a strong link between DIO and hippocampal-based learning and memory functions (Kanoski and Davidson, 2011). The hippocampus is an important brain region involved in the regulation of learning and memory processes, specifically those that are episodic, contextual or spatial in nature. Multiple studies have linked DIO to decreases in hippocampal specific learning and memory in mice, using established hippocampal-based tests such as the radial-arm maze (Valladolid-Acebes et al., 2011), the Morris water maze (Diano et al., 2006, Farr et al., 2008) and an object location task (Heyward et al., 2012). Rats fed a HFD demonstrated poorer performance on tests of hippocampal memory (Kosari et al., 2012), frontal lobe-based cortical function (McNeilly et al., 2011) and overall general intellectual function, than their normal-food counterparts (Winocur and Greenwood, 2005). The hippocampus is critical and required for the process of learning, consolidation and retrieval of long-term contextual memories (Rudy et al., 2002), and interference with any of these processes could disrupt long-term

memory function. Some studies have linked the hippocampal cognitive effects of DIO to structural and functional alterations within the hippocampus, finding decreased dendritic spine density and decreased levels of hippocampal brain-derived neurotrophic factor (BDNF)(Molteni et al., 2002, Wu et al., 2004, Park et al., 2010), the alterations of which can lead to reduced synaptic plasticity (Farr et al., 2008, Stranahan et al., 2008, Hwang et al., 2010). Synaptic plasticity is measured as a neurons ability to be modified by experience, also known as long-term potentiation (LTP), and is a process required to successfully consolidate information from short-term to long-term storage. While most studies examining the impact of DIO on cognition focus on identifying the nature of the cognitive function disrupted, a few have implicated interferences with the processes of learning (Stranahan et al., 2008, Hwang et al., 2010) and memory consolidation (Heyward et al., 2012, Boitard et al., 2014) to describe the nature of hippocampal functional decline observed with DIO.

While hippocampal function appears to be particularly *vulnerable* to the detrimental effects of DIO, it may also be an important regulatory structure involved in the *development* of obesity resulting from HFD consumption. There is an emerging theory that diet-induced alterations in hippocampal function may provide a feedback mechanism to further increase HFD intake, thus exacerbating obesity development (Davidson et al., 2007, Sellbom and Gunstad, 2012, Francis and Stevenson, 2013). This idea is supported by evidence that selective lesions of either the complete or the ventral hippocampus produce an increase in food intake and body weight (Davidson et al., 2009). Also, BDNF is a known important anorexigenic

regulator (Lebrun et al., 2006), and BDNF levels are decreased in obesity (see above) . It seems that HFD may induce neurological alterations in the hippocampus that may impair memory and regulation of impulses, leading to further consumption of HFD. While the hippocampus appears to be particularly vulnerable to the long-term effects of DIO, it may also be a brain region that is acutely sensitive to short-term changes in diet and likely a region that is initially susceptible to early diet-induced structural and functional alterations. For these reasons, the studies presented in this dissertation are aimed at observing the impact of diet on hippocampal cognitive function, as well as examining diet-induced molecular alterations, specifically within the hippocampus.

1.2. Inflammation and Diet-Induced Obesity

It is well established that obesity is a peripherally inflammatory disease. Increased markers of inflammation have been well characterized in the adipose tissue and circulation (Mito et al., 2000), and the resulting pro-inflammatory environment is known to induce many of the physiological health concerns associated with obesity (Donath and Shoelson, 2011). In addition, hormone dysregulation also commonly occurs with obesity (Ouchi et al., 2011), and as many hormones are known to possess inflammation-mediating properties, obesity progression may induce positive-feedback cycles that exacerbate inflammation (Coppack, 2001). While peripheral inflammation and neural inflammation are distinct processes, reciprocal and interconnected communication allows inflammatory signals in the body to signal the brain (Maier and Watkins, 1998).

There is emerging evidence that obesity also presents as a condition marked by altered neuroinflammation (Erion et al., 2014), which is likely a key component mediating the cognitive effects associated with the disease (Pistell et al., 2010).

1.2.1 Overview of Relevant Innate Immune Function

In order to fully understand the connection between peripheral inflammation and neuroinflammation as well as the link between neuroinflammation and the cognitive decline associated with obesity, it is important to first understand the basics of innate immune processes. In contrast to adaptive immunity, innate immune function is evolutionarily conserved across species, functions by identifying pathogenic molecules independent of prior exposure, and represents the first line of defense against infection. Innate immune processes are carried out by a number of distinct cell types, which include macrophages, dendritic cells and neutrophils in the periphery, and astrocytes (Farina et al., 2007) and microglia (Ransohoff and Cardona, 2010) within the CNS. These cells are capable of expressing multiple states of activation, with each state presenting distinct morphological and functional capacities (Mosser and Edwards, 2008). This activation process has been extensively well studied in microglia (Beynon and Walker, 2012), which will be discussed in more detail later.

In the absence of activating factors, microglia have morphologically small cell bodies with long processes that support neural function and act to monitor and survey the surrounding microenvironment (Nakajima and Kohsaka, 2001). Within the CNS, immune functions are primarily facilitated by microglia. The surface of these cells express a number of types of pattern recognition receptors (PRRs)

(Janeway and Medzhitov, 2002), such as the toll-like receptors (TLRs) (Aravalli et al., 2007), which evolved to bind and be activated by molecular patterns expressed by a variety of bacterial and viral pathogens (Park et al., 2009), which are termed pathogen associated molecular patterns (PAMPS). In addition, PRRs can be activated by danger or damage signals, called danger associated molecular patterns (DAMPs) (Bianchi, 2007). For instance, TLR2 identifies components of gram-positive bacteria, while lipopolysaccharide (LPS), a component of gram-negative bacteria, activates TLR4 (Park et al., 2009), which is a well characterized and prototypical PRR. Under non-disease states, identification by microglia of a PAMP or DAMP, induces structural alterations of the cell to support phagocytosis (Nakajima and Kohsaka, 2001, Town et al., 2005). In addition, PRR ligation initiates an internal cascade leading to the activation of transcription factors, most notably nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Kawai and Akira, 2007). NFκB induces the production and secretion of a variety of pro-inflammatory signaling molecules such as chemokines, nitric oxide, prostaglandins and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFa) and interleukin-1 beta (IL-1β)(Kawai and Akira, 2007).

IL-1β is a master regulator of neuroinflammation (Basu et al., 2004). IL-1β signaling from the periphery can induce cytokine production in the brain (Maier, 2003), and can modify the nature of an inflammatory response by inducing other pro-inflammatory cytokines, like IL-6 and TNFα, (Basu et al., 2002) or the late-phase anti-inflammatory cytokine, IL-10 (Foey et al., 1998). Effects of IL-1β signaling are

wide spread throughout the brain as both neurons (Ericsson et al., 1995) and innate immune cells express IL-1 receptors (Ban, 1994).

1.2.2. Peripheral Inflammation and Obesity

It is well established that obesity and associated health conditions, such as T2D, have a peripheral inflammatory component (Mito et al., 2000, Donath and Shoelson). This is well understood and attributed to the nature of central abdominal (visceral) adipose tissue, which has long been viewed as a passive storage reservoir for excess levels of circulating lipids. It is now understood that adipose cells are dynamic and can produce and secrete a number of physiologically active factors, known as adipokines (Cano et al., 2009). Adipokine signaling can modulate energy homeostasis, metabolism (Ouchi et al., 2011), feeding behavior, fertility, and inflammation (Kovalovsky et al., 2000). Levels of adipokine secretion are dependent on the size of adipose cells (Bergo et al., 1996, Xu et al., 2002) and are also determined by the nature of the diet (Yeop Han et al., 2010). Following DIO, adipose tissue produces significantly more adipokines such as insulin, leptin, resistin, visfatin and the pro-inflammatory cytokines IL-1β, IL-6 and TNFα (Coppack, 2001, Xu et al., 2002, Cano et al., 2009). In normal-weight individuals, this regulation is adaptive and important for subtle control over weight and metabolism (Coppack, 2001). In the case of obesity, elevated and continued adipokine signaling becomes maladaptive, fosters an inflammatory environment (Cano et al., 2009, Das, Yang et al.), and causes the body to be less sensitive to the anorexigenic properties of adipokines (Mantzoros, 1999). It is believed that this desensitization induces such

conditions as metabolic syndrome and insulin and leptin resistance (Zimmet et al., 1999, Yang et al., 2010).

Leptin is a key hormonal adipokine related to feeding and satiety regulation, and many genetic knockout models for obesity target the leptin signaling system, either by preventing leptin secretion (ob/ob), or expression of the leptin receptor (db/db)(Fernandez-Riejos et al., 2010). Leptin also has important immune regulation functions (Fantuzzi and Faggioni, 2000, Otero et al., 2006) as leptin is structurally similar to pro-inflammatory cytokines and may have similar functional signaling properties (Coppack, 2001). Leptin receptors are encoded by the diabetes gene (db) and belong to the class I cytokine receptor superfamily, which also includes receptors for IL-6 (Otero et al., 2006). Leptin has been shown to modulate inflammatory signaling in microglia by inducing IL-1β release (Pinteaux et al., 2007) and IL-6 production (Tang et al., 2007). While leptin does not appear to induce the expression of IL-6 from isolated macrophages, the presence of leptin augments the induction of IL-6 following LPS (Vaughan and Li, 2010) indicating a modulatory and 'priming' effect of leptin on inflammation. As peripheral leptin levels increase with obesity development, (Coppack, 2001), the studies presented in Chapter 2 use peripheral measures of leptin as a marker of an obese phenotype.

In addition to the inflammation induced directly by adipocytes, pro-inflammatory cytokines and other signaling factors released by adipose cells recruit and activate immune cells to fat tissue (Stofkova, 2010, Yeop Han et al., 2010). This process likely occurs through activation of TLRs (Kim et al., 2012), as TLR activation is heavily implicated in mediating adipose tissue inflammation(Fresno et al., 2011,

Konner and Bruning, 2011). This further amplifies the inflammatory environment of adipose stores (Ouchi et al., 2011) as activated innate immune cells produce and release their own set of pro-inflammatory cytokines (Harford et al., 2011). Additionally, obesity may alter vascular permeability by interacting with sympathetic nerves within perivascular adipose tissue (Guzik et al., 2007). This may allow pro-inflammatory cytokines released from adipose and innate immune cells to have easier access to the circulatory system, through which they may signal and trigger further inflammation in distal structures, such as the brain (Lee et al., 2009, Das, 2010).

1.2.3. Obesity and Glucocorticoids

In addition to the pro-inflammatory nature of obese adipose tissue, heightened inflammatory processes in obesity may also occur through dysregulation of the glucocorticoid system (McNeilly et al., 2015). Glucocortiocids (GCs) are important steroid hormone regulators of several homeostatic functions, primarily those related to metabolic (Zanchi et al., 2010) and inflammatory processes (Sorrells and Sapolsky, 2007). Originally named for its role in glucose metabolism, cortisol (CORT, cortisol in humans, corticosterone in rats) is most well known and characterized due to its role as a stress hormone (Sapolsky et al., 2000) and potent immunosuppressive properties (De Bosscher and Haegeman, 2009). The effects of CORT occur primarily through interactions with the mineralcorticoid (MR) and glucocorticoid (GR) receptors, which are distributed in varying concentrations throughout the body and brain, including on neurons and microglia (de Kloet et al., 1990, Sierra et al., 2008). Therefore, the effects of CORT are apparent within the

periphery and the CNS. It is understood that ligation of MR and GR produce different responses, with MR mediating homeostatic circadian processes, while ligation of GR induces immune-regulation and negative feedback effects (De Kloet and Reul, 1987, Lightman et al., 2008). As GR binds CORT with nearly a 10-fold lower affinity than does MR, GR activation occurs predominantly when CORT levels are particularly high (Lightman et al., 2008). Therefore, one may observe very different, potentially opposite, profiles of metabolic changes in response to CORT binding, based on which receptor class is occupied (Sorrells et al., 2009).

Due to its immunosuppressive nature, CORT therapy has been used clinically for years as a tool to curb exacerbated inflammation (McEwen et al., 1997). The literature demonstrating the anti-inflammatory effects of CORT is comprehensive and the subtle nuances of mechanisms involved are well established (Franchimont, 2004). However, as this dissertation will focus on the pro-inflammatory nature of CORT, these processes will not be discusses extensively. Briefly, intracellular GR is bound when circulating levels of CORT passively enter cells. With ligation, GRs translocate into the nucleus where they activate transcription factors known as glucocorticoid-responsive elements (GREs). Activated GREs can induce anti-inflammatory effects by inhibiting NFkB and the transcription of pro-inflammatory mediators (Kovalovsky et al., 2000, Chinenov and Rogatsky, 2007) and by activating the transcription of anti-inflammatory molecules (Almawi et al., 1996).

CORT is released when paraventricular neurons in the hypothalamus release corticotropin-releasing hormone (CRF), which stimulates the release of adrenocorticotropin (ACTH) from the pituitary, leading to the secretion of

glucocorticoids from the adrenal gland, a process known as the hypothalamic-pituitary-adrenal (HPA) axis (Carrasco and Van de Kar, 2003). Typically, serum levels of CORT follow a circadian rhythm that peaks during the active phase. In times of sickness and stress (Fleshner et al., 1995), the HPA axis induces elevated levels of CORT. CORT regulates a variety of processes related to the stress response, one of which is greater GR binding, which inhibits the HFA axis, thus helping to resolve increases in CORT (Sapolsky et al., 2000). In addition, CORT levels are regulated though interactions with 11β-hydroxysteroid dehydrogenase (11β-HSD), an enzyme that exists in dual forms, with each functioning to either activate or to neutralize circulating CORT (Seckl, 1997).

Inactive forms of CORT (cortisone in humans, 11-dehydrocorticosterone in rodents), the variants of which are not able to ligate GR and MR, are converted back into their active forms by 11β-HSD-1 (Seckl, 1997), which has been implicated in mediating the peripheral inflammatory environment in obesity (Morton, 2010, Staab and Maser, 2010). It is well established that the state of obesity and its corresponding co-morbid health issues are linked with elevated levels of peripheral CORT, specifically within adipose tissue (Harford et al., 2011). It is believed that CORT levels are increased within obese adipose stores due to an upregulation of 11β-HSD-1 (Paulsen et al., 2008, Tagawa et al., 2009).

Serum levels of CORT also appear to be altered in DIO (Wang et al., 1998, Buchenauer et al., 2009), although it is unclear if serum CORT alterations in obesity are the result of increased 11β-HSD-1 activity or through excess CORT secretion by the HPA axis in response to peripheral inflammation (Staab and Maser, 2010). CRF

release is mediated in part by hippocampal projections to the hypothalamus, so alterations in hippocampal functioning from DIO could exacerbate HPA activity (Carrasco and Van de Kar, 2003). In either case, it appears that elevated levels of CORT, particularly by signaling through GR, may mediate the insulin sensitivity (Hashimoto et al., 2013) and obesity (Marissal-Arvy et al., 2011) associated with prolonged HFD consumption as RU486/mifepristone, a GR antagonist (Schreiber et al., 1983), is able to reverse these detrimental effects following DIO (Okada et al., 1992). As obesity and linked insulin sensitivity are conditions marked by pro-inflammatory environments, evidence linking GR activity and DIO suggests that sustained elevated levels of CORT may be mediating pro-inflammatory effects, which contrasts with the typically viewed anti-inflammatory nature of CORT.

1.2.3.1. Pro-Inflammatory Nature of CORT

There is reason to doubt the commonly held belief that CORT is universally anti-inflammatory, as chronic stress-induced CORT can have substantial damaging consequences on health (Oitzl et al., 2010), and the effect of clinical CORT therapy to treat inflammation is typically not beneficial long-term (Rutgeerts, 2001). An emerging body of literature supports the idea that CORT may also induce pro-inflammatory effects (Dinkel et al., 2002, Sorrells and Sapolsky, 2007, Frank et al., 2012). An important study has recently demonstrated that the timing of CORT exposure compared to the initiation of an inflammatory response is critical for determining the immuno-modulatory nature of CORT (Frank et al., 2010c). For instance, when exogenous CORT was administered following LPS (a TLR4 agonist) the effects appear anti-inflammatory. However, if CORT is given 2 or 24 hours *prior*

to LPS, opposite cytokine profiles were measured as a result indicating a pro-inflammatory effect of CORT. Perhaps the most interesting finding of the study is that CORT was able to strongly potentiate the inflammatory response to LPS when given 24 hours prior, which allowed ample time for CORT levels to return to normal (Frank et al., 2010c). This indicates that elevated CORT may induce long-lasting functional immune alterations that persist after CORT levels subside.

The apparent dual nature of CORT-induced modulation of inflammation depending on temporal expression appears to make sense evolutionarily. As innate immune activity is energy intensive, it seems appropriate to inhibit inflammation during times of stress to reserve energy stores for other, potentially more life saving activities, such as physical exertion. However, as stressful situations may also increase the likelihood of acquiring infection or injury, the lasting effects of marked CORT elevations may have served to adaptively support a pro-inflammatory environment to later deal with such insults. However, this system no longer seems adaptive when placed in context of situations of chronic stress and disease, as continued elevated levels of CORT may exacerbate inflammatory processes past the point of being advantageous to become damaging (Dantzer et al., 2008). This concept is particularly important, as it appears that one particularly damaging effect of elevated neuroinflammatory processes is the cognitive disruption observed in obesity.

1.2.4. Peripheral and Central Inflammatory Processes are linked

It appears that obesity is heavily implicated in being peripherally pro-inflammatory, a phenomenon that may be the result of the nature of adipose tissue

and/ or also in response to the pro-inflammatory regulation of elevated CORT signaling. A discussion on the specific mechanisms potentially involved in CORT-induced mediation of lasting pro-inflammatory processes will come later. However, for purposes here, it is important to make the connection between peripheral inflammation, neuroinflammation and cognitive decline.

Obesity is marked by elevated peripheral inflammation, therefore, it is likely that the disease also has a corresponding upregulation of neuroinflammation, as peripheral inflammatory processes are able to signal and alter CNS activity (Maier and Watkins, 1998, Rosas-Ballina and Tracey, 2009). The brain and spinal cord are thought to exist in an 'immune privileged' environment, which is the result of tight junctions between astrocytic glial cells that make up the blood-brain barrier (BBB), which protects the vital yet vulnerable CNS from direct infection from pathogens. However, the brain is not void of inflammatory processes. In times of peripheral infection or injury, inflammation induced in the body signals the brain, which induces a separate inflammatory response within the CNS (Maier, 2003). This process initiates brain-regulated adaptive behavior modifications to facilitate healing. For instance, in times of sickness, there are a number of normal behavioral (reduced diet consumption and social activity), physiological (fever, increased sleep) and cognitive (brain fog) alterations that occur to facilitate recuperation, which together, are known as 'sickness responses' (Watkins and Maier, 2005). These alterations represent adaptive processes aimed at combating infection through fever, to conserve energy, and to protect the organism from harm during healing.

Peripheral innate immunity communicates with the central nervous system via multiple pathways, with bloodborne, neural, and recently discovered lymphatic (Louveau et al., 2015) routes having been identified (Ericsson et al., 1994). Signaling by IL-1β has been implicated in both blood and neural mechanisms as IL-1β binds to receptors on brain vasculature initiating an internal central response (Cao et al., 1996) and peripheral IL-1β activates vagal nerve afferent connections to initiate central inflammation (Goehler et al., 1997, Hosoi et al., 2000). As previously discussed, IL-1β is considered to be a key regulator of neuroinflammation (Basu et al., 2004) also appears to be particularly important for expression of sickness behavior (Gibertini et al., 1995, Anforth et al., 1998, Goshen and Yirmiya, 2009). In addition, vagal activation of neuroinflammation also appears to occur in response to alterations in diet (Kiecolt-Glaser, 2010) and the microbiotic environment of the gut (Montiel-Castro et al., 2013).

1.2.5. Neuroinflammation in diet-induced obesity

In light of the previous discussion on the pro-inflammatory nature of obesity and the link between peripheral and central inflammatory processes, it is not surprising that there is evidence that obesity is also marked by alterations in neuroinflammation. Interestingly, this appears to be an area of study that was, until the past few years, largely ignored by scientific researchers as initial scientific examinations linking the brain with obesity focused more on understanding neural regulation of feeding behavior and weight gain, and were less focused on how obesity changes the brain. In general, obesity appears to increase the vulnerability of the CNS through pro-inflammatory mechanisms (Bruce-Keller et al., 2009),

increased BBB permeability (Kanoski et al., 2010) and has been implicated in inducing a state of pre-mature brain aging (Uranga et al., 2010). For instance, obesity is associated with increased mortality to neurotoxicity (Choi et al., 2005) and increased oxidative stress (Zhang et al., 2005, Lu et al., 2011). In addition, obesity is associated with increased immune cell entry into the CNS (Buckman et al., 2014), indicating alterations indicative of a pro-inflammatory environment.

Due to its homeostatic regulation of feeding behavior and body weight, the hypothalamus has been the focus of many explorations into the neuroinflammatory nature of obesity (Thaler et al., 2010, Miller and Spencer, 2014). Increased levels of pro-inflammatory cytokines and NFκB have been observed in the hypothalamus (De Souza et al., 2005, Milanski et al., 2009, Thaler et al., 2012, Yi et al., 2012) and cortex (Zhang et al., 2005, Pistell et al., 2010) following HFD consumption. However, there appears to be some discrepancy, as another study observed increased IL-1β in the hippocampus, but not in hypothalamus or cortex following juvenile HFD (Boitard et al., 2014).

The previously mentioned studies report seeing basal increased levels of inflammatory markers in the brain from DIO. Interestingly, just as many studies that have examined neuroinflammation in DIO *only* observed elevated inflammation when DIO is combined with some sort of additional inflammatory insult such as stress (Yehuda et al., 2005, Alzoubi et al., 2009, Yamada-Goto et al., 2012), LPS (Scott et al., 2004, Pohl et al., 2009, Andre et al., 2014) or central infusion of IL-4 (Oh et al., 2010). In addition, multiple studies failed to observe any evidence of neuroinflammation in DIO, even after an additional peripheral LPS challenge

(Baumgarner et al., 2014, Boitard et al., 2014). The phenomenon, that neuroinflammation with DIO does not occur unless animals were additionally given an inflammatory challenge, is likely vastly important. In fact, the data presented in Chapter 2 mirror this same effect. It appears that DIO may not, itself, activate neuroinflammatory processes, but rather may induce alterations in immune cell types within the CNS to be primed such that they facilitate a potentiated inflammatory response upon an additional inflammatory signal. Note the parallels with the observations of the pro-inflammatory nature of CORT discussed previously. This data hints toward a DIO priming effect on neuroinflammatory processes, which will be discussed in more detail later.

1.2.5.1. IL-1β Mediates Hippocampal Functional Declines

The significance of neuroinflammation with DIO is important to discuss, as there is a well-established link between neuroinflammation and cognitive function, particularly for processes involved in learning and memory. As already discussed, DIO manifests with cognitive alterations, particularly for these same facets. A few studies have directly linked hippocampal-based learning and memory declines of obesity as being mediated by neuroinflammatory alterations in metabolism (Bruce-Keller et al., 2009) and increased pro-inflammatory cytokines (Pistell et al., 2010, Andre et al., 2014, Boitard et al., 2014, Erion et al., 2014). Pro-inflammatory cytokines, particularly IL-1β, are well known to mediate processes involved in learning and memory. Here, a brief overview is presented on the current understanding of the link between IL-1β and cognitive function.

Interleukin-1 is an important pro-inflammatory cytokine that naturally occurs in two distinct forms. IL-1α, which remains mostly intracellular, is not commonly found in circulation, while IL-1β, as mentioned previously, appears to act as a master cytokine regulator which can induce the transcription of additional cytokines, and mediates sickness responses, including associated cognitive disruption (Yirmiya and Goshen, 2011). IL-1β is constitutively expressed (Lopez-Castejon and Brough, 2011), however, there is tight regulation over the synthesis, processing, secretion and activity of IL-1β, which appear to occur through unique processes (Dinarello, 1998). For instance, there is a naturally occurring inhibitor of IL-1, the IL-1 receptor antagonist (IL-1RA), and receptors for IL-1 are either active (type 1) or inactive and serve as a decoy (type 2) (Dinarello, 1998). In addition, the synthesis, processing and release of IL-1 also appears unique as IL-1β lacks a signal peptide, and therefore release does not follow the classic endoplasmic reticulum-golgi pathway of other secretory proteins (Andrei et al., 1999). The specific mechanisms for IL-1β release are still not fully understood, but it appears that there are a number of methods for IL-1β to exit a cell (Piccioli and Rubartelli, 2013), and that this variability may induce altered inflammation-mediating properties of IL-1β action. In addition, prior to release, IL-1β must be enzymatically processed through cleavage from the pro-IL-1 form and it appears that a significant amount of IL-1 mRNA may degrade prior to processing (Dinarello, 1998). One mechanism of IL-1 processing is through activation of a multi-protein complex known as the inflammasome, which activates the enzyme caspase-1 to cleave IL-1β (Khare et al., 2010), but it appears that IL-1β can be processed by non-caspase-1 mechanisms as

well (Stehlik, 2009, Edye et al., 2013). A more detailed discussion of inflammasomes will come later when I discuss potential mechanism of neuroinflammatory priming.

The tight control of IL-1β levels appears to be particularly important when examining the impact of IL-1β on mediating cognitive processes. Learning events increase gene expression of IL-1 (Schneider et al., 1998) and IL-1 signaling at normal physiological levels appear to be crucial for proper hippocampal memory formation (Yirmiya et al., 2002, Avital et al., 2003). However, when levels of IL-1β become dysregulated, memory function declines (Yirmiya and Goshen, 2011) and it appears that memory function is vulnerable when levels of IL-1β are very low or very high. For instance, elevated levels of IL-1β are associated with disrupted hippocampal memory (Palin et al., 2004, Barrientos et al., 2006, Dinel et al., 2011, Erion et al., 2014). This relationship is strengthened by observations that the time course for hippocampal memory disruption is determined by the duration of IL-1β elevation (Barrientos et al., 2009a, Terrando et al., 2010), and that memory impairments can be induced by direct injection of exogenous IL-1β (Gibertini et al., 1995, Barrientos et al., 2002). Furthermore, blocking action of IL-1β by injecting IL-1RA prevents infection-induced memory impairments (Palin et al., 2004, Frank et al., 2010a), which demonstrates deficits are IL-1β mediated. Use of IL-1RA, as presented in Chapter 2, further supports this notion.

The mechanisms by which IL-1β influences memory function are not fully understood, but may occur through alterations of BDNF (Lapchak et al., 1993). BDNF is critical for memory function; levels of hippocampal BDNF mRNA are directly correlated with speed of learning (Molteni et al., 2002) and the BDNF mRNA

upregulation that occurs in the hippocampus following a learning session is crucial for learning to occur (Barrientos et al., 2004). BDNF is particularly important for hippocampal-based learning and memory functions, as hippocampal-specific deletion of BDNF impedes learning and memory (Heldt et al., 2007). HFD and obesity have been extensively observed to decrease levels of BDNF in the hippocampus (Molteni et al., 2002, Wu et al., 2004, Stranahan et al., 2008, Park et al., 2010, Yamada-Goto et al., 2012).

BDNF is a member of the neurotrophin family of signaling molecules, which can activate high-affinity tropomyosin kinase B (TrkB) receptors to induce the immediate early gene Arc (activity-dependent cytoskeletal-associated protein), which promotes cell survival and stimulates LTP and synaptic plasticity (Ying et al., 2002, Chen et al., 2009, Ohira and Hayashi, 2009, Ji et al., 2010). Interestingly, genetically induced deletion of TrkB produces an obese phenotype and decreased learning and memory (Yeo et al., 2004), which supports the notion, discussed previously, that alterations in hippocampal signaling fosters obesity development. Direct hippocampal injection of IL-1β prevents learning-induced BDNF (Barrientos et al., 2004) and IL-1RA prevents stress-induced decreases in BDNF (Barrientos et al., 2003) and blocks the decrease in Arc following peripheral infection (Frank et al., 2010a). Furthermore, in vitro cultures indicate that the presence of microglia are required for IL-1β to have detrimental impacts on BDNF (Rage et al., 2006). These studies strongly implicate microglial IL-1β as a modulator of memory function through alterations of BDNF signaling.

1.3. Diet-induced obesity and neuroinflammatory priming

Thus far, the discussion has focused on linking obesity, inflammation and cognitive decline. Unfortunately, the current DIO literature is limited beyond that scope and very little in the way of conclusions can be made as to what specific mechanisms may be at play to mediate the effects. As discussed previously, the literature suggests that, in DIO, the neuroinflammation that results may likely be less of a basal increase in response to peripheral pro-inflammatory signals, but more that the effect of DIO induces functional alterations within the brain that lead to exaggerated neuroinflammatory responses to a subsequent challenge. This phenomenon is referred to as 'neuroinflammatory priming'. The effects in DIO also mirror what is currently understood with respect to stress- and aging-induced primed neuroinflammation.

1.3.1. Stress-induced Neuroinflammatory Priming

Acute stressors induce rapid (2-6 hours) increases in pro-inflammatory cytokines (mRNA and protein) in the brain, with effects no longer apparent 24 hours after (O'Connor et al., 2003b). However, a potentiated neuroinflammatory response is observed 24 hours after acute stress, if the subjects are additionally challenged with an inflammatory stimulus, such as LPS (Johnson et al., 2002). In addition, prior stress potentiates the physiological (fever) sickness response to LPS (Johnson et al., 2003). A similar effect is observed when LPS is given after chronic unpredictable stress (Munhoz et al., 2006). It appears that acute mild stress may not induce similar priming effects, but priming can be observed with repeated mild stressor exposure (Avitsur et al., 2002). Therefore, it appears severe or chronic stress induces long-

term pro-inflammatory changes that can last 4-6 days (Johnson et al., 2002). These were the first studies describing the phenomenon of primed neuroinflammation, which is an effect that has, in addition to stress, been observed in result to a range of insults such as peripheral infection (Perry et al., 2007, Dantzer et al., 2008), advanced age (Barrientos et al., 2006, Chen et al., 2008), neonatal infection (Williamson et al., 2011) and laparotomy (Hains et al., 2011).

Obesity, or the HFD that leads to obesity, may induce neural inflammation similarly. For instance, the addition of chronic stress to HFD exposure exacerbates hippocampal memory deficits, more so than the impact of either challenge alone (Alzoubi et al., 2009), and stress and unhealthy diet are known to interact to enhance inflammation (Kiecolt-Glaser, 2010). The notion is that, in such conditions, basal cytokine levels are not elevated, but a subsequent or concomitant challenge induces the production of cytokines well above adaptive levels.

1.3.1.1. The role of Microglia

It appears that the neural innate immune cell type, microglia, may be particularly important in mediating potentiated neuroinflammatory responses (Frank et al., 2007) and microglial inhibition with minocycline prevents potentiated responses to LPS (Hains et al., 2011, Williamson et al., 2011). Furthermore, microglia are not evenly distributed within the brain but rather are found at higher concentrations in select brain areas such as the hippocampus (Lawson et al., 1990), which indicates hippocampal function may be particularly vulnerable to negative consequences of neuroinflammatory priming.

Central levels of microglia comprise three subsections of microglia types that vary in origin. Predominately, the CNS contains a resident microglial population of cells that are myeloid in origin, differentiated into monocytes during embryonic development, and migrated to the CNS where they remain throughout life (Ginhoux et al., 2010). Central immune functions are supported by perivascular microglia, which differentiate into monocytes in the bone marrow and are replenished (Aguzzi et al., 2013). In addition, peripheral macrophages can enter the brain to further facilitate neuroinflammatory responses (Mildner et al., 2007), and recall that obesity is characterized by increased immune cell entry into the CNS, which occurs as a result of peripheral macrophage entrance.

As mentioned previously, innate immune cell activation is a dynamic process and multiple activation states have been observed in microglia (Ransohoff and Perry, 2009), with multiple models existing to describe the range of microglial activation. Initially, microglial activation was thought to occur on a continuum between two extremes, with pro-inflammatory/classical/neurotoxic activation on one end (M1 type activation) and anti-inflammatory/alternate/regenerative activation (M2) on the opposite end (Town et al., 2005, Kigerl et al., 2009). The specific activation response that is initiated within a microglial cell is dependent on the nature of the triggering stimulus (Lambert et al., 2010). Classical activation of microglia is thought to occur in response to PRR ligation or by signaling from pro-inflammatory cytokines or interferon-γ (IFNγ), while the M2 state occurs in response to the IL-4 or the anti-inflammatory cytokine IL-10. However, an update of this hypothesis suggests that IL-4 may induce more of a wound-healing, rather than

regulatory, activation state (Mosser and Edwards, 2008). As resident microglia are not replenished, it is believed that the dynamic capacity of these cells to shift between activation states facilitates their ability to respond appropriately to subtle alterations in the CNS microenvironment.

Within the past decade, the understanding of microglial activation states has advanced, starting with observations first made in 2005 of an altered state of microglial activation (Cunningham et al., 2005), wherein cells appeared 'primed'. Microglia demonstrated increased surface antigen expression of major histocompatibility complex 2 (MHCII) but did not demonstrate increased pro-inflammatory cytokine secretion, but with a second 'hit' of LPS, an exaggerated inflammatory response occurred (Cunningham et al., 2005). Evidence of a primed microglial cell can be identified, even in the absence of increased production of pro-inflammatory cytokines, by upregulation of cellular protein markers such as MHCII (Frank et al., 2007) and increased CD11b (Akiyama and McGeer, 1990, Beynon and Walker, 2012). While it is likely that microglia are also responsible for mediating the effects of HFD on neuroinflammatory processes, the present series of studies do not *directly* implicate microglia in mediating the neuroinflammatory priming from HFD consumption. However, evidence of HFD-induced upregulation of CD11b mRNA observed in hippocampal tissue (data presented in chapters 3 and 4) suggests that the effects of HFD are, at least partially, due to alterations in microglial activation.

1.3.1.2. The role of CORT

Neuroinflammatory priming occurs in response to sickness and stress, however it is unclear what factor or factors are induced by stress to mediate this

process. Furthermore, the brain exists in an immune privileged environment, so even in the case of peripheral infection it is unlikely that pathogens enter the brain to target microglia to initiate the 'priming' process. As mentioned previously, CORT levels are increased as a result of stress and sickness, and as microglia express GR, CORT is a likely candidate to induce alterations in microglia indicative of neuroinflammatory priming (Sorrells et al., 2009). It appears that stress-induced neuroinflammatory priming to a secondary challenge of LPS may be attributed to the effects of CORT. Exogenous administration of CORT prior to peripheral LPS has been shown to potentiate hippocampal levels of pro-inflammatory cytokines (Frank et al., 2010c), increase activation of NFκB (Munhoz et al., 2010) and enhance sickness behaviors (Hains et al., 2011). In addition, pre-treatment with the GR antagonist RU486 prevented the stress potentiation of LPS-induced inflammatory response in hippocampus (Munhoz et al., 2006), and in isolated microglia (Frank et al., 2012). This effect was also observed when animals were adrenalectomized to prevent stress-induced CORT increases (Frank et al., 2012). Therefore, it appears that stress-induced CORT may be mediating alterations within innate immune cells leading to a primed inflammatory state (Sorrells and Sapolsky, 2010), and may do so to alert the CNS to a state of 'danger' (Frank et al., 2013).

1.3.1.3. Neuroinflammatory Priming in Aging

The systematic association between hippocampal cognitive decline, microglial neuroinflammatory priming and the mediating role of CORT is exquisitely illustrated in aging. Although the incidence of dementia increases with advanced age, many individuals maintain normal cognitive function during the aging process.

However, cognitive decline is often first observed in otherwise-healthy older individuals after systemic challenges such as peripheral infection, surgery or intense stressors, leading to a condition known as post-operative cognitive dysfunction (POCD) in humans (see (Krenk and Rasmussen, 2011) for review). The resulting cognitive modulation was initially attributed to heightened neural immune activation in response to the peripheral immune signals from these challenges (Butcher and Lord, 2004, Munhoz et al., 2006). However, it appears that the cognitive declines observed with age are more likely due to age-induced neuroinflammatory priming (Dilger and Johnson, 2008, Barrientos et al., 2010, Ownby, 2010, Norden and Godbout, 2013, Barrientos et al., 2015).

A clear association has been made linking age with a potentiated neuroinflammatory response to peripheral infection which disrupts hippocampal memory consolidation (Barrientos et al., 2006, Barrientos et al., 2009a), and exaggerates sickness responses (Barrientos et al., 2009b). Aging induces a primed microglial state as evidenced through observations of age-induced increased basal hippocampal CD11b and MHCII (Frank et al., 2006, Frank et al., 2010b), and increased pro-inflammatory cytokine response to LPS in isolated microglia (Frank et al., 2010b). In addition, aging has been shown to alter the activation phenotype of microglia (Damani et al., 2011), and age-induced alterations in microglial function have been implicated in mediating age-induced neurodegeneration (Perry and Teeling, 2013) and sickness behavior (Dilger and Johnson, 2008). As with the model of stress-induced neuroinflammatory priming, evidence suggests that CORT may also mediate age-induced primed microglia. Age is associated with elevated basal

levels of CORT in the brain (Garrido et al., 2012, Barrientos et al., 2015) and aging may also induce a greater stress-induced CORT response (Butcher and Lord, 2004). In addition, blockade of GR activation with RU486 reversed age-induced evidence of microglial priming and prevented cognitive impairments from developing following peripheral infection (Barrientos et al., 2015).

While acute stress and infection produce evidence of microglial priming, the effects don't last long-term, likely due to the short-term nature of the primary insult. However, with the case of aging, and likely with DIO, the apparent influence on microglial priming is potentially much more lasting due to the chronic nature of age and DIO. However, even age and obesity may induce non-permanent primed neuroinflammatory processes as voluntary exercise may reverse the impact of age (Barrientos, 2011) and DIO (Molteni et al., 2004) on exaggerated neuroinflammatory function. Data presented in Chapter 2 suggests similar effects may occur after DIO rats are switched to standard chow (Sobesky et al., 2014). While it appears that obesity may induce a state of pre-mature brain aging (Yehuda et al., 2005, Cohen, 2010), it is likely that the impact of obesity and age on potentiated neuroinflammation can be averted with healthy diet and regular physical activity.

1.3.2. Mechanisms of neuroinflammatory priming

The processes through which priming occurs, specifically the alterations within microglia that mediate a potentiated response to a subsequent challenge, are still not well understood. It is possible that a number of different alterations may occur to mediate priming and one possible mechanism could be an upregulation of

TLR receptors, and increased signaling through TLRs may cause the proportionally larger pro-inflammatory cytokine response to LPS (Frank et al., 2007). This notion is supported by evidence that CORT up regulates TLR2 in peripheral keratinocytes (Shibata et al., 2009) and GR activation is able to mediate the inflammatory impacts of TLR ligation (Chinenov and Rogatsky, 2007).

1.3.2.1. Activation of Caspase-1

Another potential mechanism of priming may involve alterations in the processing of IL-1β. Recall that IL-1β processing is relatively unique. The best-studied enzyme for cleaving IL-1β from pro-IL-1 is caspase-1, which cleaves the inactive 31 kilodalton (kDa) into the bioactive 17-kDa mature IL-1β. The activity of caspase-1 is heavily implicated in mediating a number of aspects of disease. For instance, caspase-1 appears to facilitate toxin-induced disease progression in rat macrophages (Muehlbauer et al., 2010). Use of the caspase-1 inhibitor Ac-YVAD-CMK increases neurogenesis (Gemma et al., 2007), is neuroprotective against ischemia-induced cell death (Rabuffetti et al., 2000), and prevents IL-1β-induced degradation of the BBB (Wu et al., 2010). Although it is the most studied, caspase-1 is only one of many potential enzymes that can cleave IL-1 (Stehlik, 2009). For instance, leptin-induced IL-1β release from microglia is not inhibited by Ac-YVAD-CMK (Pinteaux et al., 2007), indicating leptin induces IL-1β through a non-caspase-1 mechanism. The lysosomal protease cathepsin D cleaves an alternate 20-kDa form of IL-1β under acidic conditions (Takenouchi et al., 2011, Edye et al., 2013), which also appears to be caspase-1-independent. It appears that alternate versions may independently have bioactive pro-inflammatory properties as Ac-YVAD-CMK only

partially, but not entirely, ameliorates inflammatory arthritis (Stehlik, 2009). The inflammatory significance of multiple IL-1 converting enzymes and various mature IL-1β products has still not been established. While it is meaningful to recognize that the subtle nuances mediating IL-1 cleavage are still being worked out, this is likely not important for the current discussion, as the proposed method of inflammatory priming considered here focuses on mechanisms that induce caspase-1 activation.

In addition to cleaving IL-1β, caspase-1 is also a potent mediator of initiating cell death (Brough and Rothwell, 2007). All cells die, and typically, cell death occurs in one of two ways. Apoptosis is a form of programmed and systematic cell death that is not lytic and thus immunologically silent. This process is utilized in cellular reorganization during development and is adaptive. Conversely, necrosis, which occurs from unplanned or accidental damage or cellular infection, results in a lysed membrane that leaks cytosolic and nuclear proteins that can induce inflammation by acting as DAMPs (recall: danger/damage-associated molecular patterns). Pyroptosis, however, is a hybrid version of cell death that has only been observed in innate immune cells (Miao et al., 2011). This version of cell death is programmed, yet lytic, and is thus inflammatory, and is, by definition, activated by caspase-1 (Schroder and Tschopp, 2010, Miao et al., 2011, Denes et al., 2012).

1.3.2.2. The NLRP3 Inflammasome

Mechanistically, inflammatory priming could occur through assembly and activation of the inflammasome, a multi-protein complex that is responsible for activating caspase-1 (Khare et al., 2010) and represents the most characterized

method of IL-1β protein release (Martinon et al., 2009). Although there are many inflammasomes (Guarda and So, 2010, Khare et al., 2010), the NLRP3 (NOD-(nucleotide-binding oligomerization)-like receptor protein-3) type has received the most study and is reported to be present in microglia (Hanamsagar et al., 2011). Interestingly, recent research has linked HFD with up-regulation of the NLR-type proteins NLRP3 (Reynolds et al., 2012, Strowig et al., 2012) and NOD2 (Kim et al., 2011) in peripheral tissue, and activation of the NLRP3 inflammasome in adipose tissue has been identified as a mediating step transitioning obesity into a disease state (Vandanmagsar et al., 2011, Esser et al., 2013, Stienstra and Stefan, 2013). Furthermore, NLRP3 activation has been linked to functional declines in aging (Youm et al., 2013). Therefore, there is ample evidence to suggest that alterations in activation of the NLRP3 inflammasome are mediating the neuroinflammatory priming observed in the CNS from HFD consumption. However, it has not previously been established and that HFD induces increased NLRP3 in the brain. Data presented in Chapters 3 and 4 provide novel support of this phenomenon.

The NLRP3 inflammasome is unique in that it requires two distinct signals to first form and then later be activated (Latz, 2010, Hanamsagar et al., 2012, Schroder et al., 2012). The NLRP3 protein is present only at very low levels under basal conditions (Bauernfeind et al., 2009, Schroder et al., 2012), and the first signal, called a 'primer', induces the transcription and translation of NLRP3 (Hornung and Latz, 2010). This step typically requires TLR or PRR ligation to initiate downstream induction of NFκB (Hanamsagar et al., 2012), which importantly, can also lead to pro-IL-1 transcription. A second signal induces inflammasome assembly, which

leads to activation of caspace-1 and mature IL-1β production (Haneklaus et al., 2013). A variety of factors can serve as this second signal, with potassium efflux, reactive oxygen species (ROS) production (Haneklaus et al., 2013), increased intracellular calcium (Lee et al., 2012) and extracellular acidosis (Rajamaki et al., 2013) all being implicated. Interestingly, inhibition of ROS prevents NLRP3 priming, but blocking ROS does not prevent activation of the NLRP3 inflammasome (Bauernfeind et al., 2011). This suggests the pro-inflammatory environment may need to be particularly intense, or perhaps specific in nature, to actually initiate the priming step, whereas the activation step may be somewhat less sensitive, as a variety of signals can induce exacerbated inflammation from the prior priming. As evidence of neuroinflammatory priming appears to occur in response to particularly intense (sickness, stress) or chronic (age, obesity) insults, this idea makes sense.

1.3.2.3. The role of TLRs

LPS can directly induce inflammasome formation/activation, and also stimulates the priming of NLRP3 by triggering NLRP3 gene transcription (Schroder et al., 2012). This not only highlights the dual nature of NLRP3 activation, but also demonstrates the importance of activity of TLR4, the PRR that binds LPS, and a primary receptor thought to mediate the effects of inflammasome induction (Hanamsagar et al., 2012). This is supported by evidence that an intense stressor induces activation of NLRP3 in microglia (Weber et al., 2015), and that blocking activity of TLR2 and TLR4 during stress prevents stress-induced neuroinflammatory priming (Weber et al., 2013). As LPS is able to stimulate both the induction and activation of NLRP3, it appears that TLR ligation may induce a

number of effects. TLR4 has been implicated as inducing both early- and a late-phase NFκB activation (Palsson-McDermott and O'Neill, 2004), which are regulated by associated adapter proteins. Early NFκB activation occurs 30 minutes to 2 hours after TLR4 ligation (Han et al., 2002) and is dependent on MyD88 (myeloid differentiation factor 88) and MyD88-like adapter (Mal). Late-state NFκB activation (8-12 hours after) (Han et al., 2002) is MyD88-independent and regulated by TRIF and TRAM (TIR-domain-containing adapter-inducing interferon-β and TRIF-related adapter molecule, respectively) (Palsson-McDermott and O'Neill, 2004, Kawai and Akira, 2010). While both early and late phase NFκB activation induces transcription of pro-inflammatory cytokines, such as IL-1, only early phase TLR4-induced MyD88-dependent NFκB activation stimulates transcription of NLRP3 (Chilton et al., 2012).

1.3.2.4. HMGB1

Until very recently, it was still unclear how stress induces activation of TLRs to mediate neuroinflammatory priming. The endogenous DAMP (Sloane et al., 2010), high-mobility group box-1 (HMGB1), has recently been demonstrated to mediate stress-induced NLRP3 in microglia (Weber et al., 2015). HMGB1 is a ubiquitous nuclear DNA binding protein that, under normal conditions, does not exist extracellularly, but if present outside of a cell, such as following cell necrosis, can serve as a potent inflammatory alarm signal of cellular damage, a function that classifies HMGB1 as an 'alarmin' (Bianchi, 2007). HMGB1 can be passively released during necrosis, actively released from innate immune cells during pyroptosis (Magna and Pisetsky, 2014) or can be stimulated from other cells, such as neurons (Faraco et al., 2007, Klune et al., 2008, Maroso et al., 2010). Similar to IL-1β, HMGB1

lacks a secretory signal sequence so is packaged into secretory lysosomes, a process which requires acetylation (Dumitriu et al., 2005), and therefore actively released HMGB1 may have a different functional capacity than HMGB1 released passively through necrosis. In addition, alterations in the reduced or oxidized state of HMGB1 may influence its cytokine-inducing and chemoattractant properties (Antoine et al., 2014). It was originally thought that HMGB1 is not released during apoptosis, but more recent work has demonstrated that it is released during programmed cell death, but the released version is fully oxidized and thus immunologically inactive (Lee et al., 2014).

The pro-inflammatory potential of HMGB1 is vast as the molecule can activate innate immune function by binding TLR2 (Curtin et al., 2009), TLR4 (Park et al., 2006) and the receptor for advanced glycation end products (RAGE)(Hori et al., 1995, Qin et al., 2009, Rauvala and Rouhiainen, 2010) to induce NFκB activation (Yanai et al., 2012). The pro-inflammatory cytokine-like activity of HMGB1 has been well established (O'Connor et al., 2003a, Yang et al., 2005). The structure of HMGB1 consists of an anti-inflammatory A-box, a pro-inflammatory B-box and an acidic tail, and it is thought that binding sites for TLR4 and RAGE are on the B-box segment (Lee et al., 2014). While some evidence suggests HMGB1 may not initiate inflammation on its own (Sha et al., 2008), HMGB1 acts synergistically with pro-inflammatory cytokines (Sha et al., 2008, Yanai et al., 2012) and LPS (Qin et al., 2009) to enhance activation of innate cells. Furthermore, HMGB1-induced TLR4 activation of NFκB appears to occur though MyD88-dependent mechanisms (Yang et

al., 2011, Yanai et al., 2012), which indicates HMGB1 may also serve to prime innate inflammation by the induction of NLRP3.

1.4. High-fat diet and neuroinflammatory priming

1.4.1. The Impact of Short-Term HFD Consumption

A review of the literature focusing on obesity suggests that 'obesity', as defined by excess adipose tissue, is likely not the ultimate culprit that mediates the negative health and cognitive consequences associated with the disorder. While obesity is often associated with corresponding metabolic disease, they are separate conditions. A scientific conversation is only recently starting to distinguish between metabolically healthy and unhealthy obese states (Esser et al., 2013, Stienstra and Stefan, 2013) and to address the paradoxical state of 'healthy obesity' (Lavie et al., 2015), which highlights our current lack of understanding of the mediating factors regulating diet, inflammation and associated negative consequences.

Interestingly, obesity development does not occur if innate immune processes are blocked. Mice are protected from diet-induced obesity with a TLR4 loss-of-function mutation or from inhibition of TLR4 (Tsukumo et al., 2007, Milanski et al., 2009), and it appears that MyD88-dependent TLR4 signaling may be particularly important in mediating obesity development and leptin resistance to HFD (Kleinridders et al., 2009). Activity of RAGE has also been implicated in mediating DIO as deletions of RAGE inhibits diet-, but not genetic-induced obesity (Song et al., 2014). Therefore, it appears that inflammatory processes are less the 'result' of obesity development, but rather facilitate its progression in the presence

of HFD. If this is the case, then alterations in neuroinflammatory function should consequently be observed from HFD prior to obesity.

Interestingly, there are a number of dietary factors that can be directly implicated in mediating inflammation. It is well established that eating a HFD increases circulating glucose levels (Chisholm and O'Dea, 1987), that high glucose levels and free fatty acids (FFAs) may be key mediators (Calder et al., 2011) in initiating a 'postprandial inflammatory response' (Hansen et al., 1997). With increased weight and prolonged HFD consumption, chronically elevated levels of glucose (hyperglycemia) establish the foundation to develop Type-2 diabetes, a hallmark disease associated with obesity. Diets high in sugar and fat are able to amplify postprandial inflammation, which is ablated by inclusion of healthy components to the diet (such as antioxidants, vitamins and omega-3 polyunsaturated fats) (Calder et al., 2011). These studies suggest that components of the diet determine the resulting inflammation that occurs following consumption of a meal, and further support the notion that diet may regulate the inflammation that is classically associated with 'obesity'. While peripheral adipose stores, and the neural inflammation that results (Erion et al., 2014, Miller and Spencer, 2014), are not discredited by the current hypothesis, it may be that adipose 'quality' rather than 'quantity' determines the resulting impact on health, and that components of the diet may strongly influence the state of inflammation in adipose tissue as well as alter neuroinflammatory processes.

Research is starting to distinguish between the short-term influence of diet and its long-term inflammatory consequences (Lee et al., 2011), however, the body

of literature examining the impact of very short-term HFD consumption is extremely limited. In humans, cognitive functional impairments were observed following consumption of HFD for 7 days in sedentary men (Edwards et al., 2011) and 5 days in healthy subjects (Holloway et al., 2011). In mice, adipose tissue inflammation has been observed by 3 days (Lee et al., 2011, Oliveira et al., 2012) and 4 days (Ji et al., 2012, Wiedemann et al., 2013), from diets with altered compositions. Studies directly implicating the effect of short-term HFD on neuroinflammatory processes are even more limited.

Studies examining HFD consumption have found increased inflammation in hypothalamus from consumption periods that range from 11 days (Oh et al., 2010) to 16 weeks (De Souza et al., 2005). However, considering HFD-induced increased fat mass can be observed by 7 days HFD (Thaler et al., 2012), even 11 days of HFD consumption may be too long to adequately distinguish diet from 'obesity'. Perhaps the only study that observed evidence of neuroinflammatory alterations from very short-term HFD found 3 days of HFD sufficient to increase pro-inflammatory cytokine mRNA and increase microglial accumulation in the hypothalamus, but found no evidence of neuroinflammation in the hippocampus or cortex at that time point (Thaler et al., 2012). While this area is wide open for further examination of the neuroinflammatory impact of short-term HFD consumption, the evidence available suggests that 3 days of HFD may be sufficient to induce evidence of neuroinflammatory priming through increases in NLRP3. This has not yet been explored.

1.4.2. Dietary mediators of inflammation

The diet utilized in the studies discussed in this dissertation is a prototypical example of HFD used to induce obesity. While the diet is high in fat, it is also high in sugar, proportionately high in unhealthy saturated fats and the polyunsaturated fat ratio is imbalanced. Therefore, it should be noted that there might be multiple factors included in the diet that may induce any negative neuroinflammatory consequences. In particular, a number of diet-based factors such as elevated glucose and excess free fatty acids (FFAs) have been implicated in aggravated inflammation in the periphery (Inoguchi et al., 2000, Reynolds et al., 2012, Joffe et al., 2013) and the induction of peripheral HMGB1 (Rockenfeller et al., 2010). Regardless, a discussion of the inflammatory-modulatory potential of diet-induced factors is included, as well as notes on how these factors may be mediated by the specific diet used here.

1.4.2.1. Glucose

In humans, plasma glucose peaks around 1 hour after a meal, and usually returns to baseline within 2-3 hours. In normal-weight individuals, glucose peaks around 120-140 mg/dl. However, in obese people, the glucose peak can reach in excess of 200 mg/dl (Singh, 2012). In addition, diet switch is able to improve glucose homeostasis without resolving or affecting adipose tissue inflammation (Jung et al., 2013), indicating that inflammatory glucose signaling originates from the diet. Recent work has established that innate inflammatory activators (LPS and septic shock) in the presence of high-glucose levels induce apoptosis in microglial cells of rats (Wang et al., 2001), and humans (Polito et al., 2011). It is believed that high-glucose stimulates innate immune cells by inducing reactive oxygen species

and NFκB activation (Shanmugam et al., 2003, Quan et al., 2007), and persistent hyperglycemia can induce a state of 'glucose neurotoxicity' (Tomlinson and Gardiner, 2008). In addition, high-glucose levels also facilitate the activation of RAGE, as one down-stream metabolic consequence of glucose processing is the accumulation of advanced glycation end products (AGEs), for which RAGE is named.

1.4.2.2. Free-Fatty Acids

Levels of FFAs increase in the blood following a fat-rich meal (Manning et al., 2008) and levels of FFAs in the blood predict corresponding levels in the cerebrospinal fluid (CSF) in the brain (Jumpertz et al., 2012, Guest et al., 2013). Levels of FFAs are elevated in the blood (Boden, 1998) and brain during obesity (Greenwood and Winocur, 1996). Furthermore, both saturated and polyunsaturated fatty acids are able to alter inflammatory signaling.

1.4.2.2.1. Saturated Fatty Acids

Microglia produce pro-inflammatory cytokines when activated by acute saturated fat-treatment in vitro (Wang et al., 2012). Interestingly, saturated fatty acids (SFAs) are able to induce microglial activation of NFκB and pro-inflammatory cytokine expression through TLR activation (Lee et al., 2003, Milanski et al., 2009, Gupta et al., 2012, Reynolds et al., 2012), as it has been established that saturated fatty acids act as TLR2 and TLR4 agonists (Lee et al., 2003, Gupta et al., 2012, Liu et al., 2014a). The SFA palmitic acid (PA) appears to be particularly inflammatory as it has been shown to activate NFκB and cytokine expression in macrophages (Nakakuki et al., 2013, Cullberg et al., 2014), alter microglial phenotypic activation

(Tracy et al., 2013) and induce cytokine expression in cultured human microglia-like cells (Little et al., 2012).

Furthermore, saturated fatty acids have been shown to increase NLRP3 in dendritic cells (Reynolds et al., 2012), stimulate increased ACTH and CORT levels (Oh et al., 2014), and trigger necrotic-induced HMGB1 release (Rockenfeller et al., 2010). The HFD used in the studies discussed here consists of 60% fat total, 54.1% of which is saturated and consists primarily of the pro-inflammatory SFA palmitic acid (Harlan Laboratories).

1.4.2.2.2. Poly-unsaturated Fatty Acids

While saturated fatty acids are well established to be pro-inflammatory (Gupta et al., 2012, Little et al., 2012), polyunsaturated fatty acids (PUFAs) are not. Instead, omega-3 and omega-6 (ω-3, ω-6) PUFAs are involved in mediating anti-inflammatory processes, and ω-3 PUFAs have been shown to suppress TLR4 (Liu et al., 2013) and induce the anti-inflammatory cytokine IL-10 (Pascoe et al., 2011). It appears that the *ratio* of ω-3 : ω-6 fatty acid profile may be particularly important in determining the resulting impact on inflammatory processes (Liu et al., 2013). The addition of essential PUFAs to a DIO protocol ameliorated the effects of HFD on cognition (Yehuda et al., 2005), suggesting that a PUFA deficit, and not just SFA excess, may be facilitating HFD-induced neuroinflammation. While there are no established guidelines for a proper omega FA profile, many dietitians suggest that one should consume nearly 4 times the amount of ω-3s as ω-6s. The HFD employed here has ω-6 in a concentration of 9.5 times the amount of ω-3, suggesting the

negative effects of diet may, in part, be due to limited amounts of anti-inflammatory ω-3 FAs.

1.4.3. Inflammation-mediating factors induced by HFD

The purpose of this dissertation is not to evaluate what specific aspects of the *diet* are acting as mediators of inflammation, but rather, what other mechanistic factors are induced *by the diet* to mediate neuroinflammatory priming. In particular, CORT and HMGB1 appear to be key molecules worthy of consideration.

1.4.3.1 CORT

Although peripheral and central levels of FFAs were not evaluated in animals following the HFD protocol used here, evidence that FFAs increase in the periphery following fat-rich meals and that peripheral levels dictate central levels (references cited above), make it reasonable to presume that increased central levels of FFAs would be found. FFAs activate the HPA axis to induce elevations in ACTH and CORT (Oh et al., 2014), CORT has been shown to induce NLRP3 in isolated macrophages (Busillo et al., 2011), and CORT has been demonstrated to modulate TLR signaling (Chinenov and Rogatsky, 2007). Therefore, it is reasonable to theorize that CORT may influence neuroinflammatory processes following short-term HFD consumption.

1.4.3.2. HMGB1

In the periphery, HFD is known to induce adipocyte cell death (Feng et al., 2011), via FFA signaling (Rockenfeller et al., 2010), which leads to the release of high-mobility group box 1 (HMGB1) (Gunasekaran et al., 2013). In addition, peripheral hyperlipidemia induces HMGB1 from monocyte cell cultures (Haraba et

al., 2011). HMGB1 activity is strongly implicated in mediating the peripheral inflammation in disease states associated with obesity (Magna and Pisetsky, 2014), and HMGB1 activity in the brain has been shown to mediate stress-induced neuroinflammatory priming (Weber et al., 2015), it is not known if the same process occurs in the brain following HFD consumption. If action of HMGB1 is the proximate cause of diet-induced neuroinflammatory priming, than any diet that induces elevated HMGB1 could be considered unhealthy, irrespective of what specific components within are mediating HMGB1 release.

1.5. Aims, Hypotheses and General Findings.

1.5.1. Evaluation of HFD-induced obesity, hippocampal memory function and neuroinflammatory processes. Data presented in Chapter 2.

The purpose of this series of studies was to develop an appropriate DIO model with corresponding memory functional impairments for purposes of evaluating whether neuroinflammatory processes mediate cognitive disruption in obesity. Medium-fat (Med, 42% fat) and high-fat (HFD, 60%) diets were used to observe the impact on obesity development and performance on a task of hippocampal memory function, context-pre exposure fear conditioning (CPE-FC). While consumption of both diets induced comparable obesity development, only animals fed HFD demonstrated impaired performance in CPE-FC.

While there was no evidence of basally altered levels of pro-inflammatory cytokines in the brain or periphery, HFD animals demonstrated increased levels of IL-1β protein in the hippocampus following the brief shock that occurs during CPE-

FC. To evaluate if increased signaling of IL-1β mediated the observed disruption of hippocampal memory in DIO animals, IL-1RA was injected centrally prior to footshock and animals were tested for memory a week later. IL-1RA prevents DIO disruptions of hippocampal memory, suggesting IL-1β mediates the effect.

As it was not clear if 'obesity' or presence of HFD mediated the effects on hippocampal memory function, DIO animals were switched to standard chow for 4 weeks before being tested in CPE-FC. While dietary reversal (DR) animals still exhibited increased body mass and serum leptin levels indicative of obesity, DR prevented shock-induced increases in hippocampal IL-1β and eliminated evidence of impairment on CPE-FC.

1.5.2. Impact of short-term HFD on neuroinflammatory priming and response to a secondary challenge. Data presented in Chapter 3.

Prior observations suggested that obesity, in the absence of HFD, was not sufficient to induce primed neuroinflammatory responses and resulting memory impairments. Therefore, the purpose of studies presented in Chapter 3 was to evaluate the neuroinflammatory priming effect of short-term HFD consumption (ST-HFD, 3 days).

Prior data suggested DIO did not induce neuroinflammation. Rather, a secondary challenge was required for DIO alterations in neuroinflammatory function to be observed. Following 3 days HFD, peripheral LPS or vehicle was administered and hippocampus, hypothalamus and frontal cortex were analyzed. Short-term HFD was sufficient to induce a primed neuroinflammatory phenotype, but not active inflammation. Following LPS, HFD animals demonstrated a

potentiated neuroinflammatory response of IL-1β protein and mRNA and NLRP3 protein in hippocampus, but not other brain regions examined. HFD had no observable effects in frontal cortex. HFD induced neuroinflammatory alterations in hypothalamus, but data suggested that hippocampus was particularly vulnerable to impact of short-term HFD.

A brief shock was sufficient to induce primed neuroinflammatory processes in DIO. Therefore, we explored the effect of brief shock following short-term HFD. Data indicate that impact of short-term HFD alone increased markers indicative of neuroinflammatory priming, confirming prior findings. There was no effect of footshock, indicating it was not a sufficient secondary challenge to induce a potentiated inflammatory response.

1.5.3. Impact of CORT or HMGB1 blockade during HFD on the development of a primed neuroinflammatory state. Data presented in Chapter 4.

Preliminary examinations revealed short-term HFD increased CORT and HMGB1 in hippocampus. Both CORT and HMGB1 have been implicated in mediating stress-induced neuroinflammatory priming. Therefore, we sought to evaluate the relative role of CORT and HMGB1 in mediating the effects of short-term HFD, specifically within the hippocampus.

The GR antagonist, RU486 was administered during HFD consumption to observe if blockade of CORT signaling mediates neuroinflammatory priming from HFD. RU486 inhibited development of a primed phenotype from HFD consumption and prevented the potentiated inflammatory response of IL-1β and NLRP3 to LPS,

indicating HFD-induced CORT elevations mediate the impact of HFD on neuroinflammatory priming.

To evaluate if activity of HMGB1 during the 3 days HFD consumption period mediated the impact of HFD on neuroinflammatory priming, we centrally injected BoxA, an inhibitor of HMGB1, during the short-term HFD consumption period. BoxA reduced, but did not eliminate, evidence of a primed neuroinflammatory phenotype from HFD. Prior BoxA prevented HFD-induced potentiated IL-1β protein, and inhibited HFD-potentiated responses of NLRP3 but did not impact IL-1β mRNA. The data is suggestive that activity of HMGB1, at least partially, mediates neuroinflammatory alterations from short-term HFD.

Chapter 2

High-fat diet consumption disrupts memory by priming elevations in hippocampal IL-1β, an effect that can be prevented with dietary reversal or IL-1 receptor antagonism.

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Abstract

High-fat diet (HFD)-induced obesity is reaching worldwide proportions. In addition to causing obesity, HFDs also induce a variety of health disorders, which includes cognitive decline. Hippocampal function may be particularly vulnerable to the negative consequences of HFD, and it is suspected that 'primed' neuroinflammatory processes may mediate this response. To examine the link between diet, hippocampal function and neuroinflammation, male Wistar rats were fed a medium or HFD. Hippocampal memory function was measured using contextual pre-exposure fear conditioning (CPE-FC). Rats fed a HFD demonstrated impaired memory, an effect that was augmented with longer duration of HFD consumption. HFD-induced memory impairments were linked to potentiated levels of interleukin-1 beta (IL-1β) protein in the hippocampus 2 h after the foot-shock that occurs during CPE-FC. Central IL-1 receptor antagonism, with intracisterna magna (ICM) administration of hIL-1RA prior to the foot-shock prevented the diet-induced memory disruption, suggesting a critical role for IL-1β in this phenomenon. Additionally, obese animals whose diet regimen was reversed from HFD back to standard chow recovered memory function and did not demonstrate a foot-shock-

induced hippocampal IL-1β increase. Interestingly, dietary reversal neutralized the negative impact of HFD on memory and IL-1β, yet animals maintained physiological evidence of obesity (increased body mass and serum leptin), indicating that dietary components, not body mass, may mediate the negative effects on memory.

2.1. Introduction

There is a well-established link between human obesity and cognitive decline (Sellbom and Gunstad, 2012). Specifically, hippocampal-dependent functions may be particularly vulnerable (Kanoski and Davidson, 2011, Francis and Stevenson, 2013), as many studies have linked high-caloric diets with decreased contextual and spatial memory (Winocur and Greenwood, 2005, Valladolid-Acebes et al., 2011, Kosari et al., 2012, Ross et al., 2012, Yamada-Goto et al., 2012). This is most likely the result of dietary-induced alterations in hippocampal synaptic plasticity (Molteni et al., 2002, Wu et al., 2004, Stranahan et al., 2008, Hwang et al., 2010). However, the mechanisms underlying how high-caloric diets cause hippocampal dysfunction are largely unknown.

It is possible that diet and obesity may induce cognitive disruption by impacting neural inflammatory processes. Obesity is in large part a peripheral inflammatory disease (Mito et al., 2000, Das, 2010, Donath and Shoelson, 2011) and peripheral inflammation can induce neuroinflammation (Maier and Watkins, 1998), leading to the notion that obesity may also exist as a central inflammatory condition. The pro-inflammatory cytokine interleukin-1 beta (IL-1β) is required for proper hippocampal memory function (Schneider et al., 1998, Yirmiya et al., 2002, Avital et

al., 2003). However, elevated or exaggerated central levels of IL-1β are detrimental to cognitive processing (Gibertini et al., 1995, Barrientos et al., 2002, Yirmiya and Goshen, 2011) as the duration of hippocampal-based cognitive impairment mirrors the duration of elevated hippocampal IL-1β (Barrientos et al., 2009a) and inflammation-induced cognitive decline is prevented when IL-1β action is blocked with IL-1 receptor antagonist (IL-1RA) (Pugh et al., 1998, Palin et al., 2004, Frank et al., 2010a, Barrientos et al., 2012).

In addition, there is growing evidence that certain conditions, such as stress (Johnson et al., 2003, Li et al., 2008, Frank et al., 2011, Loram et al., 2011), peripheral inflammation (Perry et al., 2007, Dantzer et al., 2008, Williamson et al., 2011), and advanced age (Barrientos et al., 2006, Chen et al., 2008) sensitize or 'prime' central inflammatory responses to a secondary challenge, leading to exaggerated central IL-1β levels. Obesity may induce inflammation similarly. The notion is that, in such conditions, basal cytokine levels are not elevated, but a subsequent or concomitant challenge induces the production of cytokines well above adaptive levels.

The present series of studies aimed to examine the impact of high-fat diet (HFD) on hippocampal-dependent memory function, and to evaluate whether a primed central IL-1β response might be involved. hIL-1RA was used to observe whether blocking action of IL-1β during a learning task would prevent the negative impact of HFD on memory. Furthermore, there is a growing body of evidence to suggest that the negative inflammatory impact of obesity may be readily negated with short-term dietary reversal (Hydock et al., 2013, Reeves et al., 2013). Therefore, we also

sought to evaluate whether a switch from HFD to regular chow would induce recovery of cognitive functioning and linked neuroinflammation.

2.2. Materials and Methods

Animals

Male Wistar rats (Harlan Laboratories) were used. All animals were approximately 2 months of age and weighed between 250-275 grams at time of arrival. Subjects were pair housed in standard large cages (52cm x 30cm x 21cm; L x W x H) with food and water administered *ad libitum*. The colony room was maintained at 22° C on a 12-h light/dark cycle (lights on at 07:00h). All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

Dietary Manipulation

Animals were randomly assigned on date of arrival to receive one of three diet types (Harlan Laboratories). Control animals received standard rat chow (Reg; TD. 8640, energy density of 3.0 kcal/g; 29% calories from protein, 54% from carbohydrates and 17% from fat). Animals selected for diet-induced obesity received one of two types of adjusted calorie diets that are considered to be medium-fat (Med; TD.88137; energy density of 4.5 kcal/g; 15.2% calories from protein, 42.7% from carbohydrates and 42% from fat) or high-fat (HFD; TD.06414, energy density of 5.1 kcal/g; 18.4% calories from protein, 21.3% from carbohydrates and 60.3% from fat). Following *Experiment 1*, use of the medium-fat diet was discontinued. Upon arrival, animals were given free access to the assigned

diet and remained on that diet thereafter, unless otherwise noted. The duration of dietary consumption prior to either testing or sample collection varied across experiments, with feeding time ranging from 12-24 weeks. The specifics of dietary duration for each experiment are outlined in the *Experimental Design* section. Some animals received a dietary reversal procedure that transitioned animals from the HFD to a regular diet. For this, as the removal of palatable food is a known stressor (South et al., 2012), animals were given a one-week period of mixed HFD and regular food to ease the stress of transition before complete reversal to the regular diet. All animals were weighed on date of arrival and re-weighed bi-weekly.

Contextual Pre-Exposure Fear Conditioning Paradigm

A contextual pre-exposure fear-conditioning (CPE-FC) paradigm was chosen to assess hippocampal based memory deficits following diet-induced obesity. In this paradigm, subjects are placed in a conditioning environment, receive an immediate foot-shock, and are then removed. The immediate shock does not lead to fear conditioning to the context, putatively because the foot-shock occurs before subjects have formed a representation (hippocampal) of the context. Indeed, if the subjects are allowed to explore the environment prior to the immediate shock session (the pre-exposure experience), the immediate shock event then does condition fear to the contextual cues (Fanselow, 1990).

The CPE-FC paradigm was chosen for a number of reasons. First, studies suggest that hippocampal-based learning and memory function is particularly vulnerable to dietary influence (Kanoski and Davidson, 2011). Importantly, CPE-FC requires intact hippocampal functioning during all three phases (pre-exposure,

conditioning with shock, and retrieval memory testing; described in more detail below) of the procedure (Rudy et al., 2002). Second, it was initially unclear whether diet-based cognitive deficits would be readily apparent, so it was anticipated that the relatively long duration between conditioning and testing that occurs with CPE-FC would accentuate any dietary-induced differences.

Additionally, behavioral tests of memory that require movement could be confounded by alterations in body mass. Prior research suggests that increased body mass from high-fat dietary manipulation does not impact basal psychical activity compared to controls (Hill-Pryor and Dunbar, 2006) but other groups have observed reduced locomotor activity following HFD (Lavin et al., 2011). CPE-FC is a conservative test since memory is assessed by the presence or absence of freezing behavior (see below for further details), with more movement (less freezing behavior) indicating reduced memory. If HFD animals have reduced memory capacity, this would be exhibited as *increased* movement compared to controls.

Context Pre-Exposure Fear Conditioning (CPE-FC) Behavioral Procedures.

The CPE-FC paradigm consists of three separate and distinct components: pre-exposure, immediate shock, and testing for freezing behavior (testing occurred both within the conditioned context A and in an alternate control context B). The CPE-FC procedure used here was adapted from (Barrientos et al., 2002). The standard procedure spanned a one-week period and consisted of 4 active days.

Contextual Pre-Exposure and Immediate Shock Sessions.

For context pre-exposure (*day 1*), rats were taken two at a time from their home cage and transported to the conditioning context (context A) in a blue ice

bucket with a lid and were placed into one of two identical contextual conditioning chambers. Ice buckets were used in order to establish an association between the contextual representation and the transport cues preceding placement of the rat in the context. Animals were allowed to freely explore the conditioning chamber for 5 min, after which animals were transported back to their home cage where they remained for approximately 30 s before the next pre-exposure. This procedure was repeated 5 more times, with each additional exposure to the context lasting only 30 s. The total duration of pre-exposure to the context lasted approximately 20 min per animal pair. Immediate shock *(day 2)* in the context occurred 24 h following pre-exposure. Animals were transported via blue ice buckets to the conditioning chambers, immediately given one 2 s 1.5 mA shock, then quickly removed and returned to their home cage.

Contextual Fear Testing.

Contextual fear was assessed during two 5-min testing sessions. These occurred on *day 5* in the control context B, which was used to measure generalized freezing behavior, and on *day 7* in the conditioned context A. Following conditioning, the CPE-FC procedure did not involve further shock so it was likely that testing sessions could facilitate extinction of any observable freezing behavior. Therefore, testing in the control context B occurred prior to testing in the conditioned context A. This diminished the possibility that lack of freezing in context B could be attributed to extinguished fear acquired during the context A test, and also supported the notion that any freezing observed in Context A (but not in

context B) could be attributed to a contextually-specific fear memory for the conditioned context.

Fear behavior was assessed by placing each rat in context A or B and observing freezing behavior using a time sampling procedure in which, every 10 s, each rat was judged as either freezing or active at the instant the sample was taken. Freezing is the dominant defensive fear response for a rat and was defined as the absence of all visible movement, except for respiration. Scoring began approximately 10 s after the animal was placed into the chamber and continued for 5 min.

Whenever possible, blinded behavioral scoring was achieved by randomizing animals and altering identifying markings, which were later decoded. For some tests, it was not possible to maintain true blind scoring due to the noticeable nature of increased weight. To compensate for this possible bias, testing sessions were video recorded and a subsequent scorer later confirmed freezing behavior. Freezing scores were averaged between scorers and converted to percentages by dividing the number of positive freezing scores over the total number of sampling blocks. For context B, scores below 10 percent (%) total freezing were considered acceptable indicators of low baseline freezing behavior.

Apparatus: Context A (Conditioning context).

The conditioning chambers consisted of one of two identical Igloo ice chests (54 L x 30 W x 27 H, cm) with white interiors, which were located in a room with overhead lights on. An activated 24-V DC light bulb and mini vent fan were mounted on the ceiling of each chest. The conditioning chambers (26 L x 21 W x 24 H, cm),

placed inside each chest, were made of clear plastic. A 2 s 1.5 mA shock was delivered through a removable floor of stainless steel rods 1.5 mm in diameter, spaced 1.2 cm, center to center. Each rod was wired to a shock generator and scrambler (Coulbourn Instruments, Allentown, PA). Chambers were cleaned with a diluted Mr. Clean mixture before each animal was pre-exposed, conditioned or tested.

Apparatus: Context B (Control context).

The control context B differed substantially from the conditioning context A with respect to transport, light, tactile and smell cues. Context B consisted of identical hollow clear plastic cylinders (approximately 25 cm in diameter, 35 cm high), which were placed inside white wooden boxes (30 L x 35 W x 50 H, cm). Boxes were illuminated with a small visible red light (7.5 watt red incandescent), with overhead room lighting off. A removable plastic tray and a small amount of fresh bedding (approx 1 cup) were placed on the floor of the context. Each chamber was cleaned with a diluted mixture of alcohol and water prior to animal testing. Animals remained in their home cage during transportation to the context B testing room, then moved to the context B chambers by hand.

Tissue Collection

A rapid decapitation procedure was used for tissue collection in an effort to capture the true nature of inflammatory marker levels at the time of sacrifice. Trunk blood was collected in 10 ml glass tubes and placed on ice. Brains were rapidly extracted and the bilateral hippocampal formations dissected atop a glass plate resting on ice. Each hippocampal half was placed in a labeled 1.5 ml Eppendorf tube

and flash frozen in liquid nitrogen. After all blood samples were collected, tubes containing blood were centrifuged at 4° C at 4,000 X g for 10 min and serum was collected and placed in a clean tube. All samples were stored at -80 degrees C until further processed or used for ELISA measurement.

Tissue Processing for ELISA Protein Measurement

Levels of IL-1β in hippocampus and serum as well as serum leptin proteins were determined using commercially available rat-specific enzyme linked immunosorbant assay (ELISA) IL-1β and leptin protein kits (R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer's instructions. IL-1β was determined and is presented as picograms per 100 μg of total protein (pg/100 μg) for hippocampal samples and IL-1β and leptin are presented as picograms per milliliter (pg/ml) in serum.

Following dissection and tissue collection, hippocampal half samples were sonicated in 0.3 ml of a sonication buffer containing 50 mM Tris base and a mixture enzyme inhibitor (100 mM amino-*n*-caproic acid, 10 mM EDTA, 5 mM benzamidine HCL, and 0.2 mM phenylmethyl sulfonyl fluoride).

Tissues were mechanically homogenized using an ultrasonic cell disrupter (Thermo Fisher Scientific, Pittsburgh, PA). Sonication consisted of 20 s of cell disruption at 52 % amplitude. Sonicated samples were centrifuged at 10,000 X g at 4°C for 10 min. Supernatants were removed and stored at 4°C until ELISA was performed. Bradford protein assays were also performed on hippocampal samples to determine total protein concentrations.

Intracisternal magnal (ICM) injection of human IL-1RA

To determine the impact of blocking central action of IL-1β at the time of contextual pre-exposure fear-conditioned shock, rats received an infusion of human recombinant IL-1 receptor antagonist (hIL-1RA; Amgen) into the cisterna magna (ICM) 2 h prior to the CPE-FC shock event, as described previously (Barrientos et al., 2012). Rats were briefly anesthetized with halothane and the dorsal aspect of the skull was shaved and swabbed with 70% EtOH. A 27-gauge needle attached via PE50 tubing to a 25 μl Hamilton syringe was inserted into the cisternal magna. To verify entry into the cerebral spinal fluid (CSF), approximately 2 μl of clear CSF was drawn and gently pushed back in and a 3 μl total volume of hIL-1RA (dose of 112μg) was slowly administered and allowed to absorb for 1 min before the needle was removed. The same procedure was used and an equal volume of sterile saline was injected ICM for vehicle control animals. ICM injections were used because they do not require surgery or cannulae implantation, which are themselves inflammatory manipulations (Holguin et al., 2007) and because substances injected ICM spread readily throughout the central nervous system (CNS) (Proescholdt et al., 2000). As IL-1RA binds to but does not activate the IL-1 type-1 receptor, thereby preventing IL-1β signal transduction (Dinarello, 1998), it was anticipated that ICM IL-1RA would effectively block action of IL-1β within the CNS, including the hippocampus.

Data Analysis

Statistical analyses were conducted using StatView version 5.0 software and Prism Graphpad version 5.0d. One-way and two-way ANOVAs were used, as appropriate. CPE-FC freezing scores (across time for the 5-minute test sessions) data were analyzed using repeated measures ANOVAs. However, for simplicity,

these data are graphically presented as summed total percentage for the entire test session. Where appropriate, Tukey's post-hoc comparisons were conducted to reveal pairwise differences between groups. All data are presented as mean ± SEM with sample sizes provided. Threshold for statistical significance was set at $\alpha = .05$.

Experimental Design

Experiment 1: Impact of diet type on weight gain, cognitive performance and obesity development.

The current diet-induced obesity animal literature varies greatly with respect to use of specific components and duration of dietary manipulation. Subtle variations in diet can greatly influence resulting weight gain, as well as the linked physiological and cognitive outcomes (Winocur and Greenwood, 2005, Dziedzic et al., 2007). Therefore, the primary aim of *Experiment 1* was to establish a paradigm for the purposes of studying the impact of high-fat diet on inducing hippocampal-based cognitive function.

To establish this paradigm, Wistar rats were assigned to receive regular rat chow, the med-fat diet or HFD. After 12 weeks, weight gain progression and CPE-FC performance were assessed and compared across diet types. Lastly, serum leptin was measured and compared to final body mass as an added physiological measure of the impact of increased fat in the diet. The presence of elevated serum leptin levels is a well established component of obesity and associated metabolic alterations (Zimmet et al., 1999). Therefore, leptin measures were used to confirm that dietary manipulations produced physiological alterations (in addition to weight

gain) in a manner that is consistent with obesity, but leptin levels were not used as a determinant of obesity, nor to make claims on the metabolic status of the animals.

Experiment 2: Further characterization of the relationship between dietary manipulation, duration of HFD exposure and body mass with performance on CPE-FC.

The primary goal of *Experiment 2* was to further characterize the nature of HFD-induced weight gain and the resulting impact on memory performance. Specifically, we sought to determine whether a longer duration of feeding on a HFD (20 weeks as opposed to the 12-weeks used in *Experiment 1*) would amplify memory disruption, as well as to determine whether the influence of HFD on memory function would persist following a switch from HFD to a normal diet. Therefore, memory function was assessed in three groups of Wistar animals that had been give one of the following dietary interventions: the regular diet (20 weeks), the HFD (20 weeks) or following a 'dietary reversal' which consisted of switching animals from the HFD to regular chow (20 weeks on HFD, 4 weeks on regular diet). After the allotted time, animal groups were tested for behavior in CPE-FC.

The second portion of *Experiment 2* served as a control to validate the CPE-FC paradigm for use with animals given a HFD. It is already established that the immediate shock event alone, without benefit of the prior pre-exposure, is not sufficient to associate the context with the shock and initiate a later freezing response (Fanselow, 1990). To confirm that the pre-exposure and the shock events are both required to induce later freezing behavior under the current conditions,

rats were fed regular or HFD for 24 weeks and then given CPE-FC. Half of the animals from each diet type did not receive the pre-exposure experience (*day 1*) but did get the immediate shock on *day 2*. The remaining animals were given the established pre-exposure experience of *day 1*, but on *day 2*, animals were placed in the conditioning context and immediately removed without receiving a shock. All animals were tested for behavioral freezing in context A on *day 7*.

Experiment 3: Dietary influence on central and peripheral inflammatory response to CPE-FC shock.

The association between learning and memory deficits with elevations in hippocampal IL-1β led to the speculation that alterations in central cytokines could be mediating the observed memory decline found here. Furthermore, data from our laboratory, as well as others, suggests that inflammatory events occurring in the periphery often result in *de novo* production of pro-inflammatory cytokines in the brain (van Dam et al., 1992, Nguyen et al., 1998, Barrientos et al., 2009b) further indicating that HFD, or possibly the resulting increase in inflammatory adipose tissue in the periphery, could induce neuroinflammation. However, preliminary studies did not reveal diet-induced differences in CNS basal IL-1β or other markers of inflammation. As previously discussed, there is ample evidence to suggest that central innate immune processes are capable of being 'primed' such that constitutive levels of cytokines appear normal, yet a secondary challenge induces an amplified inflammatory response. It is believed that functional alterations in the central innate immune cell type, microglia, mediate the priming process (Frank et al., 2007). Furthermore, microglia are not evenly distributed within the brain but

rather are found at higher concentrations in select brain areas such as the hippocampus (Lawson et al., 1990), which indicates hippocampal function may be particularly vulnerable to negative consequences of inflammatory priming.

It was initially thought that the brief shock that occurs during CPE-FC would be too mild a stressor to induce inflammation in the current model for HFD-induced obesity. However, a recent study demonstrated that a brief foot shock was enough to induce elevations of IL-1β in rats that had received early life infection, an event that appears to prime microglia (Williamson et al., 2011). The purpose of *Experiment 3*, then, was to test the hypothesis that HFD leads to IL-1β increases in the hippocampus in response to a fear-conditioning shock. To test this hypothesis, animals were given contextual pre-exposure on *day 1* and sacrificed on *day 2*, 2 h after receiving either a fear-conditioning shock or being placed in the context without shock. To determine the dietary impact of immediate shock on hippocampal IL-1β levels, comparisons were made between animals that had received a regular diet (20 weeks), the HFD (20 weeks) or were given dietary reversal (20 weeks on HFD then 4 weeks regular chow). In addition, serum was analyzed to determine dietary and shock-induced alterations on IL-1β and leptin.

Experiment 4: The effect of ICM hIL-1RA injection 2 h prior to CPE-FC shock on later memory performance.

The established link between elevated IL-1β and declines in memory function, as well as the results of *Experiment 3*, suggested increased IL-1β as a primary factor mediating the memory disruption observed in HFD animals. However, it was also possible that elevated IL-1β was correlated with, but unrelated

to, diet-induced memory decline. *Experiment 4* aimed to determine whether blocking the central action of IL-1β protein at the time of CPE-FC shock would prevent diet-induced decreased fear conditioning/memory. To do this, animals were conditioned using the CPE-FC paradigm after they had been consuming a regular or HFD for 20 weeks. An ICM injection of hIL-1RA or vehicle was administered 2 hours prior to the immediate shock event of *day 2*. Animals were later scored (*day 7*) for freezing behavior within the conditioned context A.

2.3. Results

2.3.1. Experiment 1: Establishment and characterization of diet type on diet-induced obesity and CPE-FC performance following 12 weeks of dietary manipulation

Impact of diet type on weight gain.

Overall, Wistar rats gained most weight from eating an increased-fat diet as compared to the standard rat chow (n=6). Additionally, there was no difference in weight gain induced by the medium (n=7) and high-fat diets (n=6), such that both the medium and HFD induced similar weight gain (Fig. 2.1.A). A repeated measures 1-way ANOVA shows that diet type had a significant impact on weight gain ($F_{(2)}$= 6.402, p= .0325), with post hoc evaluations showing that HFD (but not medium) produced significantly elevated weight gain compared to the regular diet.

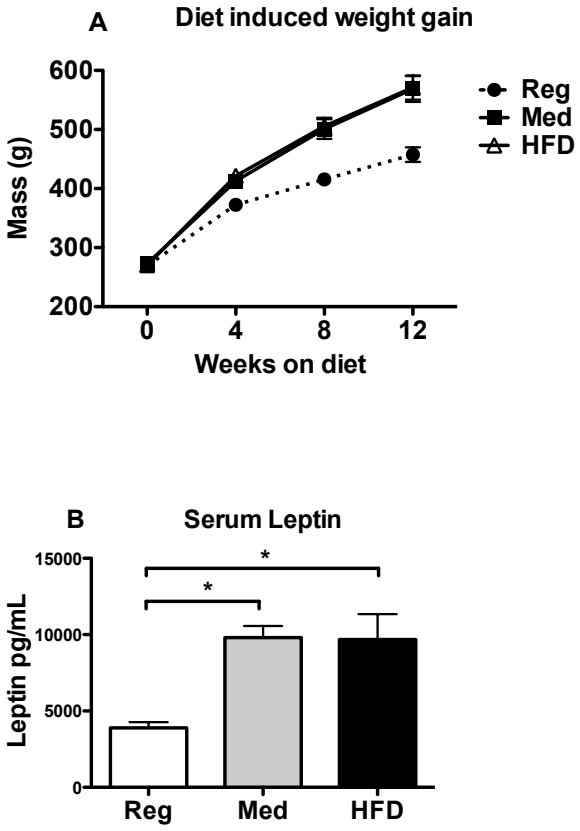

Fig.2.1. Impact of Med and HFD on weight gain and serum Leptin. Rats given the medium and high-fat diets gained significantly more body weight than regular-diet controls. There was no difference in weight gain between the medium and high-fat diet groups, (A). Serum leptin was elevated in animals fed either a medium or high-fat diet compared to regular-diet controls. *p< .05, (B)

Impact of diet type on serum leptin levels.

All animals on a Med or HFD demonstrated elevated serum leptin levels compared to regular diet controls (**Fig. 2.1.B**), and increased body mass significantly predicted increased serum leptin. A 1-way ANOVA revealed a significant effect of diet on serum leptin levels ($F_{(2)}$= 9.752, p= .0019), such that consumption of high-caloric diets induced higher leptin levels. Post hoc tests demonstrated that animals fed either a medium or HFD had elevated leptin compared to controls.

In addition, there was a very strong correlation between body mass and leptin levels ($R_{(16)}$=.7068, p< .0001), such that increased raw body mass is an adequate predictor of elevated leptin and is thus a strong indicator of a physiological state associated with obesity (data not presented graphically).

Impact of dietary manipulation on CPE-FC performance: Conditioned Context A.

Rats fed a HFD demonstrated memory declines as measured by decreased freezing behavior during exposure to context A in CPE-FC as compared to regular-diet controls (Fig. 2A). Merged total freezing scores for both test sessions are presented in Figure 2. A 1-way repeated measures ANOVA comparing the impact of diet on freezing scores (across the 5-min test session) revealed a significant effect of diet ($F_{(2)}$= 6.449, p=.0215) such that HFD animals (n=6) froze significantly less than regular-diet animals (n=6), as indicated by post hoc evaluations.

Impact of dietary manipulation on CPE-FC performance: Control Context B.

As anticipated, there was very little overall freezing behavior in the control context B (Fig. 2B), for all diet groups observed, indicating that the substantial freezing observed in Context A could not be attributed to generalized freezing. A 1-way repeated measures ANOVA demonstrated no diet-induced differences in freezing between groups ($F_{(2)}$= 1.108, p= .3762). Overall group averages were below the pre-established baseline level of 10% for freezing in Context B, indicating that no groups expressed generalized freezing behavior to a non-fear conditioned context. Therefore, data for context B are not presented hereafter.

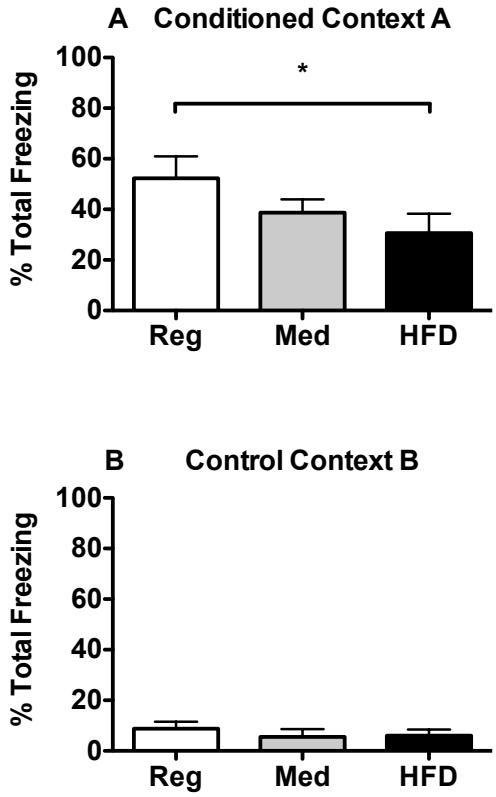

Fig. 2.2. Impact of diet on CPE-FC performance. Animals fed HFD had reduced memory as measured by total freezing behavior within conditioned context A (A) compared to regular-diet controls. *p< .05. There was very low freezing behavior observed in control context B (B), with no differences between diet groups, indicating lack of generalized fear behavior to a non-conditioned context.

A primary purpose of *Experiment 1* was to establish the diet that would most robustly produce obesity as well as demonstrate a hippocampal-specific cognitive deficit. Based on the results of *Experiment 1*, use of the medium-fat diet was discontinued for all subsequent studies. As *Experiment 1* established that 12 weeks of consuming HFD was sufficient time for animals to demonstrate reduced conditioned fear memory, *Experiment 2* aimed to determine whether prolonged HFD consumption beyond 12 weeks, and the resulting additional weight gain, would further amplify the deficit in memory function.

2.3.2. Experiment 2: Impact of duration of diet, type of dietary intervention, and overall body mass with performance on CPE-FC.

CPE-FC performance following 20 weeks of HFD.

As anticipated, animals that consumed a HFD for 20 weeks (N=6) demonstrated significantly lower freezing behavior in context A of CPE-FC (**Fig. 2.3**) compared to the average freezing of regular diet controls (N=6; $t_{(10)}$=2.431,

p=.0354). This demonstrates that the memory decline that occurs from HFD persists with prolonged or long-term consumption.

Impact of dietary reversal on CPE-FC performance.

Animals previously fed HFD then given a short-term dietary reversal (DR) paradigm completely recovered hippocampal memory function as measured by CPE-FC (**Fig 2.3**). A repeated measures 1-way ANOVA comparing DR animals to regular and HFD groups on freezing scores across the 5-min testing session confirmed that diet impacted freezing behavior ($F_{(2)}$=17.33, p=.0012). Post-hoc tests revealed that HFD animals froze significantly less compared to both regular diet and DR animal groups, which were not significantly different from each other. Therefore, DR enabled a complete recovery of cognitive function equivalent with regular diet controls.

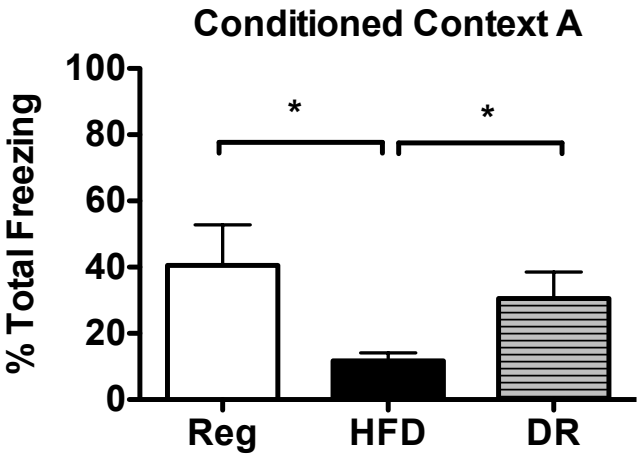

Fig. 2.3. Impact of dietary reversal on CPE-FC. Animals fed HFD for 20 weeks froze significantly less overall compared to regular diet controls when tested in context A of CPE-FC. Animals that experienced the dietary reversal paradigm (DR) demonstrated freezing comparable to regular diet animals, indicating that discontinuing HFD consumption enabled recovery of memory function. *p< .05.

The HFD and DR animals were from the same feeding cohort and were allowed to consume the HFD for the same duration. After 20 weeks, animals were randomly assigned to receive immediate CPE-FC or else were given DR and switched to the standard chow (4 additional weeks) before being tested on CPE-FC.

Therefore, it is safe to presume that the DR animals, if also tested after 20 weeks of HFD, would have demonstrated memory deficits similar to that observed in the HFD group. This indicates that, despite having received HFD for 20 weeks, the relatively brief switch from HFD to regular chow was able to completely *reverse* memory deficits, thereby negating the damaging impact of the prior high-fat diet consumption.

Comparison of 12 and 20 weeks of HFD duration on CPE-FC performance.

In order to determine whether a longer duration of HFD feeding augmented the reduction of freezing behavior in CPE-FC, the freezing scores of animals given HFD for 12 weeks (*Experiment 1*) were compared with freezing scores from rats given HFD for 20 weeks (*Experiment 2*; comparison not shown graphically). An evaluation of average total freezing times revealed that animals on the HFD for 20 weeks froze significantly less compared to animals that consumed HFD for only 12 weeks ($t_{(10)}$=2.743, p=.0207).

The overall freezing scores of control regular-diet animals at 12 weeks (52.2% ± 8.592, n=6) were not statistically different from values observed at 20 weeks (39.4% ± 12.31, n=6; $t_{(10)}$= .8510, p= .4147). However, there was a trend for a longer feeding duration (on regular diet) to induce lower overall freezing behavior. In an effort to be conservative and account for this trend, raw freezing scores of HFD animals at 12 and 20 weeks were transformed to percent of same-duration regular-diet controls and again compared. Percent of control values from animals on the HFD for 12 weeks (58.5% ± 14.5) are significantly higher than percent of control values of HFD animals at 20 weeks (23.2% ± 4.72; $t_{(10)}$=2.313, p=.0433). Therefore,

prolonged HFD consumption further reduced freezing behavior on CPE-FC, such that the longer the duration of HFD feeding, the worse was the resulting hippocampal-dependent memory deficit.

These results indicate that the most pronounced hippocampal-dependent cognitive decline occurs after a prolonged duration (20+ weeks) of high-fat dietary manipulation. Therefore, for all subsequent experiments, duration of dietary intervention lasted in the range of 20-24 weeks.

Comparison of dietary intervention with body mass and performance on CPE-FC.

Based on the results from *Experiment 1*, it was unclear whether reduced freezing in CPE-FC was due to components of the diet or if it was a side effect of increased body mass. Therefore, an additional aim of *Experiment 2* was to address this uncertainty. Animals that had been fed HFD and were designated for dietary reversal were the same weight (737.3g ± 41.5, n=6) as were animals that remained on the HFD (735.3g ± 52.9, 6) at the time of the diet switch (20 weeks). As anticipated, the dietary reversal caused animals to stop gaining weight and instead facilitated weight loss (**Fig. 2.4**), such that those animals had slightly lower overall average body mass at the time of CPE-FC at 24 weeks (678.3g ± 37.8, 6). However, the body mass of the DR group at the time of CPE-FC was not significantly lower when compared to the same animals at 20 weeks or when compared to the HFD animals tested on CPE-FC at 20 weeks. Therefore, the DR animals demonstrated improved memory performance while still exhibiting increased body mass. This suggests that dietary component, not reduction in body mass, was likely the primary cause for the recovered memory function observed in the DR animals.

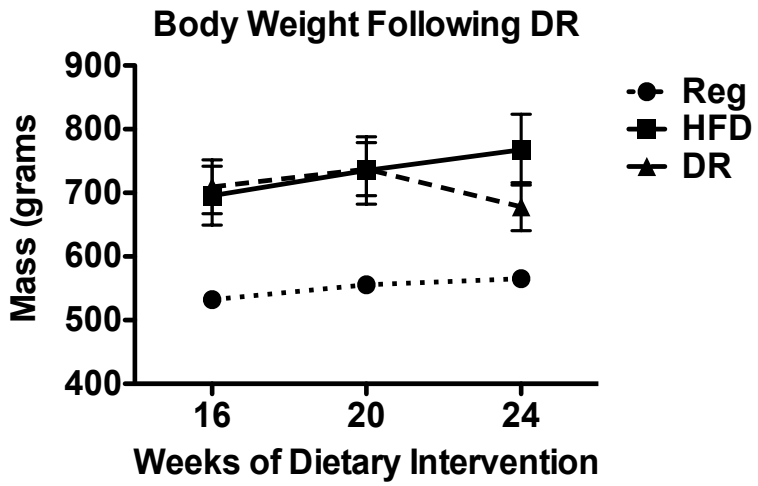

Fig. 2.4 Impact of dietary reversal on body weight. Animals that experienced the dietary reversal (DR) feeding paradigm lost weight as a result of the diet switch, however average body mass for the DR group was not significantly less than the HFD animals at 24 weeks. Also, DR animals still weighed more than regular-diet controls, indicating that the memory function recovery in the DR group was not due to obesity reversal.

Evaluation and establishment of CPE-FC parameters for use with HFD-induced obese rats.

Within the CPE-FC paradigm, the immediate shock event, without benefit of the prior exposure to the context, is not typically an adequate experience on its own to condition fear to the context. It has been argued that the failure to conditioning occurs because when the shock happens immediately, there has not been sufficient time to form a hippocampal-based representation of the context that could be associated with the shock (Fanselow, 1990). To verify that this is true with the current model, a control group of regular and HFD animals (24 weeks feeding duration) were given the standard CPE-FC procedure with either the pre-exposure session or shock event eliminated. All animals (both regular diet and HFD, n=4 per group), that had pre-exposure but did not receive an immediate shock, showed no freezing behavior at all during the later test in context A (0%; data not shown graphically). For animals that had received immediate shock (with no context pre-exposure), very low freezing behavior was observed for HFD (4.2% ± 3.3) and

regular diet (7.5 ± 3.4; data not shown). These levels were all below 10% freezing, which was the pre-set level to indicate lack of generalized freezing behavior. These data confirm that both the pre-exposure experience and shock event, separately, are required to form a contextual representation of the conditioning context and then to associate that representation with the foot shock.

2.3.3. Experiment 3: Dietary influence on central and peripheral inflammatory response to CPE-FC shock

***Hippocampal IL-1β protein*.**

An initial comparison was done examining samples collected on day 2 of CPE-FC, 2 h after exposure where regular and HFD animals were given either no shock (baseline) or the mild shock that typically occurs during conditioning. HFD did not increase basal (non-shock) levels of IL-1β in hippocampus (**Fig. 2.5.A**) compared to regular diet animals, nor did the shock event alone elevate IL-1β within regular diet animals. However, consumption of HFD combined with the foot-shock lead to an increase in IL-1β protein levels. A 2-way ANOVA comparing HFD-induced alterations in hippocampal IL-1β protein levels following shock or no shock revealed a significant effect of diet ($F_{(1,23)}$= 7.923, p= .0098, n=7), shock ($F_{(1,23)}$=8.326, p=.0082) and an interaction of diet and shock ($F_{(1,23)}$=9.434, p= .0054) such that only the HFD animals that also received a shock displayed elevated IL-1β protein levels above the other groups. Post-hoc tests revealed there were no baseline (non-shock) dietary differences in levels of hippocampal IL-1β protein between regular and HFD animal groups (p>.05) and regular diet animals did not

demonstrate altered IL-1β levels in response to a mild shock compared to non-shock controls (p>.05).

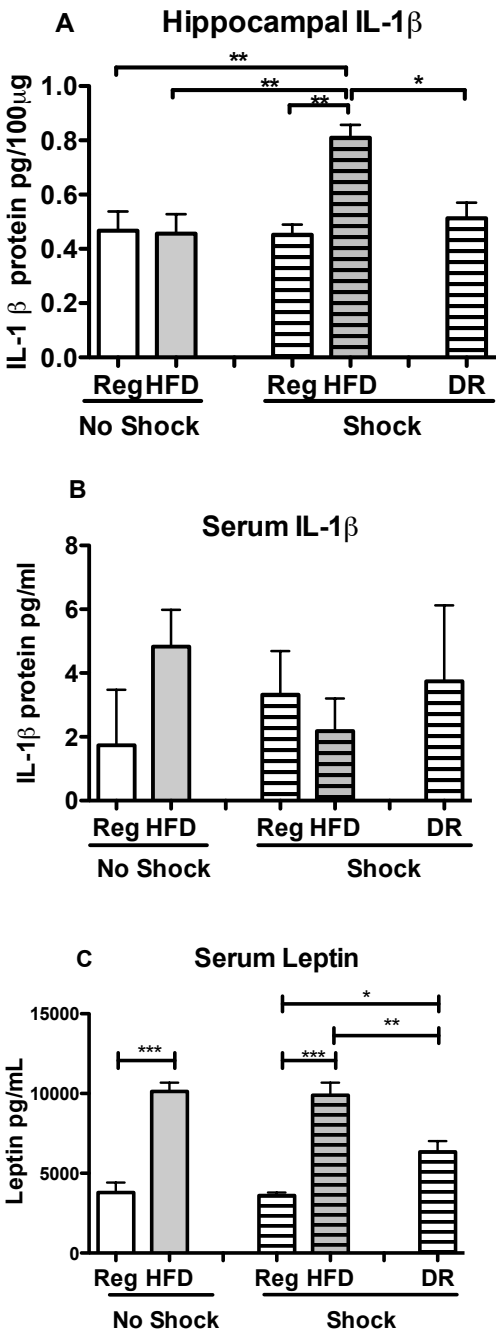

Fig. 2.5. Impact of shock and diet protocol in hippocampus and serum. Samples collected 2 h after CPE-FC with exposure to either a shock or no shock revealed: IL-1β protein levels in hippocampus were unaffected by diet when animals are not exposed to shock, however HFD combined with a shock event produced an IL-1β protein increase. DR animals do not demonstrate this priming as they had IL-1β protein levels comparable to regular-diet controls. ** p< .01; * p< .05, (A). Serum IL-1β protein levels were not affected by diet nor shock, with no group differences in serum IL-1β protein, thereby discounting the likelihood that the HFD-induced priming seen in HC is a result of peripheral contamination, (B). Serum leptin levels were altered by diet, but not shock, with animals on HFD demonstrating significantly elevated leptin levels. Leptin levels in DR animals were reduced compared to HFD, but also still significantly elevated compared to the regular-diet group indicating physiological evidence of obesity had not resolved. *** p< .001; ** p< .01; * p< .05, (C).

The shock event was required to see evidence of diet-induced alterations on hippocampal IL-1β. Therefore, in an effort to conserve animal use, a dietary reversal (DR) non-shock control group was not included. The impact of shock on the DR group demonstrates that a return to the control diet clearly reversed any potentiating effects of the shock event on IL-1β production.

This conclusion was confirmed by a 1-way ANOVA comparing post-shock IL-1β protein levels from regular-diet, HFD and DR groups ($F_{(2)}$= 16.453, p= .0001), such that only HFD animals demonstrated elevated IL-1β protein. Post-hoc analyses show that IL-1β protein levels in the hippocampus are significantly higher in HFD animals compared to both regular diet and dietary reversal groups (p< .05). In addition, dietary reversal animals demonstrated IL-1β protein levels that were not significantly different from regular diet animals (p>.05). Thus, the DR procedure was able to reverse the impact of prior consumption of HFD and prevent the shock-induced pro-inflammatory response in the hippocampus.

Serum IL-1β protein.

A potential difficulty with regard to the hippocampal IL-1β measurements is that animals were sacrificed using a rapid decapitation method to reduce stress- and pentobarbital-induced increases in the pro-inflammatory cytokine IL-1β. This prevented the use of intra cardiac saline perfusion to remove peripheral immune cells from the central nervous system vasculature. Therefore, the elevation of observed hippocampal IL-1β protein could have been partly, or completely, caused by differences in circulating IL-1β. If this were true, the pattern of group differences in hippocampal IL-1β would be mirrored in circulating IL-1β. To determine if similar pro-inflammatory expression would be observed peripherally, serum samples were analyzed to determine IL-1β protein levels (**Fig. 2.5.B**). Clearly, neither the shock nor the diet altered peripheral IL-1β. A 1-way ANOVA comparing regular, HFD and DR serum samples following both shock or no shock revealed no significant

differences between any groups ($F_{(4)}$=1.052, p= .3877). These results indicate that the observed levels of IL-1β in the hippocampal tissues were not produced by peripheral contamination.

Serum leptin.

Serum leptin was also examined for any diet or shock-induced alterations (**Fig. 2.5.C**). As can be observed, diet, but not foot-shock, altered leptin levels. A 2-way ANOVA comparing regular and HFD animals post shock or no-shock (DR post-shock group excluded) revealed only a significant effect of diet ($F_{(1,24)}$= 111.2, p< .0001), such that all HFD animals, regardless of shock, had elevated serum leptin levels. There were no effects of shock, or an interaction of diet and shock (p>.05). A 1-way ANOVA comparing regular diet, HFD and DR on serum leptin levels post-shock demonstrated a highly significant effect of diet ($F_{(2)}$=28.4, p<.0001). As expected, HFD animals had significantly elevated leptin compared to regular diet animals (p<.001). In addition, DR leptin levels were higher than regular diet animals (p<.05), but were also significantly reduced compared to the HFD group (p< .01). Additionally, across groups, body mass was significantly correlated with serum leptin ($R_{(20)}$=.7653, p<.0001). It is important to note that DR animals sustained physiological evidence of an obese state (as indicated by persistent increased body mass and elevated leptin levels), yet no longer showed a primed central immunological response to the shock.

2.3.4. Experiment 4: Impact of hIL-1RA blockade of IL-1β at time of CPE-FC shock on diet-induced cognitive function

As expected, HFD strongly interfered with expression of memory for contextual fear. However, this diet-induced memory deficit was completely blocked by preventing the action of IL-1β at the time of CPE-FC shock with an ICM injection of hIL-1RA (**Fig 2.6**). A 2-way repeated measures ANOVA comparing freezing levels (across the 5-min test session) of animals fed regular or HFD and given either saline or hIL-1RA 2 hours prior to CPE-FC shock revealed neither a main effect of drug ($F_{(1,32)}$= 2.74, p= .1076) nor a main effect of diet ($F_{(1,32)}$= 3.383, p= .0751) but did illustrate a significant interaction of diet type with drug treatment ($F_{(1,32)}$= 5.003, p= .0324), such that only HFD animals that were given saline had significantly decreased freezing scores compared to the other three groups. As expected, there was also a significant main effect of time ($F_{(4,128)}$=26.632, p<.0001) such that all animal groups demonstrated a reduction in freezing behavior as the testing sessions progressed. In addition, there was an interaction of time and drug ($F_{(4, 128)}$= 2.471, p= .0478) indicating that hIL-1RA facilitated fear extinction during the test session in both the regular and HFD animals compared to their saline-treated same-diet controls. There was not a significant 3-way interaction between diet, drug and time (p>.05), nor a 2-way interaction of diet and time (p>.05). Post hoc analyses of overall freezing scores by group show that HFD animals given saline had reduced freezing compared to all regular diet animals (saline: p= .0083; hIL-1RA: p= .0167) and HFD animals given hIL-1RA (p= .0041). Also, HFD animals given hIL-1RA were not significantly different from either of the regular diet animals, regardless of drug treatment (p>.05), indicating that the presence of hIL-1RA at the time of the shock eliminated the detrimental effect of HFD.

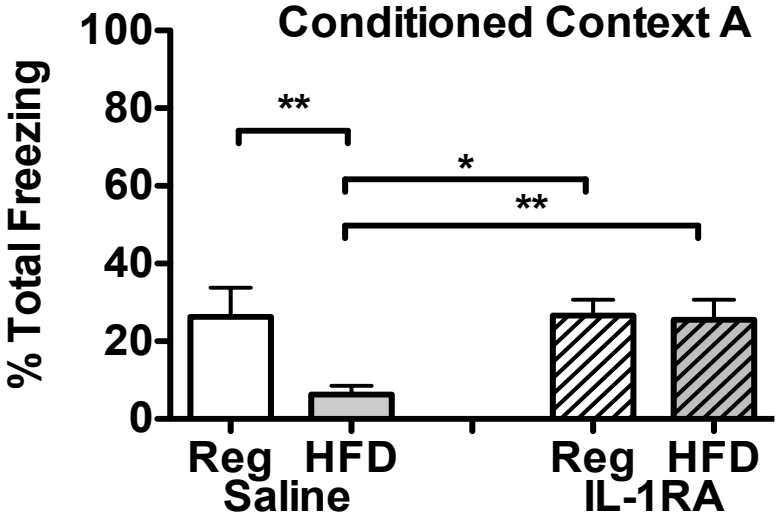

Fig. 2.6. Impact of IL-1RA on CPE-FC performance. Animals that consumed HFD had decreased freezing behavior in context A of CPE-FC compared to regular-diet controls. Antagonism of IL-1β action with an ICM injection of hIL-1RA prior to CPE-FC shock eliminated the HFD-induced decrease in behavior indicating the memory deficit is mediated by action of IL-1β at the time of CPE-FC shock. ** P< .01; * p< .05.

These data demonstrate a number of important outcomes. First, blockade of IL-1 receptors at the time of shock does not inhibit memory formation in regular diet animals, yet allowing the full action of elevated IL-1β in the HFD animals does. This suggests that the action of *excess* IL-1β during the conditioning experience leads to memory disruption. Furthermore, the pre-exposure experience is as important for fear conditioning as is the shock event and there was no drug manipulation until 24 hrs after the pre-exposure experience in these animals. The observation that HFD animals given hIL-1RA learned equally well compared to regular-diet controls suggests that HFD does not alter how animals learn from or respond to the pre-exposure experience.

2.4. Discussion

In sum, the present series of experiments clearly show that HFD increased body mass and disrupted hippocampal memory function in wistar rats. The data suggest that the diet produced memory deficits by priming a shock-induced increase in hippocampal IL-1β protein, which at elevated levels disrupted later behavioral memory expression. The negative impact of diet was prevented by either blocking action of IL-1β with hIL-1RA or by preventing the primed stress-induced increase in IL-1β using a short-term dietary reversal prior to conditioning. Furthermore, the data suggest that the primary factor mediating cognitive disruption was the continued intake of HFD, not increased body mass from prior HFD consumption.

The diets used in the present study are routinely labeled 'medium-fat' and 'high-fat', however, it should be noted that they differ from the standard diet in many other ways. Indeed, they might also be considered high-sugar diets. The medium-fat diet contains 341.46g/kg of sucrose while the high-fat diet has 90g/kg sucrose and 160g/kg maltodextrin as sweeteners. Therefore, while the fat content is elevated above standard chow, these diets are also more palatable and the overall caloric content is denser. It is possible that any number of alterations in the macronutient composition of the diets could have led to the observed negative cognitive consequences. It should also be noted that 60% calories from fat may not be considered by some to be 'high-fat', as ketogenic diets can contain up to 85-95% fat, and may actually be beneficial for health (Ruskin et al., 2009, Krikorian et al., 2012, Mobbs et al., 2013).

Animals fed the high-fat diet demonstrated elevated IL-1β protein levels in the hippocampus following a brief foot-shock that occurred during CPE-FC. The regular-diet animals showed no shock-induced changes in IL-1β protein. This suggests that HFD produces an altered response to a mild stressor by inducing neural inflammatory processes. This notion is supported by observations that waist circumference in humans is linked to augmented physiological stress responses (Brydon, 2011) and that obesity induces increased vulnerability of the CNS to the negative impacts of stress and injury (Yehuda et al., 2005, Bruce-Keller et al., 2009).

Dietary reversal was able to eliminate the negative impacts of prior HFD consumption on memory disruption and elevated hippocampal IL-1β in response to footshock. However, while the DR animals lost weight and had reduced leptin levels, these animals continued to exhibit increased body mass and leptin levels compared to regular diet controls. Therefore, the benefits of dietary reversal occurred prior to the *complete* resolution of either increased body mass or leptin.

It should be noted that no conclusions are being made about obesity or the metabolic status of the animals in the current studies. Rather, the goal is to distinguish between HFD consumption and the resulting physiological impacts of such consumption with respect to cognitive and neural inflammatory processing. Due to the well-established inflammatory nature of enlarged adipose stores, it is possible that increased peripheral adiposity must occur simultaneously with continued consumption of HFD in order for the cognitive effects and neural inflammatory priming to be observed. The present data indicate that the presence of elevated body mass and leptin are not sufficient alone (in absence of HFD

consumption) to produce those negative effects. What is not known, and is beyond the scope of the current paper, is how quickly the benefits of DR can be observed following diet switch, or if any amount of weight loss, even minimally, must occur in order to observe those effects.

The effects of DR, in the present study, are mirrored in a short-term diet and exercise intervention study in humans, in which significant improvements in inflammatory markers of metabolic health were observed prior to obesity reversal (Izadpanah et al., 2012). In addition, (Kosari et al., 2012) found that a 60% fat diet altered spatial memory, but the effect was unrelated to body weight, while (Badman et al., 2009) found improved health markers, but not altered body weight, of genetically-induced obese mice fed a ketogenic diet. In addition, very short-term consumption of a high-fat diet (4 days) is sufficient to induce pro-inflammatory changes in adipose tissue (Ji et al., 2012) prior to inducing significant weight gain. The evidence suggests that, perhaps, health and cognitive consequences are mediated less in the long-term by adipose stores but rather in the short-term by macronutrient composition of the diet. Although 'high-fat diet consumption' and 'obesity' are typically viewed as synonymous, as they typically occur simultaneously, distinguishing between the two may be important for future mechanistic research in this area. Although it is interesting and important to note that diet alteration can improve the negative consequences of HFD on memory function, it does not address what, mechanistically, may be occurring to mediate diet-induced memory dysfunction.

An injection of hIL-1RA prior to the shock experience of CPE-FC prevented diet-induced memory disruption, strongly suggesting that IL-1β mediates the effect of HFD on memory performance. Overall, animals in this experiment showed reduced freezing compared to other cohorts run through CPE-FC, but proportionately, the dietary impact within the saline animals remained clear, as did the recovery of function in HFD animals given hIL-1RA. It is possible that the brief anesthetization during the saline/hIL-1RA ICM injections may have had a later impact on freezing behavior expression. Furthermore, all groups of rats were un-treated during the pre-exposure phase, with the only intervention at that point being diet, as the injection procedure didn't occur until just prior to the immediate shock event on day 2. Because the hIL-1RA injection did not occur until after pre-exposure, it is clear that HFD animals learned normally during the pre-exposure session as HFD animals treated with hIL-1RA demonstrated memory equivalent with controls. The only group that demonstrated impaired memory in Experiment 4 was the HFD/saline group. Therefore, it was the action of potentiated levels of IL-1β, as induced in the HFD animals by the immediate shock, which acted as the primary facilitating factor in the ensuing memory impairment. Thus, HFD does not appear to interfere with all facets of memory function, or even hippocampal-dependent memory in all contexts. Rather, it seems that in order to observe the impact of a HFD on memory disruption, the learning experience may need to coincide with a secondary challenge, such as stress or infection.

The single foot-shock did not increase levels of IL-1β in serum, thus diet-induced elevations in IL-1β protein in hippocampus after HFD must have originated

centrally. Therefore, there is reason to believe that the primed IL-1β increase is the result of diet-induced changes to cells within the CNS. It is well established that functional changes in microglia can mediate primed central inflammatory processes (Combrinck et al., 2002, Frank et al., 2007, Ransohoff and Perry, 2009). Furthermore, the primed neural inflammation that occurs with aging is mediated by microglia (Barrientos et al., 2010) and there are many similarities between obesity and age-induced cognitive and health impairments (Cohen, 2010, Uranga et al., 2010), suggesting that obesity may induce neural inflammatory priming via similar mechanisms.

It is generally understood that IL-1β protein production is complex and requires multiple steps, and it is not yet clear how a HFD alters central innate immune function such that a sensitized overproduction of IL-1β occurs upon stimulation. Interestingly, saturated fatty acids are able to stimulate microglia to induce NFkB and pro-inflammatory cytokine expression (Lee et al., 2003, Milanski et al., 2009), and levels of free fatty acids are known to be elevated centrally with obesity (Greenwood and Winocur, 1996). Taken together, this evidence suggests that consumption of a HFD may induce 'primed' CNS innate immune cells, and this process may occur via fatty acid signaling. However, this has not yet been demonstrated.

Chapter 3

Short-term high-fat diet consumption induces a primed neuroinflammatory phenotype and potentiates the neuroinflammatory response to subsequent lipopolysaccharide.

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Abstract

Inflammatory processes partially mediate weight gain and disease progression indicative of obesity. While the neuroinflammatory environment of obesity is still under investigation, there is reason to suggest that high-fat diets (HFD), which facilitate obesity development, may directly induce neuroinflammatory processes prior to obesity development. In an effort to dissociate effects of HFD from obesity, we examined the impact of short-term (3 days) HFD consumption (60% fat) on neuroinflammatory processes to subsequent challenges of either lipopolysaccharide (LPS, 10μg/kg, IP) or a single footshock (2 s, 1.5mA). Protein and gene expression for markers of inflammation and inflammatory priming were assessed in brain and periphery of wistar rats 2 h after secondary challenge. 3 days of HFD induced alterations indicative of a primed neuroinflammatory phenotype, as determined by increased hippocampal levels of the endogenous danger signal HMGB1, the inflammasome-associated protein NLRP3 and increased gene expression of hippocampal IκBα mRNA (a marker of NFκB activity). Increased expression of the microglial antigen CD11b was observed in hippocampus and hypothalamus. Corticosterone (CORT) was increased by HFD in

hippocampus and serum. In addition, HFD potentiated the inflammatory response to LPS, as observed by elevations in hippocampal levels of the pro-inflammatory cytokine IL-1β (protein and mRNA), increased hippocampal NLRP3 protein, and increased IκBα in hypothalamus. Consuming HFD for 3 days was not sufficient to elevate body weight or induce an inflammatory response to footshock. The present results demonstrate that short-term HFD sensitizes neuroinflammatory processes, particularly in the hippocampus, and is the first to indicate that HFD increased levels of HMGB1 and NLRP3 in the brain.

3.1. Introduction

High-fat diet-induced obesity (DIO) is a condition that has a well-established peripheral inflammatory component but the nature of neuroinflammation in DIO is still being explored. We previously reported that rats fed high-fat diet (HFD), but not regular chow (Reg), for 20 weeks exhibited increased hippocampal IL-1β protein in response to a single brief footshock. Furthermore, when DIO rats were switched back to Reg chow for 4 weeks, they failed to demonstrate increased shock-induced hippocampal IL-1β despite their maintained obese status (Sobesky et al., 2014). This suggests that macronutrient properties of the diet, rather than obesity *per se*, mediate the resulting altered neuroinflammatory responses.

In obesity, metabolic disruption is facilitated in part by increased activity of the nod-like receptor protein 3 (NLRP3) inflammasome (Coppack, 2001, Vandanmagsar et al., 2011), which mediates release of the pro-inflammatory cytokines interleukin-1 beta (IL-1β) and IL-18. Furthermore, the endogenous

danger signal high-mobility group box-1 (HMGB1) is increased in adipose tissue with obesity and further mediates peripheral inflammation (Magna and Pisetsky, 2014). Inflammation appears necessary for weight gain as obesity does not develop following HFD in genetic knock-outs of RAGE (Song et al., 2014) and TLR4 (Tsukumo et al., 2007, Davis et al., 2008), which mediate inflammation, partially through interactions with HMGB1 (Hori et al., 1995, Park et al., 2006, Rauvala and Rouhiainen, 2010), to induce the transcription factor NFκB (Yanai et al., 2012). Therefore, inflammation may mediate, rather than result from, obesity (Thaler et al., 2010), and such, alterations in neuroinflammatory processes may occur upon initiation of HFD consumption, long before obesity develops.

It is possible that short-term HFD consumption does not induce neuroinflammation directly, but that it might potentiate the pro-inflammatory effect of a subsequent secondary challenge. In this scenario, the resident innate immune cells of the central nervous system, microglia, can enter an altered state of activation known as 'primed', wherein basal markers of inflammation are not increased, but an exacerbated response occurs when a secondary challenge is presented (Cunningham et al., 2005). Stress is able to induce primed neuroinflammatory processes (Johnson et al., 2002), and it has recently been established that stress-induced microglial priming is mediated by glucocorticoids (CORT)(Frank et al., 2012) and HMGB1-induced regulation of NLRP3 (Weber et al., 2015). Furthermore, microglia exist in particularly high concentrations in the hippocampus (Lawson et al., 1990), so evidence of altered neuroinflammatory function from short-term HFD may be initially apparent in the hippocampus. The few studies that have examined

very short-term HFD consumption reported induced inflammatory effects peripherally within 3-4 days (Lee et al., 2011, Ji et al., 2012, Oliveira et al., 2012, Wiedemann et al., 2013), and elevated pro-inflammatory cytokine gene expression and increased microglia in the hypothalamus after 1-3 days HFD (Thaler et al., 2012). However, the neuroinflammatory impact of short-term HFD in the hippocampus is still unexplored. The present series of studies aims to evaluate the influence of short-term HFD on hippocampal neuroinflammatory processes and potentiated neuroinflammatory responses to secondary challenges. Furthermore, we sought to evaluate the effect of HFD on central HMGB1 and NLRP3, as any such effects are currently unknown.

3.2. Materials and Methods

Animals

Male wistar rats (Harlan Laboratories) were used. All animals were approximately 2 months of age and weighed between 250-275 grams at time of arrival. Following arrival, animals were allowed to acclimate to the facility for at least a 7d period prior to diet modifications. Subjects were pair housed in standard large cages (52cm x 30cm x 21cm; L x W x H) with food and water administered *ad libitum*. The colony room was maintained at 22° C on a 12-h light/dark cycle (lights on at 07:00h). All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

Diet

Animals were randomly assigned to either continue consuming regular chow (Reg; TD. 8640, energy density of 3.0 kcal/g; 29% calories from protein, 54% from carbohydrates and 17% from fat) or an adjusted calorie high-fat diet (HFD; TD.06414, Harlan Laboratories, energy density of 5.1 kcal/g; 18.4% calories from protein, 21.3% from carbohydrates and 60.3% from fat). All rats were weighed on date of diet change and on date of tissue collection, 3 days later.

Experimental Design

Impact of 3 days HFD consumption on the inflammatory response to peripheral lipopolysaccharide (LPS).

To evaluate the impact of short-term HFD on neuroinflammatory processes, a known inflammatory agent, LPS, was administered peripherally following 3 days of HFD. For this, rats consumed Reg or HFD, and then 3 days later rats were given an intraperitoneal (IP) injection of saline or LPS. Animals were then sacrificed and tissue collected 2 hrs later. To evaluate if neuroinflammatory processes occurred widespread throughout the brain or were isolated to hippocampus, serum, hippocampus, hypothalamus and frontal cortex samples were collected and analyzed for markers of inflammation. All brain tissue samples were separated by hemisphere, with half used for steroid or protein analysis (ELISA: CORT and IL-1β, western blot: NLRP3 and HMGB1), with the other half processed to determine expression of inflammatory genes (pro-inflammatory cytokines: IL-1β and IL-6, IκBα, a measure of NFκB activation, CD11b, a microglial marker, NLRP3 and GILZ, a marker for glucocorticoid receptor activation) by RT-PCR.

Impact of 3 days of HFD consumption to a subsequent footshock.

Prior data (Chapter 2) indicated that prolonged HFD consumption was able to induce a neuroinflammatory response to a brief footshock, while animals fed Reg chow did not demonstrate an inflammatory response to the relatively mild stimulus. Here we aimed to determine if the brief footshock used previously would also induce an inflammatory response after only 3 days HFD. To determine if short-term HFD is sufficient to, itself, alter neuroinflammatory processes and to potentiate such responses, we gave animals Reg or HFD, then 3 days later exposed the animals to the shock chamber where they either received a single, 1.5 mA 2s footshock or no shock. Two hours post shock-apparatus exposure, serum and hippocampal tissue was collected and processed to measure mRNA and protein markers of inflammation. Comparisons of hippocampus, hypothalamus and frontal cortex from the previous study indicated the hippocampus to be particularly vulnerable to the impact of HFD. Therefore, here we only examined the impact of HFD and shock within hippocampal and serum samples.

LPS Injections

Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4, Sigma (St. Louis, MO)), a potent TLR4 agonist, was used to induce an inflammatory response. LPS was administered IP at a dose of 10μg/kg, or saline served as the vehicle control. The dose of LPS was selected as it has shown to induce a sub-threshold pro-inflammatory response in the hippocampus (Johnson et al., 2002).

Shock Exposure and Chamber Apparatus

For shock administration, rats were taken two at a time from their home cage and transported to the shock chambers in a blue ice bucket with a lid and were

placed into one of two identical chambers. The shock chambers were each located inside one of two identical Igloo ice chests (54 L x 30 W x 27 H, cm) with white interiors, which were located in a room with overhead lights on. An activated 24-V DC light bulb and mini vent fan were mounted on the ceiling of each chest. The shock chambers (26 L x 21 W x 24 H, cm) were made of clear plastic. A 2 s 1.5 mA shock was delivered through a removable floor of stainless steel rods 1.5 mm in diameter, spaced 1.2 cm, center to center. Each rod was wired to a shock generator and scrambler (Coulbourn Instruments, Allentown, PA). Immediately following the 2 s shock (or after 2 s without shock exposure), each animal was removed, placed back into the ice bucket with their cage mate, and transported back to their home cage. Shock-rod floors, chambers and ice buckets were cleaned with a diluted Mr. Clean mixture before and between each animal pair. All shock/no shock exposure occurred on day 3 of HFD consumption, with tissue collection occurring 2 hrs later.

Tissue Collection

Two hours following either LPS injection or shock exposure, animals were briefly anesthetized with Isoflurane and injected IP with a lethal dose of sodium pentobarbital until unresponsive (as evident by lack of blink to an eye touch and lack of flinch to a foot pinch). Blood samples were taken from cardiac puncture just prior to transcardial perfusion with ice cold 0.9% saline for 3 minutes. Following saline perfusion, brains were extracted from skull and placed on a clean glass dish inverted on ice wherein brain tissue was dissected, placed into pre-labeled 1.5 ml Eppendorf tubes and flash frozen in liquid nitrogen. Blood samples were centrifuged

(4° C, 14,000g, 10 min), and serum collected. All samples were stored at -80° C until further processed.

PCR

RNA Isolation from whole tissue samples.

RNA was isolated from whole tissue utilizing a standard method of phenol:chloroform extraction (Chomczynski and Sacchi, 1987). Briefly, tissue samples were rapidly homogenized in Trizol reagent (1 ml for hippocampus and frontal cortex, 500μl for hypothalamus; Invitrogen, Carlsbad, CA). Whole tissue samples were homogenized using a Tissue Tearor homogenizer. After incubation at room temperature for 5 min, chloroform was added to supernatant, vortexed 2 min, and centrifuged (4°C, 12,000g, 15min) to achieve phase separation of nucleic acid. Isopropyl alcohol (0.5 volume of Trizol volume) was added to precipitate nucleic acid. Samples were briefly vortexed and incubated at room temperature for 10 min followed by centrifugation (4°C, 12,000g) for 10 min. Nucleic acid precipitate was washed in 75% ethanol (1 ml) and centrifuged (4°C, 7500g, 5 min). The ethanol was gently poured out, the RNA pellet allowed to dry, followed by resuspension with 40 μl of nuclease-free water (Ambian).

cDNA Synthesis of Whole-Tissue Derived RNA.

Total RNA was reverse transcribed into cDNA using the SuperScript II First Strand Synthesis System for RT-PCR (Invitrogen). A standard amount of sample was added to nucleic acid-free water to equate 11 μl. This RNA was incubated for 5 min at 65°C in a total reaction volume of 13 μl containing random hexamer primers (5 ng/μl) and dNTPs (1 mM). Samples were chilled on ice for at least 1 min. A cDNA

synthesis buffer (6 μl) was added to the reaction and incubated at 20°C for 2 min. Reverse transcriptase (1 μl; 200 units SuperScript II) was added to the reaction and incubated at 25°C for 10 min followed by 42°C for 50 min. Reaction was terminated by heating to 70°C for 15 min.

Primer Specifications.

cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Qiagen Oligo Analysis & Plotting Tool (www.oligos.quiagen.com/oligos/toolkit.php?) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen and primer specificity was verified by melt curve analysis. Gene function and primer sequences of the genes of interest are presented in **Table 3.1**.

Table 3.1 PCR Primer Description and Sequences

Gene	Primer Sequence: 5' -> 3'	Function
β-Actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	Cytoskeletal protein (housekeeping gene)
IL-1β	F: CCTTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA	Pro-inflammatory cytokine
IL-6	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCCTGGTTATATCC	Pro-inflammatory cytokine
IκBα	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Activated by NFκB to inhibit NFκB function
CD11b	F: CTGGGAGATGTGAATGGAG R: ACTGATGCTGGCTACTGATG	Microglial antigen marker
GILZ	F: CAGGCCATGGATCTAGTGAA R: AGCGTCTTCAGGAGGGTATT	Glucocorticoid immunomodulation
NLRP3	F: AGAAGCTGGGGTTGGTGAATT R: GTTGTCTAACTCCAGCATCTG	Inflammasome protein

Table 3.1. Abbreviations: IL: interleukin, IκBα: nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha, CD: cluster of differentiation, GILZ: glucocorticoid-induced leucine zipper, NLRP3: nod-like receptor protein 3.

Quantitative Real Time PCR.

PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Quiagen, Valencia, CA). cDNA (1 μl) was added to a reaction master mix (25 μl) containing 2.5 mM $MgCl_2$, HotStar Taq DNA polymerase, SYBR Green I, dNTPs, fluorescein (10 nM) and gene specific primers (500 nM of each of forward and reverse primer). For each experimental sample, triplicate reactions were conducted in 96-well plates (BioRad, Hercules, CA). PCR cycling conditions consisted of a hot-start activation of HotStart Taq DNA polymerase (94°C, 15 min) and 40 cycles of denaturation (95°C, 15 sec), annealing (55-58°C, 30 sec), and extension (72°C, 30 sec). A melt curve analysis was conducted to assess uniformity of product formation, primer dimmer formation, and amplification of non-specific products. PCR product was denatured (95°C, 1 min) and annealed (55°C, 1 min) prior to melt curve analysis, which consisted of incrementally increasing reaction temperature (0.5°C/10 sec) from 55 to 95°C.

Real-Time Detection and Relative Quantification of PCR Product.

Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Fluorescence of SYBR Green I was captured at 72°C. Threshold for detection of PCR product above background was set at 10x the standard deviation of mean background fluorescence for all reactions. Background fluorescence was determined from cycle 1 to 5 cycles prior to exponential amplification of product and subtracted from raw fluorescence of each reaction/cycle. Threshold for detection of PCR product fell within the exponential

phase of amplification for each reaction. Threshold cycle (C_T; number of cycles to reach threshold of detection) was determined for each reaction.

Relative Quantitation of Gene Expression.

Relative gene expression was determined using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Mean C_T of triplicate measures was computed for each sample. Sample mean C_T of the internal control (β actin) was subtracted from the sample mean C_T of the respective gene of interest (ΔC_T). The sample with the absolute highest mean ΔC_T was selected as a calibrator and the mean ΔC_T of each experimental sample ($\Delta\Delta C_T$) was subtracted from this value. $2^{\Delta\Delta C_T}$ yielded fold change in gene expression of the gene of interest normalized to the internal control gene expression and relative to the calibrator sample. Relative gene expression for each sample was calculated and data are presented as percent of Reg diet controls.

Tissue Processing for ELISA and western blot

In preparation for assays, tissue half samples were sonicated in 0.3 ml (0.25 ml was used for hypothalamic tissue half-samples) of a sonication buffer containing 50 mM Tris base and a mixture enzyme inhibitor (100 mM amino-*n*-caproic acid, 10 mM EDTA, 5 mM benzamidine HCL, and 0.2 mM phenylmethyl sulfonyl fluoride). Tissues were then mechanically homogenized using an ultrasonic cell disrupter (Thermo Fisher Scientific, Pittsburgh, PA). Sonication consisted of 20 s of cell disruption at 52 % amplitude. Sonicated samples were centrifuged (4°C, 10,000g, 10 min) and supernatants were removed and stored at 4°C until ELISA or western blots were performed. Bradford protein assays determined total protein concentrations of sonicated tissue.

ELISA.

Levels of IL-1β protein and CORT were determined using commercially available rat-specific enzyme linked immunosorbant assay (ELISA) IL-1β (R&D Systems, Minneapolis, MN) and corticosterone kits (Enzo Life Sciences, Plymouth Meeting, PA). The assays were performed according to the manufacturer instructions. IL-1β was determined and normalized to total protein.

Western Blot.

Samples were heated to 75° C for 10 min then loaded into a standard polyacrylamide Bis-Tris gel (Invitrogen, Carlsbad, CA). SDS-PAGE was performed in MOPS running buffer (Invitrogen) at 175 V for 75 min. Protein was transferred onto a nitrocellulose membrane using the iblot dry transfer system (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and incubated with a primary antibody in blocking buffer overnight at 4° C. The following day, the membrane was washed in 1X PBS containing Tween 20 (0.1%) and then incubated in blocking buffer containing either goat anti-rabbit or goat anti-mouse (LI-COR, Lincoln, NE) IRDye 800CW secondary antibody at a concentration of 1:10,000 for 1 h at room temperature. Primary antibodies included: mouse anti-rat HMGB1 monoclonal antibody (1:4,000, Abcam, Cambridge, MA), rabbit anti-rat NLRP3 monoclonal antibody (1:1,000, Millipore, Billerica, MA) and mouse anti-rat β-actin (1:200,000, Sigma Aldrich, St. Louis, MO). Protein expression was quantified using an Odyssey Infrared Imager (LI-COR, Lincoln, NE) and normalized to the housekeeping protein value for that sample, and data are presented as percent of the within-blot Reg diet control samples.

Data Analysis

All data are presented as means ± SEM. All statistical comparisons were computed using Graphpad Prism version 5 (Graphpad Software, Inc. La Jolla, CA). A 2-way ANOVA was computed for each marker, followed by Bonferroni post hoc tests. Threshold for significance was set at $\alpha = 0.05$.

3.3. Results

3.3.1. Short-term HFD consumption not sufficient to increase body mass.

To evaluate if 3 day of HFD consumption was sufficient to induce increased body mass compared to regular diet controls, animals were weighed on day of diet switch (day 0) and day of tissue collection (day 3). Group mass averages for the regular and HFD groups are presented as mean ± SEM, in grams. On day of diet switch, the regular diet group mass average was 367.5g ± 5.8g and the HFD group mass average was 368.1g ± 6.8g. Following 3 days of dietary intervention, the regular diet animals gained on average 8.2g and exhibited a final group mass average of 375.7g ± 6.1g. The animals given HFD gained on average 18.8g over the course of the 3 days, with a final mass average of 386.9g ± 7.9g. A 2-way ANOVA comparing the dietary impact on weight gain over the course of the 3-day experiment revealed a significant effect of time ($F_{(1, 22)}=89.66$, $p< .0001$) but not of diet, indicating that rats fed HFD did not gain significantly more weight over the course of the 3-day experiment (data not shown graphically). Therefore, we conclude that 3 days HFD is an appropriate duration wherein impacts of diet can be

distinguished from impact of 'obesity' as significantly increased body mass in not apparent following 3 days HFD.

3.3.2. Impact of short-term HFD on inflammatory response to subsequent LPS

Short-term HFD and LPS: Protein and CORT

Hippocampus:

Hippocampal CORT was increased both by HFD ($F_{(1,20)}$=8.55, p= .008) and LPS ($F_{(1,20)}$=34.57, p= < .0001) but HFD did not potentiate the CORT response to LPS. HFD interacted significantly with LPS with regard to hippocampal IL-1β, HMGB1 and NLRP3, where HFD potentiated the IL-1β response to LPS (IL-1β: interaction: $F_{(1,27)}$=4.641, p= .0403), NLRP3 was only increased by the combination of HFD and LPS (NLRP3: interaction: $F_{(1,20)}$=4.667, p= .04), and the diet-induced increase in HMGB1 was negated with LPS (HMGB1 interaction: $F_{(1,20)}$=17.45, p= .0005). Data presented in **Fig. 3.1**.

Serum:

Peripheral IL-1β was increased by both HFD ($F_{(1,27)}$=8.791, p= .0063) and LPS ($F_{(1,27)}$=153.3, p< .0001) but prior HFD did not significantly potentiate the serum IL-1β protein response to LPS (interaction: $F_{(1,27)}$=3.916, p= .058, NS). HFD and LPS interacted to potentiate serum levels of CORT (interaction: $F_{(1,28)}$=10.45, p= .0031). Data presented in **Fig. 3.1**, B and F.

Hypothalamus and Frontal Cortex:

Of the markers measured in hypothalamic and frontal cortical tissue (IL-1β, HMGB1, NLRP3), the only effect occurred on IL-1β in response to LPS (hypothalamus: $F_{(1,27)}$=25.7, p< .0001); frontal cortex: $F_{(1,27)}$=25.7, p< .0001), data

presented in **Fig. 3.1**. There were no HFD-induced effects on protein levels in hypothalamus or frontal cortex, and no LPS-induced alterations in hypothalamic or frontal cortical levels of HMGB1 or NLRP3 protein (data not presented).

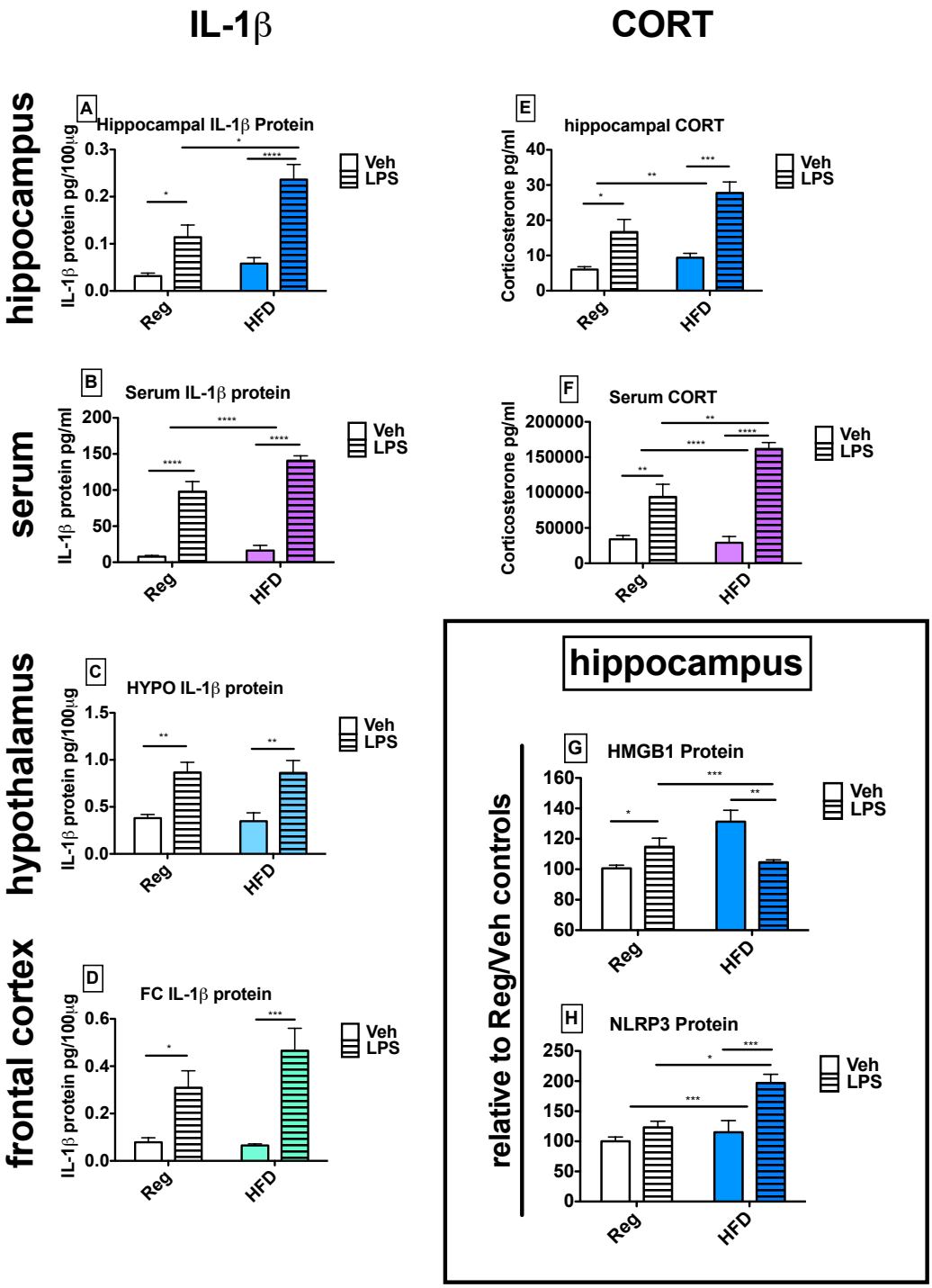

Fig. 3.1. Effect of short-term HFD consumption on brain and serum protein responses to LPS. Following 3 days of Reg or HFD, animals were given IP injection of LPS (10μg/kg) or saline, tissue collected 2 hrs later. Significant effects of HFD observed on IL-1β in hippocampus (A) and serum (B), but not hypothalamus (C) or frontal cortex (D). Significant effects of HFD also observed on CORT in hippocampus (E) and serum (F). LPS increased all markers, except hippocampal HMGB1 (G). **HFD potentiated LPS-induced hippocampal IL-1β (A), NLRP3 (H) and serum CORT (F). LPS negated the HFD-induced increase in hippocampal HMGB1 (G)**. Data presented as mean ± SEM, with data in G and H presented as percent of Reg chow/veh controls. p< .05* .01** .001***

Short-term HFD and LPS: mRNA

Hippocampus:

There were significant HFD-induced increases in all genes examined except for NLRP3, where no group differences were observed (diet (df: 1,24): IL-1β: (F=4.714, p= .04); IL-6: (F=6.835, p= .01); IκBα: (F=4.987, p= .03); CD11b: (F=6.682, p= .02); GILZ: (F=2.964, p=.02)). As anticipated, LPS (df: 1,24) increased hippocampal IL-1β (F= 34.06, p< .001), IL-6 (F=27.41, p< .001) and IκBα (F=25.79, p< .0001), but did not induce effects on CD11b, NLRP3 or GILZ. Additionally, there were significant interactions of diet and LPS on IL-1β, where HFD potentiated the hippocampal inflammatory response to LPS (interaction: $F_{(1,24)}$=5.242, p=.03) and CD11b, where HFD increased CD11b, but this effect was negated with LPS (interaction: $F_{(1,23)}$=6.695, p= .02). Although prior HFD amplified the hippocampal IL-6 mRNA response to LPS, the interaction trended but was not significant ($F_{(1,24)}$=3.710, p= .06, NS). All mRNA data (except NLRP3) presented in **Fig. 3.2.**

Hypothalamus:

The effect of HFD (df: 1,28) was observed on hypothalamic increased gene expression of IκBα (F=6.55, p= .02), CD11b (F=4.798, p=.04) and GILZ (F=5.661, p=.02). There were no effects of HFD or LPS on NLRP3 gene expression (not presented). LPS (df: 1,28) induced increased gene expression for IL-1β (F=15.09, p=.0006), IL-6 (F=16.36, p= .0004) and IκBα (F=37.74, p< .0001), which also interacted significantly with HFD (IκBα interaction: $F_{(1,28)}$=4.48, p= .04), such that HFD potentiated the inflammatory response of IκBα to LPS. Data presented in **Fig. 3.2**.

Frontal Cortex:

No effects of HFD or interactions of diet and LPS were observed on gene expression in the frontal cortex, for any of the genes examined. Consistent with the inflammatory nature of LPS, increased gene expression of all genes except CD11b were observed following LPS injection (LPS (df: 1,28) IL-1β: ($F=97.74$, $p< .0001$), IL-6: ($F=43.3$, $p< .0001$), IκBα: ($F=127.2$, $p< .0001$), NLRP3: ($F=8.57$, $p= .007$), GILZ: ($F=6.135$, $p=.02$)). Data in **Fig. 3.2**.

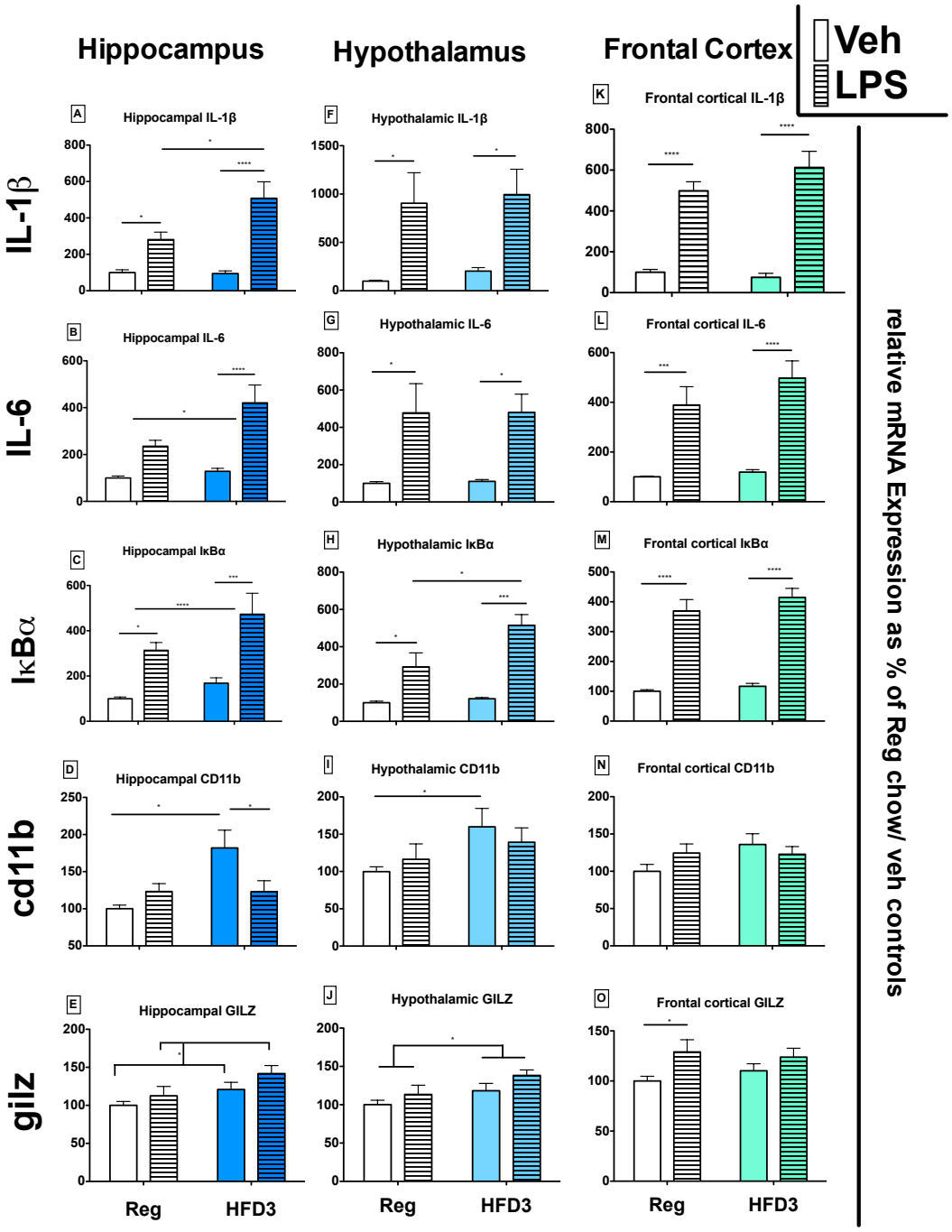

Fig. 3.2. Impact of short-term HFD on LPS-induced gene expression in hippocampus, hypothalamus and frontal cortex. Rats were fed Reg chow or HFD for 3 days then given an IP injection of LPS (10μg/kg) or vehicle. Tissue collected 2 hrs later. Significant effects of HFD observed in all genes in hippocampus (A-E), and in hypothalamic IκBα (H), CD11b (I) and GILZ (J). HFD did not have any effect on any genes measured in frontal cortex (K-O). LPS induced increased gene expression in all brain regions for IL-1β (A,F,K), IL-6 (B,G,L) and IκBα (C,H,M), and increased frontal cortical NLRP3 (not shown) and GILZ (O). **HFD consumption primed LPS-induced hippocampal IL-1β (A) and hypothalamic IκBα (H)**, and increased, but did not potentiate, the LPS response of hippocampal IL-6 (B). Data presented as mean ± SEM, as relative expression as percent of Reg chow/Veh controls. p< .05* .01** .001*** .0001****

3.3.3. Impact of short-term HFD to a subsequent footshock

Of all the markers examined, the only effect of footshock was observed in hippocampal HMGB1 ($F_{(1,28)}$=5.192, p=.03). The interaction of diet and footshock on HMGB1 was not significant, indicating that short-term HFD did not potentiate shock-induced levels of HMGB1. Data presented in **Fig. 3.3**.A. Three days of HFD consumption increased hippocampal protein levels of NLRP3 ($F_{(1,28)}$= 6.643, p= .02) and HMGB1 ($F_{(1,28)}$=5.334, p= .03). There were no effects of diet on hippocampal or serum levels of IL-1β protein (data not presented). CORT was increased by HFD consumption, both within the hippocampus ($F_{(1,28)}$=5.439, p= .03) and in serum ($F_{(1,28)}$=4.870, p= .04). Data presented in **Fig. 3.3**.

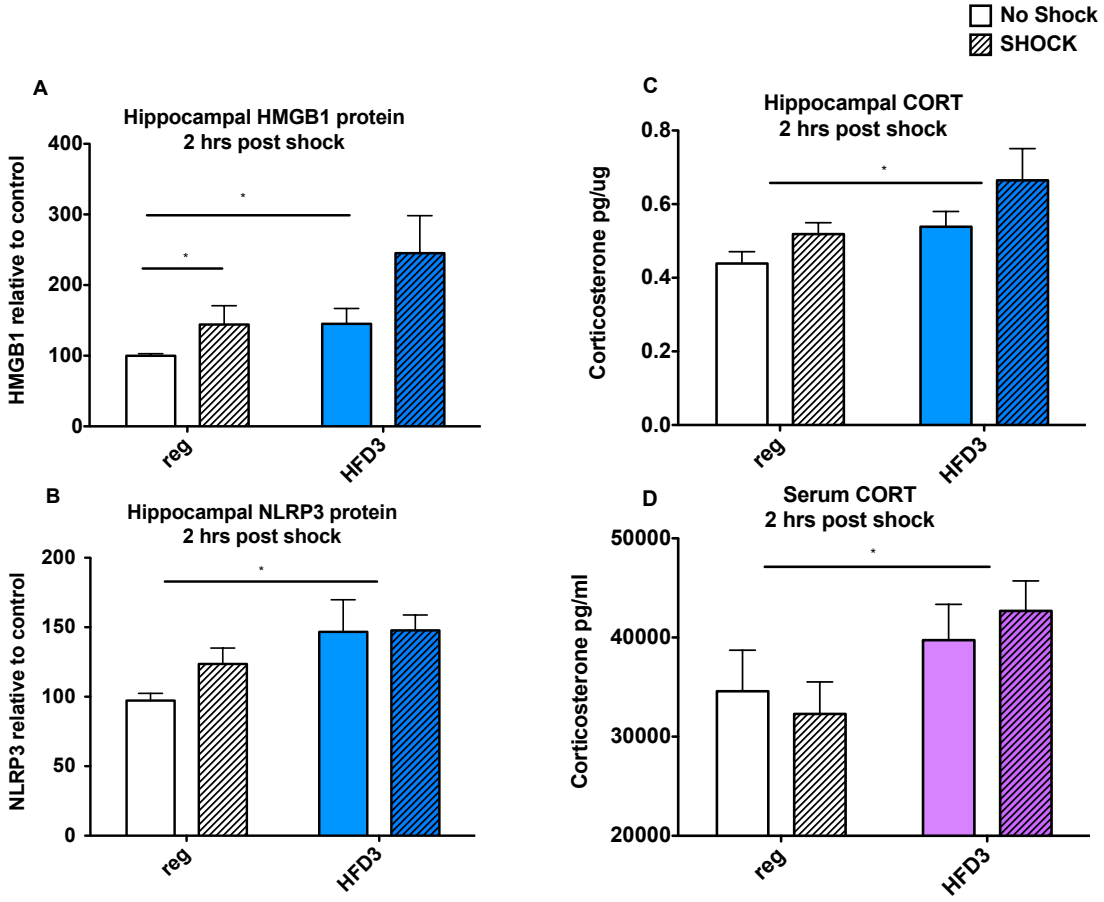

Fig 3.3. Effect of 3 days of HFD prior to footshock in hippocampus and serum. Samples collected 2 hours post shock. Shock elevated hippocampal HMGB1 (A), but did not alter levels of other proteins. **HFD increased hippocampal HMGB1 (A) NLRP3 (B) and CORT (C) and serum CORT (D)**. There were no interactions of HFD and shock exposure on protein measures. Data presented as mean ± SEM. Data in A and B are presented as percent of Reg chow/No shock controls. p< .05*.

mRNA.

Significant effects of diet on hippocampal gene expression were observed for CD11b ($F_{(1,19)}$=4.401, p< .05) and IκBα ($F_{(1,19)}$=5.690, p= .03). There were no effects of diet or footshock observed for IL-1β, IL-6, NLRP3 or GILZ hippocampal gene expression (data not shown). Significant effects are presented in **Fig. 3.4**.

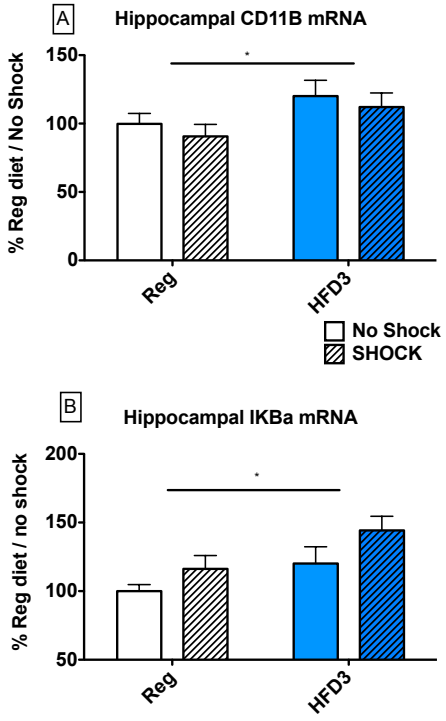

Fig. 3.4. Effect of 3 days HFD consumption prior to shock on hippocampal gene expression. Samples collected 2 hours post shock or no shock exposure. **HFD increased hippocampal CD11b (A) and IκBα (B)**. There were no effects of shock exposure on hippocampal mRNA levels. Data presented as mean ± SEM, as relative expression compared to Reg chow/no shock controls. p< .05*.

3.4. Discussion

The data from the present series of studies demonstrate that 3 days of HFD consumption induces alterations indicative of a primed neuroimmune phenotype, and potentiates the neuroinflammatory response to subsequent LPS. HFD did not increase inflammation directly, as levels of the pro-inflammatory cytokine IL-1β were not increased from HFD in the brain or in serum, yet LPS induced exaggerated levels of IL-1β in HFD-fed rats, (compared to the effect of LPS in regular chow controls), suggesting diet altered the neuroinflammatory environment to sensitize inflammation to the subsequent LPS administration. This

phenomenon is known as neuroinflammatory 'priming' and the central innate immune cell type microglia are thought to be responsible for mediating this effect (Cunningham et al., 2005, Frank et al., 2007).

One mechanism by which such HFD-induced neuroinflammatory sensitization could occur is through upregulation of NLRP3. NLRP3 is a structural component of one type of inflammasome, which are a multi-protein complexes that regulate IL-1β cleavage and release through activation of caspase-1 (Khare et al., 2010). The NLRP3 inflammasome, in particular, is of interest to the concept of neuroinflammatory priming, as it requires two independent signals to become activated (Latz, 2010). A primary signal initiates the transcription and translation of inflammasome products, and a second signal initiates assembly and activation of the inflammasome (Hornung and Latz, 2010, Haneklaus et al., 2013). At least one way NLRP3 priming is induced is through activation of pattern recognition receptors such as TLR4 (Hanamsagar et al., 2012). The data presented here demonstrates that short-term HFD increased levels of hippocampal NLRP3 protein, indicating HFD induced the initial step for NLRP3 inflammasome formation.

In addition to elevated NLRP3, further evidence that HFD primed a neuroimmune phenotype was indicated by elevated hippocampal gene expression of IκBα and CD11b. IκBα is induced by the pro-inflammatory transcription factor NFκB to inhibit its own activity, and is therefore a marker for NFκB induction. Increased CD11b, an antigenic marker of microglia which is increased when microglia become reactive, (Akiyama and McGeer, 1990), indicates that HFD induced alterations in microglia suggestive of sensitization. HFD also increased

CD11b mRNA in hypothalamus, but not in frontal cortex. HFD increased HMGB1 and NLPR3 protein levels in hippocampus, but not in hypothalamus or frontal cortex. Furthermore, there were no HFD-induced alterations in protein or mRNA levels in frontal cortex. Therefore, with the HFD consumption protocol used here, we observed regional differences in priming, such that the hippocampus appears most vulnerable to the effects of HFD, the hypothalamus less so, and the frontal cortex not at all. One possible explanation for this trend is the relative abundance of microglia in the hippocampus (Lawson et al., 1990).

It may be difficult to interpret the present data within the context of current related literature, as the current studies are only the second to examine the neuroinflammatory impact of very-short term HFD, the first to examine the impact of short-term HFD in the hippocampus, and the first to use LPS to evaluate short-term HFD-induced potentiated neuroinflammation.

Other groups have reported increased basal neuroinflammatory environments from prolonged consumption of HFD, having observed HFD-induced effects such as increased pro-inflammatory cytokines (Pistell et al., 2010, Maric et al., 2014) and NFκB (Zhang et al., 2005). However, these studies use protocols of extended (8-24 weeks) HFD feeding, and as HFD-induces observable increased fat mass within 7 days of HFD (Thaler et al., 2012), these studies are not able to isolate the effects obtained to the impact of HFD due to the confounding influence of developed obesity.

The use of LPS to reveal evidence of neuroinflammatory alterations is a widely used technique in animal models of obesity and multiple studies have

reported obesity-induced altered neuroinflammatory responses to LPS. However, general conclusions on the nature of obesity-induced neuroinflammatory processes are currently difficult to make, as the overall data trends from this body of literature appear contradictory. For instance, DIO increased pro-inflammatory cytokine mRNA responses to LPS in the hypothalamus of rats (Pohl et al., 2009), and in hippocampus and hypothalamus of mice (Andre et al., 2014). However, other groups reported no alterations following DIO on LPS-induced brain cytokines (Boitard et al., 2014) or even showed *decreased* pro-inflammatory cytokine responses to LPS in hippocampus (Baumgarner et al., 2014) and hypothalamus (Lawrence et al., 2012). The present series of studies are the first to isolate the influence of short-term HFD, not obesity, on subsequent responses to LPS. Here, we show that short-term HFD potentiates LPS-induced hippocampal IL-1β protein and mRNA, NLRP3 protein, and hypothalamic IκBα mRNA, and induces a nearly significant effect on LPS-induced hippocampal IL-6 mRNA.

Prolonged HFD protocols make it impossible to distinguish between the effects of diet and obesity. Here, we report that 3 days HFD consumption was not sufficient to induce increased body mass. Therefore, we feel confident that observed effects on neuroinflammatory priming and LPS-induced inflammation are mediated by factors from the diet, not obesity.

It is conceivable that diet may directly induce neuroinflammatory processes as saturated fatty acids (FAs) stimulate microglia through TLR4 (Milanski et al., 2009, Wang et al., 2012, Tracy et al., 2013), and FAs have been shown to increase NLRP3 in the periphery via TLR4 (Reynolds et al., 2012). As peripheral FAs show a

postprandial increase (Manning et al., 2008), and central FA levels shadow blood concentrations (Jumpertz et al., 2012, Guest et al., 2013), this provides a direct mechanism linking HFD with neuroinflammatory function.

Furthermore, observations that HFD induced elevated levels of hippocampal HMGB1 and CORT, and increased hippocampal GILZ mRNA (a marker for glucocorticoid receptor activation), are particularly important for understanding mechanisms by which HFD may induce a primed neuroinflammatory phenotype. Intense stress, such as an inescapable stress (IS) protocol that uses 100 X 5-s, 1.6mA tail shocks, primes the neuroinflammatory response to LPS (Johnson et al., 2002), and both CORT (Frank et al., 2012) and HMGB1-induced NLRP3 (Weber et al., 2015), have been implicated as mediators of IS-induced microglial priming. Therefore, it is possible that HFD-induced hippocampal CORT and HMGB1 mediate the neuroinflammatory priming observed following short-term HFD consumption. However, this has not yet been demonstrated, and is an area for further study. It should be noted that we report that footshock increased hippocampal HMGB1, but did not observe any other footshock-induced alterations in other markers measured. We attribute the general lack of inflammatory effects from footshock to the brief and comparatively mild nature of the shock protocol used here, which is a single, 2-s, 1.5mA footshock.

Overall, we conclude that HFD, alone, does not induce basal neuroinflammation, but does prime a neuroinflammatory phenotype, which is expressed in response to a subsequent inflammatory challenge of LPS. The data presented here are the first to examine the impact of very short-term HFD to a

subsequent LPS challenge, the first to examine the neuroinflammatory nature of short-term HFD within the hippocampus and the first to demonstrate elevations of HMGB1 and NLRP3 centrally following HFD.

Chapter 4

The primed neuroinflammatory response to lipopolysaccharide induced by short-term high-fat diet consumption is mediated by corticosterone and HMGB1.

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Abstract

As established previously, only 3 days consumption of a 60% fat, high-fat diet (HFD) is sufficient to induce elevations of hippocampal corticosterone (CORT) and the endogenous danger signal, HMGB1. The primed neuroinflammatory phenotype from 3 days of HFD potentiates the neuroinflammatory responding to a peripheral immune challenge with lipopolysaccharide (LPS) in Wistar rats. As both CORT and HMGB1 have been implicated in mediation of stress-induced inflammatory priming, here we evaluate the respective influence of CORT and HMGB1 in mediating increased neuroinflammation from HFD. Either the glucocorticoid receptor antagonist, RU486, or the HMGB1 inhibitor, BoxA, was administered during 3 days of HFD. The impact of these agents was evaluated on HFD-induced neuroinflammatory priming and HFD-potentiated response to LPS challenge in the hippocampus. Examination of markers previously demonstrated as increased by HFD showed partial or complete inhibition by RU486. Additionally, the potentiated inflammatory impact of HFD to subsequent LPS was also eliminated by prior RU486. The data strongly implicate CORT in mediating the primed hippocampal neuroinflammatory environment produced by HFD. BoxA reduced evidence of a

HFD-mediated primed phenotype and prevented HFD potentiation of IL-1β to subsequent LPS, which indicates HMGB1 also mediates the impact of HFD on neuroinflammatory processes.

4.1. Introduction

Microglia, the primary innate immune cells of the central nervous system (CNS), can exhibit an intermediate state of activation, known as 'primed', wherein cells express upregulation of markers for activation but do not exhibit active inflammation. However, when in this state, they show exaggerated inflammatory responding to a subsequent inflammatory stimulus (Cunningham et al., 2005). Increased expression of microglial activation markers, such as MHC II (Frank et al., 2007) and CD11b (Akiyama and McGeer, 1990), that appear without simultaneous evidence of active inflammation, such as increased protein or gene expression of pro-inflammatory cytokines, like interleukin-1 beta (IL-1β), would indicate a primed phenotype. We previously observed that 3 days high-fat diet (HFD) consumption induced a primed neuroinflammatory phenotype within the hippocampus, as evidenced by increased hippocampal gene expression of CD11b and IκBα (a marker of activation of the pro-inflammatory transcription factor NFκB), but not IL-1β protein or mRNA from HFD. However, HFD led to a potentiated neuroinflammatory response to a subsequent lipopolysaccharide (LPS) challenge as indicated by elevated levels of IL-1β protein and mRNA, as well as through increased NLRP3 protein (data presented in Chapter 3).

Inflammatory processes are mediated, in part, through activation of multi-protein complexes known as inflammasomes, of which NLRP3 is the structural component of one type (Khare et al., 2010). The NLRP3 inflammasome, in particular, could be involved in the mediation of inflammatory priming. This is because multiple steps are required to first induce, and then assemble and active the inflammasome (Latz, 2010). Activation of NFκB appears to mediate the primary NLRP3 production step (Hanamsagar et al., 2012), which, interestingly, also induces IL-1 mRNA. Therefore, increases in NLRP3 indicate priming as well as facilitate potentiated inflammation. Exaggerated levels of NLRP3 protein were observed in HFD animals given LPS, however, we have not consistently observed significant increases in hippocampal NLRP3 from HFD alone. As such, our prior data suggests that NLRP3 may mediate, at least in part, the primed and potentiated neuroinflammatory environment from HFD consumption.

In prior studies, three days of HFD increased levels of the immune-modulatory steroid hormone, corticosterone (CORT), as well as the endogenous danger signal, HMGB1, in hippocampus, the brain region that showed strongest neuroinflammatory evidence of impact of HFD. Interestingly, both CORT (Frank et al., 2012) and HMGB1 (Weber et al., 2015) have been implicated in mediating stress-induced neuroinflammatory priming to subsequent LPS. Furthermore, induction of NLRP3 has been attributed to the activity of both HMGB1 (Weber et al., 2015) and CORT (Busillo et al., 2011). Therefore, there is evidence to support the notion that CORT and/or HMGB1 may mediate the neuroinflammatory effects of HFD, possibly through induction of NLRP3. The present series of studies aims to evaluate the

respective roles of CORT and HMGB1 in mediating the HFD-induced primed neuroimmune phenotype as well as mediating HFD-induced potentiated neuroinflammatory response to LPS, specifically within the hippocampus.

4.2. Methods and Experimental Design

Animals

Male wistar rats (Harlan Laboratories) were used. All animals were approximately 2 months of age and weighed between 250-275 grams at time of arrival. Animals were allowed to acclimate to the facility for at least a 7d period prior to diet modifications. Subjects were pair housed in standard large cages (52cm x 30cm x 21cm; L x W x H) with food and water administered *ad libitum*. The colony room was maintained at 22° C on a 12-h light/dark cycle (lights on at 07:00h). All experiments were conducted in accordance with protocols approved by the University of Colorado Animal Care and Use Committee.

Diet

Animals were randomly assigned to either continue consuming regular chow (Reg; TD. 8640, energy density of 3.0 kcal/g; 29% calories from protein, 54% from carbohydrates and 17% from fat) or an adjusted calorie high-fat diet (HFD; TD.06414, Harlan Laboratories, energy density of 5.1 kcal/g; 18.4% calories from protein, 21.3% from carbohydrates and 60.3% from fat). All rats were weighed on date of diet change and on date of tissue collection, 3 days later.

Experimental Design

General Considerations.

As a primed phenotype was observed after 3 days HFD (refer to Chapter 3), it is likely that HFD-induced priming processes were initiated soon after start of HFD. Here, we evaluate the impact of glucocorticoid receptor (GR) blockade (RU486) or HMGB1 inhibition (BoxA) *during HFD consumption*, on HFD-induced primed phenotype and potentiated response to LPS. For this, either RU486 (Experiment 1) or BoxA (Experiment 2), or the appropriate vehicle, was administered (2x) during HFD consumption, at 24 and 48 hrs after HFD initiation. For both Exp.1 and 2, LPS or Veh was administered 72 hrs after HFD onset (thus, 24 hrs post 2nd injection of RU486, BoxA or Veh) and hippocampal samples were collected 2 hrs later.

We previously concluded (Chapter 3) that the hippocampus is particularly susceptible to effects of HFD, with other regions showing little to no effect of HFD. Therefore, observations herein will focus only on the hippocampus. Hippocampal markers of inflammation that we previously demonstrated were increased from either HFD alone (primed phenotype) or were amplified by HFD to subsequent LPS (potentiated neuroinflammation) will be used to evaluate the potential impact of GR or HMGB1 blockade on HFD-induced neuroinflammatory alterations. Markers of interest are listed in **Table 4.1.** Prior data suggest CORT and IκBα gene expression were increased by HFD but not potentiated in response to LPS, and NLRP3 has not consistently been increased by HFD alone, but was potentiated by HFD following LPS. Regardless, CORT, NLRP3 protein and IκBα gene expression are included in examinations of both priming and potentiated inflammatory responses.

Table 4.1. Markers of Interest

		Function
Primed Phenotype:	HMGB1	Endogenous danger signal
(Induced by HFD)	NLRP3 (*)	Component of inflammasome
	CORT	Immunomodulatory hormone
	IκBα mRNA	Marker for transcription factor NFκB activity
	CD11b mRNA	Antigen marker of microglia
	GILZ mRNA	Marker for GR activation
Inflammatory Response to LPS:	IL-1β	Pro-inflammatory cytokine
(Potentiated by HFD)	NLRP3	Component of inflammasome
	CORT (*)	Immunomodulatory hormone
	IL-1β mRNA	Pro-inflammatory cytokine
	IL-6 (*) mRNA	Pro-inflammatory cytokine
	IκBα (*) mRNA	Marker for transcription factor NFκB activity

Table 4.1. Abbreviations: HMGB1: high-mobility group box 1; NLRP3: nod-like receptor protein 3. IL: interleukin, IκBα: nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha, CD: cluster of differentiation, GILZ: glucocorticoid-induced leucine zipper. (*) Denotes markers that did not significantly or consistently reveal impact of HFD in prior work, but are included in the present evaluations.

Proper observations of a primed neuroinflammatory environment would require the absence of an additional inflammatory-triggering stimulus. Therefore, in an effort to conserve animal numbers, the data evaluating the impact of RU486 or BoxA (presented in 4.3.1. and 4.3.3, respectively) on the HFD-induced primed phenotype, were collected from rats that received saline-vehicle injections on day 3. The assumption is that the IP saline injection will not be a sufficient challenge/stress to trigger active neuroinflammatory responding, so those data should still be reflective of a primed, but not active, neuroinflammatory state.

Experiment 1. Effect of the glucocorticoid receptor antagonist, RU486, during 3 days of HFD consumption on HFD-induced neuroinflammatory primed phenotype and potentiated response to subsequent LPS.

Glucocorticoids facilitate the neuroinflammatory priming that occurs from stress (Frank et al., 2012) and aging (Barrientos et al., 2015). We previously observed that 3 days of HFD consumption increased both hippocampal and serum levels of CORT (Chapter 3), and induced a primed neuroinflammatory phenotype, as well as an exaggerated response to subsequent LPS. Therefore, we explore the idea that increased signaling by CORT during HFD consumption mediates the impact of HFD on neuroinflammation. To evaluate if blockade of glucocorticoid receptors (GR) during HFD would inhibit HFD-induced priming, as well as prevent HFD-induced potentiated inflammatory responses to LPS, we administered peripheral injections of the GR receptor antagonist RU486 (Schreiber et al., 1983) or vehicle (propylene glycol) to rats fed Reg or HFD. Although the goal was to block GR signaling in hippocampus, peripheral administration was deemed satisfactory as peripheral RU486 administration at this dose has previously demonstrated to be effective in preventing CORT-mediated neuroinflammatory priming within hippocampus (Frank et al., 2012). Injections were given 24 and 48 hrs after initiation of diet. On the 3rd day of HFD consumption, rats were given either LPS or a saline vehicle injection and hippocampal tissue was collected 2 hrs later.

Experiment 2. Effect of HMGB1 antagonist, BoxA, during 3 days of HFD consumption on HFD-induced neuroinflammatory primed phenotype and potentiated response to subsequent LPS.

HMGB1 is a constitutive DNA binding protein that remains intracellular unless released through non-programmed cell death or through active release by innate immune cells in response to infection (Magna and Pisetsky, 2014). As HMGB1

would only be found extracellularly in times of sickness or stress, this protein also possesses the ability to serve as an endogenous signal for damage or danger (Sloane et al., 2010). HMGB1 can mediate a primed neuroinflammatory phenotype that induces a potentiated response to a subsequent inflammatory challenge, at least in part, through activation of NFκB (Lee et al., 2014) and induction of NLPR3 (Weber et al., 2015). Our prior observations of the neuroinflammatory environment that results after 3 days of HFD consumption revealed that HFD increases levels of both HMGB1 and NLRP3 proteins in the hippocampus. Therefore, we sought to block activity of HMGB1 with BoxA, during HFD consumption, to observe whether HMGB1 blockade had effects on HFD-induced priming and inflammation.

BoxA, the anti-inflammatory subcomponent of the larger HMGB1 structure, serves as a competitive antagonist to HMGB1 by binding, but not activating, receptor targets of HMGB1 (Yang et al., 2004). The half-life of BoxA is not known, so we utilized two repeated doses of BoxA during the 3 days of HFD so as to parallel the experiment above. BoxA was administered via ICM injections to restrict effects to the CNS, and this method of BoxA administration has previously been effective in preventing stress-induced neuroinflammatory priming (Weber et al., 2015). Injections occurred 24 and 48 hrs after initiation of dietary intervention, with LPS or saline vehicle administered on the 3rd day, and hippocampal tissue collected 2 hrs later.

Drug Administration

RU486. To block activity of CORT, RU486 (mifespristone, Sigma, St. Louis, MO) was dissolved in 100% propylene glycol and administered subcutaneously (s.c.) at a dose of 50mg/kg/ml. 100% propylene glycol was used for vehicle controls.

BoxA. For each BoxA administration, rats were anesthetized with isoflurane, and held under mild sedation that lasted approximately 3 min per rat per injection. The dorsal aspect of the skull was shaved and swabbed with 70% EtOH, a 27-gauge needle, attached via PE50 tubing to a 25µl Hamilton syringe, was inserted into the cisterna magna (ICM). BoxA injection consisted of 10 µg BoxA (HMGBiotech, Milan, Italy, certified LPS free) suspended in 5 µl pyrogen free, sterile water. For vehicle control animals, 5 µl sterile water was injected ICM following the same procedure. Each injection was verified to determine entry into the ICM. For this, approximately 2µl of clear CSF was drawn and gently pushed back followed by 5µl of drug or water. If evidence of blood was observed during verification, the needle was removed, cleaned or replaced, re-inserted and re-verified for clear CSF. Following drug infusion, injection needles were held in place for approximately 30 s to allow drug to diffuse into the CSF. Careful notes were taken during ICM injection sessions and it was recorded if initial injection was bloody or if there were any issues that resulted in a rat being held under isoflurane for a longer duration then average. BoxA was injected ICM as neuroinflammatory process are of primary interest here, and because it would not have been cost-effective to inject peripherally. Although BoxA administration required multiple ICM injections, this procedure was used to avoid implanting cannulae, a process that induces lasting neuroinflammation (Holguin et al., 2007), and because directly injecting into the cisterna magna is an effective

method for administration of a drug into and throughout the CNS (Proescholdt et al., 2000).

LPS. Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4, Sigma, St. Louis, MO), a potent TLR4 agonist, was injected peripherally to induce an inflammatory response. All LPS injections occurred 72 hrs after initiation of HFD. LPS was administered IP at a dose of 10μg/kg. The dose of LPS was selected as it has shown to induce a sub-threshold pro-inflammatory response in the hippocampus (Johnson et al., 2002). Each IP injection was verified by pulling back air into the syringe to confirm placement in the intraperitoneal cavity before injecting LPS or vehicle. Vehicle control injections consisted of of sterile 0.9% saline.

Tissue Collection

Two hours following LPS or saline injection, animals were briefly anesthetized with Isoflurane and injected IP with a lethal dose of sodium pentobarbital until unresponsive (as evident by lack of blink to an eye touch and lack of flinch to a foot pinch), followed by transcardial perfusion with ice cold 0.9% saline for 3 minutes. Following saline perfusion, brains were extracted from skull and placed on a clean glass dish inverted on ice wherein hippocampal tissue was dissected, separated by hemisphere, placed into pre-labeled 1.5 ml Eppendorf tubes and flash frozen in liquid nitrogen. One half of each hippocampus collected was used to measure gene expression via RT-PCR, and the other half processed for protein or CORT measures. All samples were stored at -80° C until further processed.

Real Time RT-PCR Measurement of Gene Expression

A detailed description of the PCR protocol as been published previously (Frank et al., 2006) cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Qiagen Oligo Analysis & Plotting Tool (www.oligos.quiagen.com/oligos/toolkit.php?) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from Invitrogen and primer specificity was verified by melt curve analysis. Gene function and primer sequences of the genes of interest are presented in **Table 4.2**. PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Quiagen, Valencia, CA). Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative gene expression was determined by taking the expression ratio of the gene of interest to β-actin, and each value was computed to relative percent of average of Reg diet/Veh controls.

Table 4.2. PCR Primer Description and Sequences

Gene	Primer Sequence: 5' -> 3'	Function
β-Actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	Cytoskeletal protein (housekeeping gene)
IL-1β	F: CCTTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA	Pro-inflammatory cytokine
IL-6	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCCTGGTTATATCC	Pro-inflammatory cytokine
IκBα	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Activated by NFκB to inhibit NFκB function
CD11b	F: CTGGGAGATGTGAATGGAG R: ACTGATGCTGGCTACTGATG	Microglial antigen marker
GILZ	F: CAGGCCATGGATCTAGTGAA R: AGCGTCTTCAGGAGGGTATT	Glucocorticoid immunomodulation

Table 4.2. Abbreviations: IL: interleukin, IκBα: nuclear factor kappa light chain enhancer of activated B cells inhibitor alpha, CD: cluster of differentiation, GILZ: glucocorticoid-induced leucine zipper.

Tissue Processing For ELISA and western blot

In preparation for assays, hippocampal tissue was sonicated in 0.3 ml of a mixture containing extraction buffer (Invitrogen, Carlsbad, CA) and protease inhibitors (Sigma, St. Louis, MO). Samples were then mechanically homogenized using an ultrasonic cell disrupter (Thermo Fisher Scientific, Pittsburgh, PA) while being held on ice. Sonication consisted of approximately 20 s of cell disruption at 52 % amplitude. Sonicated samples were centrifuged (4°C, 10,000g, 10 min) and supernatants were removed and stored at 4°C until ELISA or western blots were performed.

ELISA

Levels of IL-1β protein and CORT were determined using commercially available rat-specific enzyme linked immunosorbant assay (ELISA) IL-1β (R&D Systems, Minneapolis, MN) and corticosterone kits (Enzo Life Sciences, Plymouth Meeting, PA). The assays were performed according to the manufacturer instructions. IL-1β levels were determined and normalized to total protein, as measured by Bradford protein assays.

Western Blot

Samples were heated to 75° C for 10 min then loaded into a standard polyacrylamide Bis-Tris gel (Invitrogen, Carlsbad, CA). SDS-PAGE was performed in MOPS running buffer (Invitrogen) at 175 V for 75 min. Protein was transferred onto a nitrocellulose membrane using the iblot dry transfer system (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln,

NE) for 1 h and incubated with a primary antibody in blocking buffer overnight at 4° C. The following day, the membrane was washed in 1X PBS containing Tween 20 (0.1%) and then incubated in blocking buffer containing either goat anti-rabbit or goat anti-mouse (LI-COR, Lincoln, NE) IRDye 800CW secondary antibody at a concentration of 1:10,000 for 1 h at room temperature. Primary antibodies included: mouse anti-rat HMGB1 monoclonal antibody (1:4,000, Abcam, Cambridge, MA), rabbit anti-rat NLRP3 monoclonal antibody (1:1,000, Millipore, Billerica, MA) and mouse anti-rat β-actin (1:200,000, Sigma Aldrich, St. Louis, MO). Protein expression was quantified using an Odyssey Infrared Imager (LI-COR, Lincoln, NE). In an effort to minimize variability in measured values across gel runs, all hippocampal tissue for each study was process through western blot simultaneously, and samples from each group were distributed evenly across gels. Infrared values for proteins of interest were normalized to infrared values of the housekeeping protein (β-actin) value for that sample by taking the ratio of target protein to β-actin. Target protein/β-actin values were then computed to percent relative to the average of the Reg diet/Veh control ratio values of controls in the same blot. Values were then merged and group data are presented as percent of the Reg/Veh control.

Data Analysis

All data are presented as means ± SEM. All statistical comparisons were computed using Graphpad Prism version 5 (Graphpad Software, Inc. La Jolla, CA). A 2-way ANOVA was computed for each marker, followed by Bonferroni post hoc tests. Threshold for significance was set at $\alpha = 0.05$.

4.3. Results

4.3.1. Impact of RU486 during HFD on development of a primed neuroinflammatory phenotype.

Previously, we established that 3 days of HFD is sufficient to increase levels of CORT in the hippocampus, as well as increase a number of hippocampal markers for inflammation that, together, indicated that HFD primed a neuroinflammatory phenotype but did not actively induce inflammation. To evaluate if elevated CORT signaling during HFD consumption mediates the impact of HFD on priming, we administered the GR antagonist, RU486, 24 and 48 hrs after initiation of HFD and collected hippocampal samples on the third day. Presented here is an examination of the protein and mRNA markers that previously indicated evidence of the HFD-induced primed phenotype. Data are presented graphically in **Fig. 4.1**.

Protein and CORT

As revealed by 2-way ANOVA and consistent with prior findings, HFD significantly increased levels of hippocampal HMGB1 (diet: $F_{(1,22)}$=5.078, p=.0345, **Fig. 4.1.A**) and NLRP3 protein (diet: $F_{(1,22)}$=10.10, p=.0034, **Fig. 4.1.B**). In contrast to prior findings, CORT levels in HFD animals were elevated, but not significantly, compared to Reg chow (p>.05, **Fig. 4.1.C**). HMGB1 levels revealed a significant interaction of HFD with RU486 (interaction: $F_{(1,22)}$=8.503, p=.0080) such that RU486 increased HMGB1 levels in Reg chow rats, but decreased HMGB1 in HFD. Therefore, RU486 completely blocked the HFD-induced increase in HMGB1. A similar interaction trend was observed in NLRP3, however the overall interaction did not

quite reach significance (F=3.291, p=.08, NS). An overall effect of RU486 was observed on hippocampal CORT ($F_{(1,22)}$=13.28, p=.0014, **Fig. 4.1.C**), as RU486 decreased CORT levels in both Reg and HFD rats, and RU486 completely prevented HFD-induced increases in CORT such that the HFD/RU486 group was equivalent with Reg diet controls.

mRNA

Gene expression of both IκBα and CD11b were increased by HFD compared to Reg diet controls, and both genes illustrated interactions of diet and RU486 such that RU486 mildly increased expression in Reg diet animals, yet decreased gene expression when given with HFD. RU486 reduced HFD-induced CD11b (interaction: $F_{(1,27)}$=7.424, p=.0112, **Fig. 4.1.E**) and eliminated HFD-induced IκBα gene expression (interaction: $F_{(1,27)}$=13.06, p=.0012, **Fig. 4.1.D**). It was not anticipated that HFD alone would influence GILZ gene expression, as we have not observed prior evidence of such a change. As such, GILZ expression is not considered a marker of a primed phenotype from HFD. However, analysis of GILZ was included here as RU486 is a GR antagonist and GILZ is a marker for GR activation. The only significant influence on GILZ mRNA was RU486 ($F_{(1,28)}$=9.781, p=.0041, **Fig. 4.1.F**), which, interestingly, increased GILZ expression in both diet groups, inducing a partial increase in HFD and a significant increase ($p<.05$) in Reg diet controls.

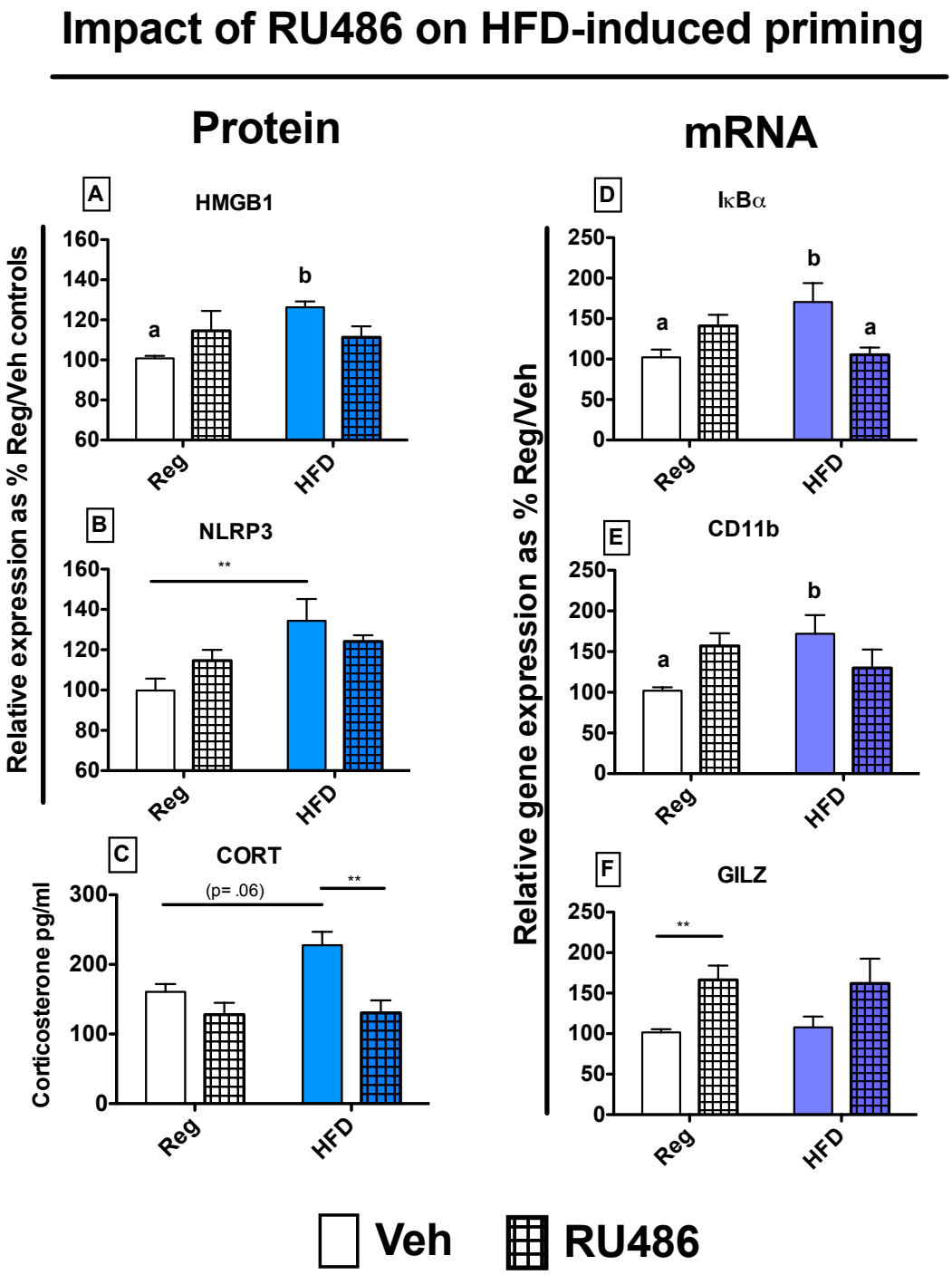

Fig. 4.1. Impact of GR blockade with RU486 during HFD consumption on the HFD-induced primed neuroinflammatory phenotype in hippocampus. Data that revealed significant interactions of HFD and RU486 (A,D,E) are indicated by use of letters to illustrate means that are significantly different (p<.05). HFD increased HMGB1 (A), NLRP3 (B), IκBα mRNA (D) and CD11b mRNA (E), and hippocampal CORT was elevated, but not significantly (C). **RU486 completely blocked HFD effects on HMGB1 (A) CORT (C) IκBα (D) and CD11b (E) and partially (p> .05) inhibited HFD increases in NLRP3 (B).** GILZ expression was not altered by diet (F), but was increased by prior RU486. p< .01**.

4.3.2. Impact of RU486 during HFD on response to LPS

The data presented in the preceding section indicates that GR blockade during HFD consumption reduces or inhibits the development of a primed neuroinflammatory phenotype from HFD consumption. The nature of a primed phenotype is such that, while not actively inflammatory, an exaggerated response occurs if a subsequent inflammatory challenge is presented. To further evaluate the impact of RU486 on HFD-induced neuroinflammatory processes, we repeated the diet/RU486 protocol used previously, followed with a peripheral injection of LPS on day 3, 2 hrs prior to tissue collection. The appropriate saline vehicle controls were included in the test design and evaluation of markers for inflammation discussed here. However, those data merely serve to confirm LPS induced an inflammatory response, so for simplicity, only measures reflecting impact of LPS are presented in **Fig. 4.2**.

Protein and CORT

Replicating prior observations, HFD rats that did not receive RU486 exhibited a potentiated (compared to Reg diet controls) hippocampal inflammatory response to LPS, as seen through increased IL-1β protein (diet: $F_{(1,20)}$=5.113, p=.035, **Fig. 4.2.A**) and NLRP3 protein (**Fig. 4.2.B**). The HFD-induced potentiated response of NLPR3 to LPS was eliminated by RU486 (interaction: $F_{(1,26)}$=4.552, p=.0425, **Fig. 4.2.B**), while RU486 had no influence in Reg diet controls. In addition, the response of IL-1β protein to LPS in both Reg and HFD groups was reduced by RU486 ($F_{(1,20)}$=8.070, p=.0101, **A**). While hippocampal CORT has not previously demonstrated a potentiated response to LPS in HFD, the analysis was included here

due to the direct impact of RU486 on CORT signaling. Consistent with prior observations, here we demonstrate that CORT was not potentiated by HFD to LPS, however a significant overall effect of RU486 on CORT was observed (RU486: $F_{(1,22)}$=6.014, p=.0226, **C**) such that RU486 inhibited CORT levels in HFD animals, but the same effect was not significant in Reg diet controls.

mRNA

All genes examined (mRNA: IL-1β, IL-6, IκBα, GILZ, **Fig. 4.2. D-F**) demonstrated significant interactions of diet and RU486, such that RU486 had no significant impact on the LPS response in Reg diet animals, but decreased HFD responses to LPS (Interaction: IL-1β ($F_{(1,26)}$=6.027, p=.0211, **D**), IκBα ($F_{(1,27)}$=4.612, p=.0409, **F**) and GILZ: ($F_{(1,27)}$=4.308, p=.0476, not shown). The interaction of RU486 and HFD was also significant for IL-6 ($F_{(1,27)}$=5.315, p=.0305), however, RU486 did not significantly decrease IL-6 gene expression (compared to HFD/Veh: p=.056, **Fig. 4.2.E**). Although IκBα mRNA gene expression was not previously, or here, potentiated by HFD to LPS, analysis of this marker is included due to the role of NFκB in mediating transcription of pro-inflammatory cytokines and upregulation of NLRP3, two markers which are potentiated by HFD. RU486 significantly decreased the IκBα response to LPS in HFD animals; however, the HFD/veh group was not significantly increased compared to Reg diet controls.

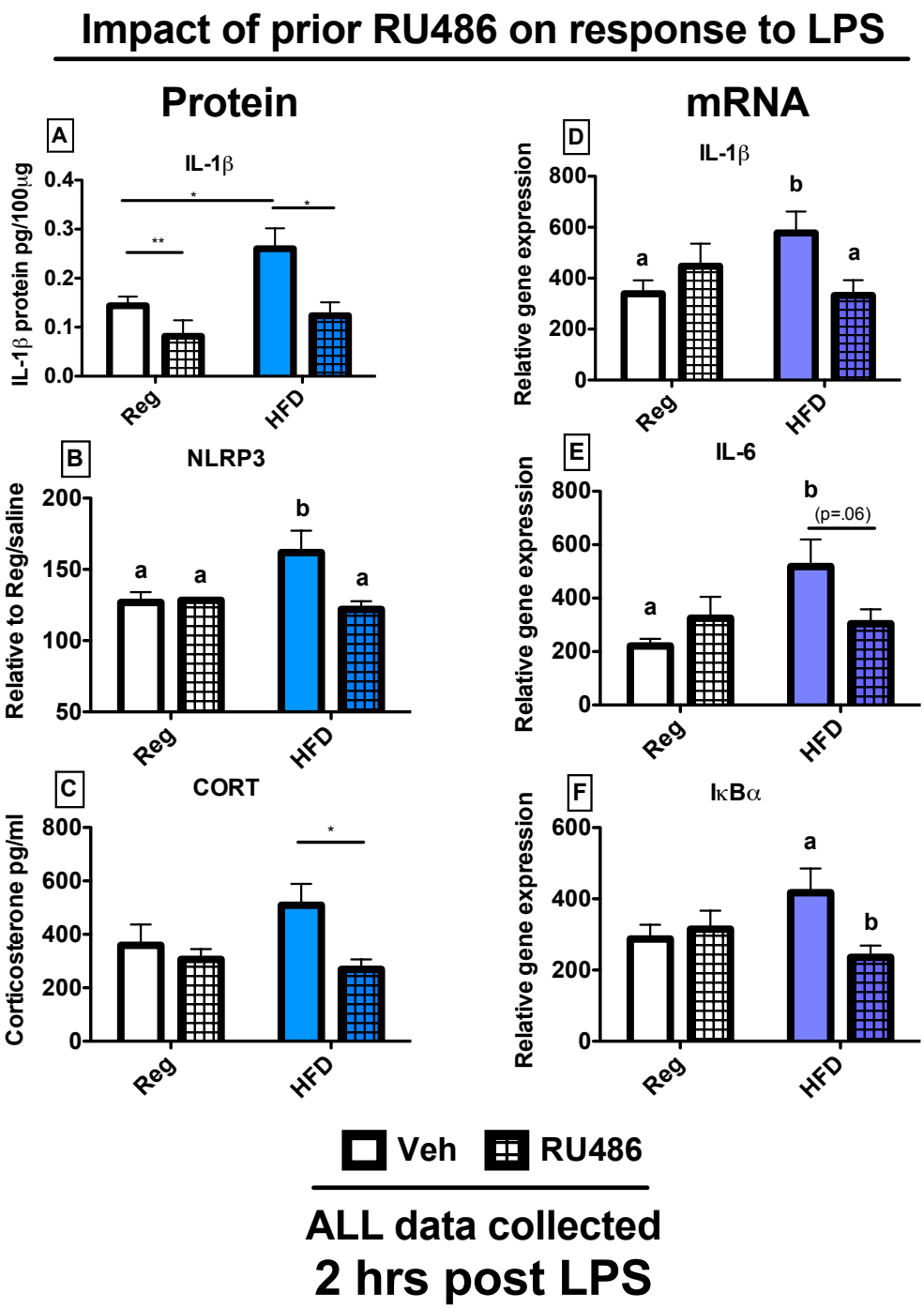

Fig. 4.2. Effect of RU486 on HFD-induced potentiated inflammatory response to LPS. All data were collected 2 hrs after peripheral LPS administration. HFD potentiated the inflammatory response to LPS on: IL-1β protein (A), NLRP3 protein (B), and gene expression of the pro-inflammatory cytokines IL-1β (D) and IL-6 (E). RU486 decreased levels of CORT (C) and IκBα mRNA (F), neither of which were increased by HFD. **RU486 negated HFD-induced potentiated responses to LPS in IL-1β protein and mRNA (A, D), NLRP3 protein (B) and prevented the HFD increase of IL-6 (E).** For data sets where significant interactions of diet/RU486 were observed, group mean differences ($p > .05$) are indicated by different letters, otherwise, differences indicated by $p < .05$*, .01**.

4.3.3. Impact BoxA during HFD on the development of a primed neuroinflammatory phenotype.

In addition to increasing CORT, 3 days of HFD also increases hippocampal levels of the endogenous danger signal HMGB1. To evaluate the relative impact of HMGB1 signaling during HFD on mediating a primed neuroimmune phenotype, BoxA was administered 24 and 48 hrs after start of HFD consumption and hippocampal tissue collected on day 3. Data discussed here is presented in **Fig. 4.3**.

Protein and CORT

Of protein markers examined to evaluate the role of BoxA administration during HFD consumption on mediating a primed neuroimmune phenotype, the only effect of BoxA was observed on HMGB1 protein (**Fig. 4.3.A**) where the increase in HMGB1 from HFD (diet: $F_{(1,21)}$=24.53, p<.0001) was ameliorated with BoxA (interaction: F=8.737, p=.0075), while BoxA had no effect in Reg diet controls. No effects on diet or BoxA were observed for NLRP3 protein (**Fig.4.3.B**), however HFD has elicited variable increases in NLRP3 previously, so this lack of a diet-induced difference is not inconsistent with prior observations. Similarly to what has been observed previously, hippocampal CORT levels were increased by HFD ($F_{(1,21)}$=8.461, p=.0084, Fig. **4.3.C**), but were not altered by BoxA.

mRNA

As previously established, gene expression of both IκBα and CD11b were increased by HFD (IκBα: $F_{(1,17)}$=4.474, p=.0495, Fig. **4.3.D**; CD11b: $F_{(1,18)}$=19.35, p=.0003, **Fig. 4.3.E**), with BoxA inducing no alterations in CD11b, and decreasing IκBα in HFD but not Reg, however the interaction was not significant (p> .05). As

Exp. 1 utilized a GR antagonist reversal approach, GILZ gene expression (a marker for GR activity) was included in that analysis (4.3.1) even though GILZ expression has not previously been altered by HFD. To maintain consistency, observations of GILZ gene expression are included here (**Fig. 4.3.F**), however there were no statistical effects of HFD or BoxA on GILZ expression.

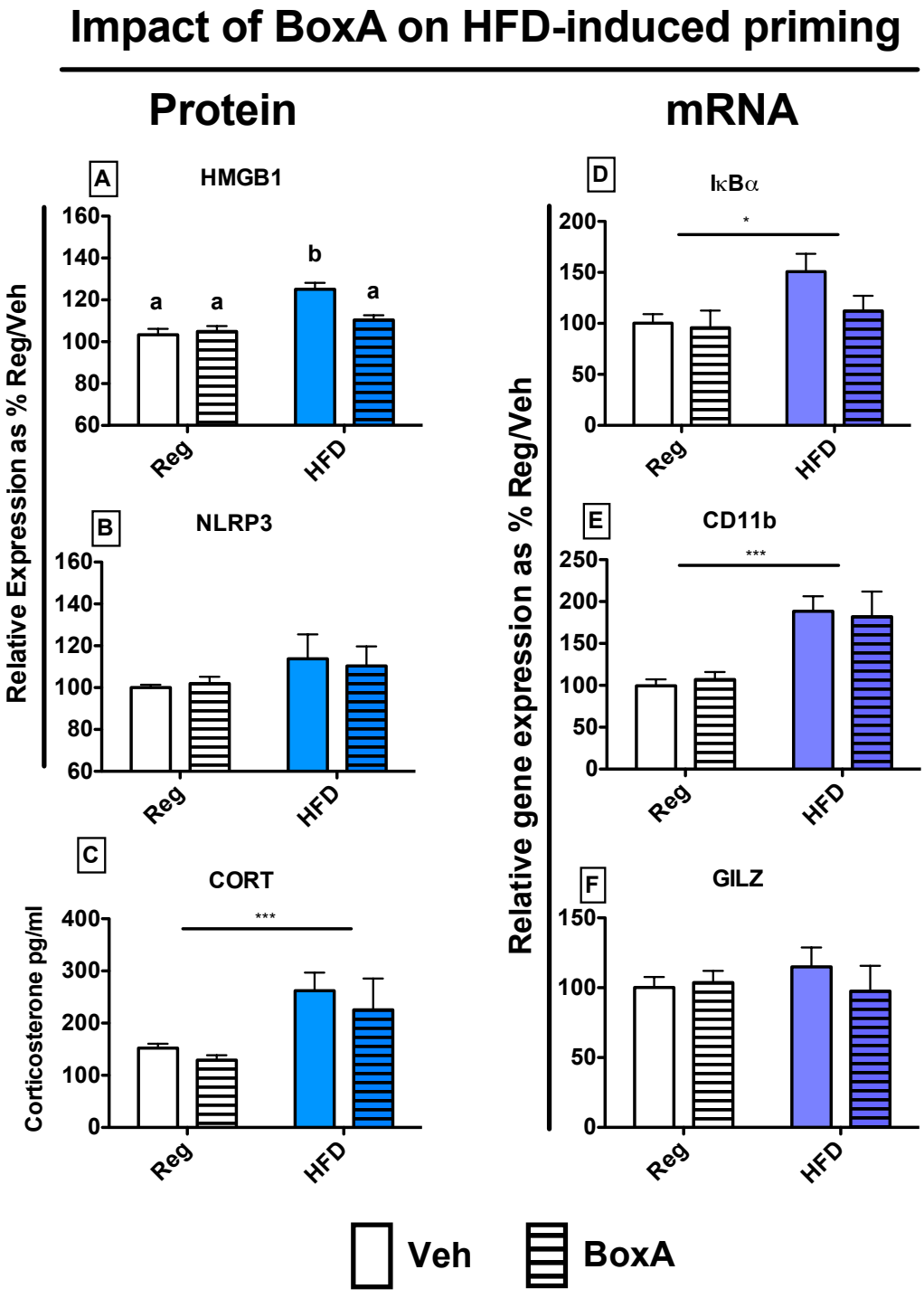

Fig. 4.3. Effect of BoxA on the measured impact of HFD in mediating a neuroinflammatory primed state. The only significant effect of BoxA was observed in HMGB1 protein (A), where BoxA administration during HFD prevented the HFD-induced increase yet had no effect on Reg diet animals. HFD increased CORT (C) and mRNA of IκBα (D) and CD11b (E). No effects on NLRP3 protein (B) or GILZ mRNA (F) were observed. Data that revealed a significant interaction of HFD and BoxA (A), the means are designated by letters, with different letters indicating means that are significantly different ($p<.05$). $p< .05$*, .01**, .001***

4.3.4. Impact of BoxA during HFD on response to LPS

To examine the impact of BoxA on HFD-induced potentiated inflammatory response to LPS, dual injections of BoxA were administered during HFD with LPS administered on day 3. Hippocampal tissue collected 2 hrs after LPS was analyzed for the protein and mRNA markers that have previously indicated HFD-potentiation to LPS. (Data discussed here are presented in **Fig. 4.4**.). Overall, the only statistically significant impact of BoxA was seen for IL-1β protein, where prior BoxA reduced the response to LPS in HFD animals (IL-1β: BoxA: $F_{(1,20)}$=5.690, p= .02, Fig. **4.4.A**). The IL-1β protein response to LPS in the HFD/Veh group was nearly significantly potentiated compared to Reg controls (diet: F=3.937, p= .06, NS). Therefore, these data support, but do not statistically replicate, prior observations of HFD potentiation of IL-1β protein to LPS. Replication of prior evidence of HFD-potentiated responses to LPS were seen in NLRP3 (diet: $F_{(1,25)}$=8.386, p= .0077, **Fig. 4.4.B**) and IL-1β mRNA (diet: $F_{(1,17)}$=11.62, p=.0033, **Fig. 4.4.D**). Observations of hippocampal CORT (**Fig. 4.4.C**) and IκBα mRNA (**Fig. 4.4.E**) further verify prior observations of lack of HFD-potentiation to LPS, and here demonstrate no alterations from BoxA. In addition, IL-6 gene expression was not altered by diet or BoxA (data not presented). Overall, these data suggest BoxA reduced the impact of HFD on potentiating an inflammatory response to LPS, by eliminating IL-1β protein potentiation and reducing potentiated NLRP3, but did not influence IL-1β gene expression.

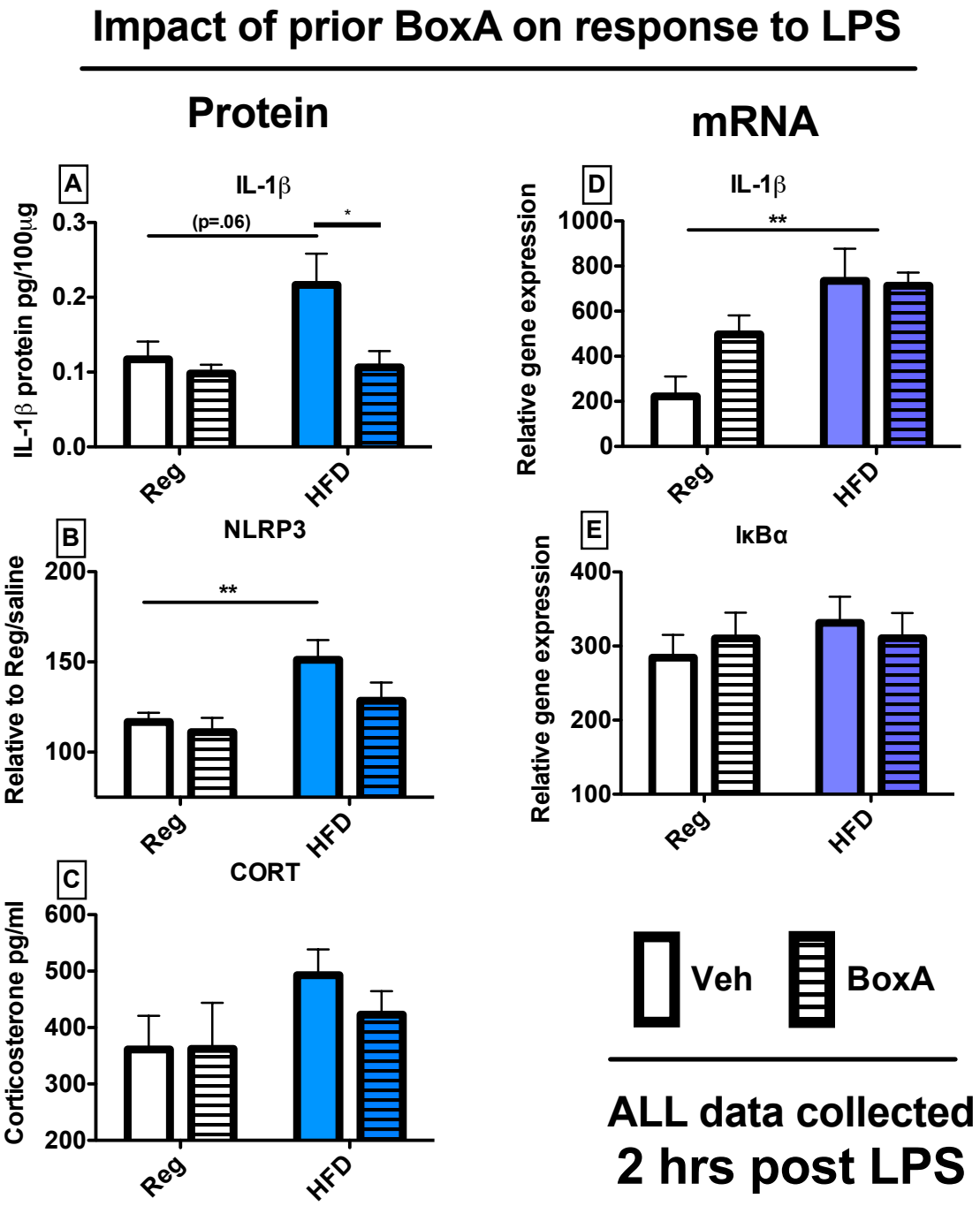

Fig. 4.4. Impact of BoxA or Veh during HFD consumption on the inflammatory response to subsequent LPS. Dark borders on all data columns indicate all data is in response to LPS administration. **The only effect of BoxA was observed in IL-1β protein (A),** where the nearly significant potentiated response to LPS in HFD animals was blocked by prior BoxA. HFD induced potentiated LPS-induced responses in NLRP3 protein (B) and IL-1β mRNA (D). Although HFD-potentiated responses have not been observed previously in CORT (C) and IκBα mRNA (E), data from these markers are included and indicate no effects of diet or BoxA. P< .05*, .01**

4.4. Discussion

Overall, the data presented here strongly implicates a role for CORT, as a mediator of HFD-induced neuroinflammatory priming and potentiated inflammatory responses to LPS. Inhibition of CORT signaling through GR blockade with RU486 nearly or completely prevented a primed phenotype in HFD animals, and abolished the pro-inflammatory impact of HFD on responding to LPS. Evaluations from the HMGB1 blockade experiment produced data that are generally equivocal to what was observed following CORT antagonism, however, strong conclusions cannot be drawn given that the study used only one dose of BoxA and one dosing regiment. Given the current protocol, BoxA administration during HFD prevented HFD-induced HMGB1 protein, and BoxA reduced gene expression of IκBα, but not significantly. Furthermore, prior BoxA eliminated the HFD potentiated LPS response of IL-1β protein, and reduced elevated NLRP3 in HFD animals given LPS, but did not impact IL-1β gene expression from LPS.

Both CORT (Busillo et al., 2011) and HMGB1 (Weber et al., 2015) have been implicated in the induction of primed neuroinflammatory states by inducing NLRP3. NLRP3 induction can occur from NFκB activation (Hanamsagar et al., 2012), but it is not known if this is the only mechanism of NLRP3 induction. Most of what is understood about CORT regulation of NFκB activity is anti-inflammatory, and involves direct inhibition of NFκB by GR (Nelson et al., 2003), or through regulating the phosphorylation state of IκB (Tahera et al., 2006), which exerts inhibitory control over NFκB activity (Majdalawieh and Ro, 2010). Therefore, the mechanism of CORT induction of NLRP3 is not clear.

One potential mechanism by which CORT may indirectly induce NLRP3 is through stimulation of an intermediary factor, such as HMGB1, which then induces NLRP3. HMGB1 can mediate induction of NLRP3 (Yanai et al., 2012) through signaling of TLR4 (Park et al., 2006) and other receptors (RAGE, TLR2), or by binding and synergizing the activity of pro-inflammatory cytokines or other TLR ligands such as LPS (Sha et al., 2008). However, it is not established whether CORT directly induces HMGB1. During the early phase of an acute stress response, HMGB1 has demonstrated to move from the nucleus to the cytoplasm in thymocytes (Billing et al., 2012), however it is not clear if the effect on HMGB1 was mediated by CORT. The data presented here demonstrates that GR blockade with RU486 prevents HFD-induced elevated levels of HMGB1 in hippocampus, and also prevents the impact of HFD on potentiating NLRP3 and IL-1β to subsequent LPS. Furthermore, HMGB1 inhibition with BoxA inhibited HFD potentiation of IL-1β and reduced NLRP3 responses to LPS. While these data are not definitive, they provide strong support linking GR activation with mediating HMGB1, and also linking activity of HMGB1 with mediating NLRP3 and potentiated inflammation to LPS.

Classically, the immune-modulatory impacts of CORT have been viewed as primarily anti-inflammatory (De Bosscher and Haegeman, 2009), however this notion is being updated as prior stress potentiates pro-inflammatory functions to subsequent LPS (Johnson et al., 2002, Munhoz et al., 2006), and the pro-inflammatory effects of stress have been linked to CORT signaling (Munhoz et al., 2010, Frank et al., 2012). It appears that timing of CORT elevations are important for determining the resulting anti-or pro-inflammatory function, as CORT elevations

prior to a secondary challenge increase the response to LPS, but CORT signaling after the inflammatory challenge dampens the response (Frank et al., 2010c). Our examinations revealed increased hippocampal CORT from 3 days of HFD, indicating that CORT was elevated prior to the time of LPS administration. As the data presented here indicate HFD potentiated the inflammatory response to LPS, the current studies provide evidence supporting the notion that prior CORT elevations mediate pro-inflammatory effects.

We observed RU486 decreased CORT significantly in HFD animals, and reduced CORT in Reg diet controls. Due to the nature of GR-induced HPA axis inhibition (Carrasco and Van de Kar, 2003), we anticipated GR blockade with RU486 would result in CORT elevations. Therefore, this finding was surprising and contrary to expectations. However, while the half-life of RU486 is around 18 hours, and tissue was collected 26 hours after the second RU486 injection. Therefore, it is possible the GR-blocking effects of RU486 were beginning to wear off and GR signaling was again enabled at the time tissue was collected. This is indicated by elevations in GILZ mRNA in both diets. If this were the case, it may explain the observations of reduced CORT levels, as GR activation can relatively quickly inhibit CORT (Lightman et al., 2008). Furthermore, reduced CORT signaling, such as following adrenalectomy, has resulted in increased GR expression in hippocampus (Reul et al., 1987). It is possible that RU486 may have altered GR similarly, however this was not examined.

As HMGB1 may both induce NLRP3 as well as synergize an inflammatory signal through binding with cytokines or LPS, it is not clear if the effects of HMGB1 in mediating neuroinflammation with HFD can be attributed to HMGB1 facilitating

the priming process or to aggravating inflammation during the LPS challenge. The data generated from the BoxA study, while not conclusive, indicates HMGB1 contributes to HFD-induced neuroinflammatory alterations. Recall that intense stress induces neuroinflammatory priming to LPS given 24 hours later, when stress-induced CORT elevations have subsided (Frank et al., 2010c), however, stress-induced HMGB1 levels remain elevated in hippocampus for at least 24 hrs (Weber et al., 2015). Therefore, stress-induced HMGB1 levels would still be elevated when LPS is given 24 hrs later. Therefore, it is not clear if the impact of HMGB1 on mediating neuroinflammatory priming is through increased levels prior to, or during, the LPS challenge. We found prior BoxA inhibited HMGB1 as well as prevented HFD-potentiated response of IL-1β protein to LPS, but did not eliminate the impact of HFD on potentiating LPS-induced NLRP3 or alter LPS-induced IL-1β mRNA.

Recall that NLRP3 activation requires 2 steps, the first of which may involve NFκB activation to induce NLRP3 transcription, a process that also induces pro-IL-1. In addition to inducing transcription of pro-inflammatory mediators such as pro-inflammatory cytokines and NLRP3, NFκB also increases IκBα mRNA as a mechanism of auto-inhibition. Here, we used IκBα gene expression as a means to evaluate NFκB activity, and use this rational to conclude that HFD increases NFκB activity. Therefore, a primed cell that demonstrates increased NLRP3 protein would conceivably also contain elevated levels of non-active pro-IL-1. Upon further stimulation, assembly and activation of the NLRP3 inflammasome would activate caspase-1, the enzyme primarily implicated in cleaving pro- into mature-IL-1β. If the phenotype of a primed cell includes increased pro-IL-1 in the cytosol, then

activation of caspase-1 would cleave available pro-IL-1, inducing a potentiated primary IL-β protein signal. LPS is able to induce IL-1β inflammation directly, without requiring a priming signal to first increase NLRP3, as LPS signaling through TLR4 is able to both induce NFκB and activate inflammasomes through independent mechanisms (Schroder et al., 2012). Therefore, as HFD animals given BoxA showed an absence of the HFD-induced potentiated IL-1β protein response, but BoxA did not prevent the LPS-induced increased gene expression of IL-1 mRNA, it is conceivable that HMGB1 inhibition with prior BoxA acted to prevent accumulation of pro-IL-1. However, this is speculation.

Taken together, while both CORT and HMGB1 appear to mediate the neuroinflammatory nature of HFD, it appears that CORT may, proportionately, exert pro-inflammatory influence by inducing long-lasting changes indicative of a primed state, which may include induction of HMGB1. Overall, the results presented confirm prior findings that HFD increases hippocampal protein and mRNA markers consistent with a primed neuroimmune phenotype. In addition, administration of RU486 during HFD partially or completely decreased all markers of a primed neuroinflammatory phenotype in HFD and prevented the HFD-induced potentiation to subsequent LPS. These effects were mirrored by, although less robustly, BoxA. The data presented here implicates CORT and HMGB1 in mediating neuroinflammatory alterations from 3 days HFD consumption.

Chapter 5: General Discussion

5.1. Overview of main experimental results

5.1.1. General summary of all data

The overall purpose of this dissertation is to establish a link between high-fat diet (HFD) and neuroinflammation, and to evaluate both the nature and mechanisms by which HFD influences neuroinflammatory processes. The body of data presented here serves to demonstrate a number of important characteristics connecting HFD with neuroinflammation. These are: 1) HFD induces obesity and corresponding hippocampal memory impairment, 2) disruptions in hippocampal memory function from HFD are mediated by primed neuroinflammatory processes, 3) very short-term HFD consumption induces the same primed and potentiated neuroinflammatory processes observed from prolonged HFD, and 4) the impact of HFD on neuroinflammation appears to be mediated through elevations in hippocampal CORT and HMGB1.

The data presented in Chapter 2 focuses on evaluating the impact of prolonged HFD consumption on mediating hippocampal memory function through neuroinflammatory processes. Specifically, long-term (3-5 months) HFD consumption alters the neuroinflammatory phenotype to become hyper-stress responsive and initiate hippocampal inflammation (increased IL-1β protein) to a brief footshock. This neuroinflammatory response mediates a resulting disruption in hippocampal memory, as we found that blocking action of IL-1β protein signaling, with IL-1RA, during the footshock, inhibited the IL-1β mediated memory disruption.

Furthermore, previously fed HFD animals switched to regular chow showed no evidence of altered neuroinflammation or impaired memory, yet maintained physiological evidence of an obese state. We concluded continued consumption of HFD diet, not obesity, mediated the neuroinflammatory alterations that led to hippocampal functional decline. The results presented in Chapter 2 indicate long-term HFD induces a hyper-stress responsive and neuroinflammatory primed state, which mediates resulting hippocampal memory disruption. These data suggest that potentiated neuroinflammatory processes may also mediate obesity-induced cognitive disruption in humans.

A theme that emerged from the initial investigations of HFD and obesity was that the effects of diet appeared unrelated to evidence of obesity, so we hypothesized the diet alone might have mediated the neuroinflammatory effects. The goal of studies presented in Chapter 3 was to evaluate if similar neuroinflammatory processes would be evident following very short-term HFD consumption. For this, animals were given Reg chow or HFD followed by a peripheral inflammatory challenge of lipopolysaccharide (LPS) or footshock on the third day. Results were consistent with our prior observations that HFD does not induce active neuroinflammation, as evidenced by lack of increased IL-1β protein from diet alone. However, 3 days of HFD increased gene expression of CD11b and IκBα in hippocampus, which indicated alterations indicative of a primed neuroinflammatory phenotype. Furthermore, HFD animals demonstrated a potentiated neuroinflammatory response to subsequent LPS, with exaggerated levels of IL-1β and NLRP3 protein observed in hippocampus, but not hypothalamus

or frontal cortex. 3 days of HFD did not induce an inflammatory response to footshock, but HFD animals given shock demonstrated increased hippocampal HMGB1. The general lack of inflammatory response following shock indicated 3 days HFD induced a primed, but not hyper stress-responsive, neuroinflammatory state. Furthermore, HFD increased levels of CORT and HMGB1 in hippocampus. The results presented in Chapter 3 indicate that short-term HFD is sufficient to induce a primed neuroinflammatory phenotype, a potentiated neuroinflammatory response to LPS, and that the hippocampus appears particularly vulnerable to the effects of HFD.

We aimed to further evaluate the nature of HFD on neuroinflammation by exploring mechanisms by which 3 days HFD induces primed and potentiated neuroinflammatory responses. As HFD increased levels of CORT and HMGB1, which have both been implicated in mediating the pro-inflammatory impact of intense stress (Frank et al., 2012, Weber et al., 2015), the studies presented in Chapter 4 evaluate the impact of CORT or HMGB1 blockade during HFD consumption in mediating the neuroinflammatory impact of HFD. In separate groups of animals, either the glucocorticoid receptor (GR) antagonist, RU486, or the HMGB1 inhibitor, BoxA, were administered during 3 days HFD. On the third day, an injection of LPS or vehicle was given, and hippocampal samples collected 2 hours later. RU486 prevented HFD-induced increase in IκBα mRNA, as well as prevented the potentiation by HFD to LPS through normalized levels of both IL-1β protein and mRNA as well as NLRP3 protein. Prior BoxA prevented HFD-induced increased HMGB1 protein, decreased HFD induction of IκBα, but did not impact CD11b mRNA.

Prior BoxA prevented the HFD potentiated response of IL-1β protein to LPS and reduced HFD-increased NLRP3 protein responding to LPS. BoxA had no impact on altering the influence of HFD on LPS induction of IL-1β mRNA. Overall, the results implicate both CORT and HMGB1 in mediating the development of a primed neuroinflammatory environment from HFD.

5.1.2. Impact of prolonged HFD consumption

HFD induces obesity and hippocampal memory disruption

As discussed previously, obesity is a condition that is extensively well studied to be damaging in both the body and the brain. The collection of negative health and metabolic consequences of obesity are attributed to peripheral inflammation (Mito et al., 2000), that, at least in part, results from the pro-inflammatory nature of obese adipose tissue (Coppack, 2001). While less work has examined the neuroinflammatory environment with obesity, the literature suggests that obesity also presents with alterations in central inflammatory processes (De Souza et al., 2005). Research examining obesity in humans reveals that disrupted cognitive function is common, with hippocampal functioning appearing particularly vulnerable (Gunstad et al., 2010). Similar hippocampal cognitive disruption has been observed in aging (Barrientos et al., 2006) and attributed to age-induced alterations in neuroinflammatory processes (Barrientos et al., 2009a). Therefore, the research discussed within this dissertation was initiated with the intention of evaluating whether obesity induces alterations in neuroinflammatory processes to mediate memory impairments, in a manner similar to what has been observed in aging.

The initial goal was to establish a model of obesity that also demonstrated hippocampal functional disruption. As the recent trend of obesity development in western societies is likely influenced by alterations in diet and activity levels (Archer and Mercer, 2007, Dwyer-Lindgren et al., 2013), and not due to genetic influences, we did not consider use of genetic models of obesity, and instead focused on developing a model of diet-induced obesity (DIO). The animal literature on DIO varies greatly with respect to species and strain used, as well as type of diet employed to induce obesity (Buettner et al., 2006). Initial observations compared obesity development in Sprague Dawley (data not presented) and Wistar strains from consumption of two dietary interventions, medium-fat (42% fat, Med) and high-fat (60% fat, HFD) diet. We found that Sprague Dawley rats did not gain weight as readily from either diet compared to Wistars, but also did not demonstrate evidence of hippocampal functional disruption (measures explained in next section). In addition to observations of body mass, serum leptin levels were measured to further confirm that diet induced physiological alterations indicative of an obese state, as elevated leptin is a common component of obesity in humans (Otero et al., 2006). Interestingly, wistar rats gained equivalent weight and demonstrated comparable serum leptin increases whether they consumed Med or HFD (data presented in Chapter 2).

To evaluate if our model of DIO also induced observable alterations in hippocampal memory function, a model of fear conditioning that uses a contextual pre-exposure (CPE-FC) protocol was selected as all aspects of the procedure (pre-exposure, conditioning and memory retrieval), require intact hippocampal

functioning (Rudy et al., 2002). This test of hippocampal memory involves a significant pre-exposure period of the rat to a specific context, followed 24 hours later with re-exposure to the same context with the immediate presentation of a footshock, and immediate removal. The notion is that the animal must have solidified a representation of the context from the previous day to associate the same context with the shock, as the immediate shock event is not sufficient to form a representation of the context (Fanselow, 1990). Animals were tested for evidence of memory (as expressed through freezing behavior, an innate behavioral expression of fear) to the conditioning context 7 days later. Thus, if a rat had associated the context with the shock event, increased freezing behavior would be observed when tested within the same context. As presented in Chapter 2, animals that had consumed HFD, but not Med fat diet, demonstrated reduced freezing behavior in the conditioned context, indicating reduced contextual memory. Initial observations of memory function were conducted after 12 weeks of Med or HFD, with additional observations conducted after 20 weeks of HFD. Data suggested that the degree of hippocampal memory functional decline was exaggerated with longer duration of HFD feeding.

Prolonged HFD primes, but does not induce, neuroinflammation

Following CPE-FC (in animals tested after 12 weeks diet), samples were collected and analyzed for evidence of neuroinflammatory alterations. There were no differences between diet groups on hippocampal protein levels of the pro-inflammatory cytokines IL-1β, IL-6 or TNFα (data not shown). These results were discouraging, as it appeared that neuroinflammatory alterations were not mediating

the observed effects of diet on disrupted hippocampal memory function. However, in aging, neuroinflammatory alterations and corresponding hippocampal disruption are not observed from influence of age alone, but potentiated responses occur with an additional inflammatory challenge (Barrientos et al., 2006). Due to the brevity of footshock, it did not seem likely that the footshock experience during CPE-FC was a sufficient secondary challenge to induce a potentiated neuroinflammatory response in HFD animals. Yet, tissue collected 2 hours after shock, revealed HFD animals demonstrated a potentiated hippocampal inflammatory response of IL-1β protein (Chapter 2, Fig. 2.5). Interestingly, the Reg diet animals did not display an inflammatory response to the shock, suggesting that the HFD animals had become hyper stress-responsive.

Dietary reversal eliminates impact of prior HFD

While central and peripheral inflammatory processes are generally distinct, there are a number of ways in which inflammation in the body can signal and induce neuroinflammation (Watkins and Maier, 2005). As adipose tissue becomes inflammatory with obesity development, it was not initially clear if the neuroinflammatory response to footshock in HFD animals was the result of inflammatory signaling from peripheral adipose tissue, or from specific alterations occurring within the CNS. To evaluate whether neuroinflammatory-mediated disrupted memory function was influenced by dietary factors or from weight gain, HFD animals were evaluated following dietary reversal (DR). For this, rats that had fed HFD for 20 weeks were switched back to regular chow for 4 weeks. The DR animals demonstrated normal CPE-FC memory function and showed no evidence of

hippocampal neuroinflammatory response to footshock. However, DR animals maintained significantly elevated body mass and serum leptin levels compared to regular diet controls. This suggested aspects of the diet, rather than increased adipose tissue, were mediating the neuroinflammatory and hippocampal functional disruption observed with HFD.

HFD-induced potentiated IL-1β mediates hippocampal memory disruption following footshock

To further establish whether elevations in shock-induced hippocampal IL-1β were mediating the memory disruption observed from HFD, we sought to block action of IL-1β signaling during the shock event. For this, hippocampal memory was again assessed with CPE-FC, however, prior to the conditioning shock experience on day 2, animals were centrally injected with IL-1RA, a naturally occurring competitive antagonist of IL-1 receptors (Dinarello, 1998), and were tested for contextual memory 7 days later. Results indicate that blocking action of IL-1β with IL-1RA eliminated the impact of HFD on disrupting hippocampal memory function. Furthermore, as HFD rats given IL-1RA demonstrated normal memory function, and as the IL-1RA injection did not occur until the second day of CPE-FC, this indicates all animals, including those on HFD, demonstrated effective learning of the context during the pre-exposure period. Therefore, the impact of potentiated neuroinflammation appeared to interrupt consolidation of the memory linking the context to the shock event. These data indicate neuroinflammatory-mediated memory impairments from DIO are similar in nature to observations in aging (Barrientos et al., 2009a).

5.1.3. Impact of Short-term HFD consumption

The results from the dietary reversal study discussed previously indicated the neuroinflammatory alterations induced by prior prolonged HFD do not persist following discontinuation of HFD, and evidence of an obese state, in absence of HFD, does not demonstrate a neuroinflammatory primed phenotype or corresponding memory impairment. These results indicate that obesity, in absence of HFD, is not sufficient to induce neuroinflammation, and instead suggest HFD consumption was the primary mediator of altered neuroinflammatory function. If this is true, then similar alterations in neuroinflammatory function might be observed following much shorter durations of HFD consumption, prior to obesity development. As all rats gain weight throughout adulthood, and rats that consume HFD may develop significantly increased fat mass as soon as 7 days after starting HFD (Thaler et al., 2012), we sought to examine the impact of very short-term HFD, in an effort to further distinguish effects of HFD from obesity.

Previously, we did not observe basal alterations in neuroinflammation from prolonged HFD, but did observe alterations following a brief footshock. Therefore, for studies presented in Chapter 3, two separate secondary challenges were utilized to evaluate the impact on neuroinflammatory responses from HFD: LPS and the same footshock administered with CPE-FC. Animals either continued on Reg chow or were switched to HFD. 3 days later, rats received either an injection of LPS (10μg/kg) or saline or were exposed to footshock (1.5 mA, 2 s) or the shock chamber without shock (the full CPE-FC protocol was not run, only the shock experience). Tissue was collected 2 hrs following either challenge.

Other groups examining DIO have observed altered neuroinflammatory responses to LPS in hippocampus (Andre et al., 2014) and hypothalamus (Pohl et al., 2009). The neuroinflammatory environment following very short-term HFD consumption (days) appears to be limited to a single study, which focused on alterations within the hypothalamus (Thaler et al., 2012). However, our examinations of prolonged HFD revealed alterations in hippocampal neuroinflammation, and the human obesity literature suggests that hippocampal function may be particularly disrupted by prolonged HFD (Cohen, 2010). Furthermore, the hippocampus is a brain region particularly high levels in microglia (Lawson et al., 1990), the primary cell type implicated in mediating neuroinflammatory priming (Frank et al., 2007). Therefore, we sought to examine the impact of short-term HFD on hippocampal neuroinflammatory processes. While our primary intention was to evaluate the hippocampus, samples were also collected from hypothalamus and frontal cortex to evaluate whether short-term HFD induced widespread or regionally specific neuroinflammatory alterations.

Short-term HFD induces a primed neuroinflammatory phenotype

Initial observations indicated short-term HFD increased gene expression of CD11b in hippocampus and hypothalamus. CD11b is an antigen marker of microglia (as well as other peripheral leukocytes, which would not be present in tissue collected from the CNS), and expression is increased in reactive cells (Akiyama and McGeer, 1990). While the data presented do not definitively implicate microglia as mediating the neuroinflammation from HFD, observations of increased CD11b indicate an increase in hippocampal microglial activity from HFD. Furthermore,

IκBα gene expression was increased by HFD in hippocampus but not hypothalamus. IκBα gene expression is a marker for NFκB activity, and observation that HFD-induced IκBα mRNA was interpreted as evidence of neuroinflammatory priming. IκB protein, when bound with NFκB, prevents NFκB activation (Majdalawieh and Ro, 2010), upon stimulation of the cell, such as through PRR ligation, an internal cascade is initiated which results in phosphorylation of IκB, leading to activation of NFκB. In addition to regulating the transcription of a number of pro-inflammatory mediators such as cytokines (Han et al., 2002) and components of the inflammasome, such as NLRP3 (Schroder et al., 2012), the transcription of IκB mRNA is also induced by NFκB, as a mechanism of auto-inhibition. NLRP3 is a structural component of one type of inflammasome, which are multi-protein complexes that regulate the cleavage and release of IL-1β (Khare et al., 2010, Schroder and Tschopp, 2010). This process occurs through activation of caspase-1, which is discussed in detail in Chapter 1. The NLRP3 inflammasome is particularly implicated in mediating inflammatory priming, as activation of this inflammasome requires multiple steps (Latz, 2010). Induction of NFκB (Bauernfeind et al., 2009), which occurs in response to TLR4 and other receptor activation (Hanamsagar et al., 2012), is typically associated with mediating the NLRP3 priming step. However, it is not known if NFκB is required for NLRP3 induction.

HFD also increased levels of the proteins NLRP3 and HMGB1 in hippocampus but not in hypothalamus or frontal cortex. Recall that HMGB1 is an endogenous danger signal that can stimulate inflammation through interactions with a number of pattern recognition receptors such as TLR2, TLR4 and RAGE (Yanai et al., 2012)

to facilitate NFκB activity (Su et al., 2011). In addition, HFD was observed to induce elevated levels of CORT in hippocampus and serum. Recall that CORT has both anti-inflammatory and pro-inflammatory modulatory capabilities, with timing of CORT elevations mediating the resulting effect (Frank et al., 2010c). Taken together, these data strongly suggest HFD increases a number of factors that are associated with, or directly influence, neuroinflammatory function. However, we did not see increased IL-1β protein from HFD alone, indicating HFD primes, but does not actively induce, neuroinflammation.

Short-term HFD potentiates the neuroinflammatory response to LPS but not footshock

Following 3 days of HFD, a peripheral injection of LPS induced a potentiated hippocampal response of both IL-1β (protein and mRNA) and NLRP3. Interestingly, LPS reduced CD11b mRNA and decreased HMGB1 levels. Lastly, hippocampal IL-6 mRNA was nearly potentiated, but the effect was not significant. However, IL-6 induction tends to peak later than IL-1β, so it is likely that if tissue had been collected more than 2 hour post LPS, potentiation of IL-6 from HFD may also have occurred. Evaluations of serum CORT and IL-1β protein revealed that while HFD potentiated the neuroinflammatory IL-1β protein response to LPS, the same effect was not observed peripherally, as serum IL-1β was not potentiated. While the impact of HFD on peripheral markers was not of primary interest, short-term HFD is known to induce alterations peripherally (Ji et al., 2012, Wiedemann et al., 2013). Therefore, our evaluations of serum served to evaluate whether inflammatory alterations from HFD were similar peripherally and centrally.

Following footshock, no effects of shock-induced inflammation were observed in any of the markers examined, however HFD animals given shock demonstrated elevated hippocampal HMGB1 protein. Previous data suggested prolonged HFD consumption (5 months) sensitized the neuroinflammatory environment to induce inflammation in response to the footshock. However, 3 days of HFD was not sufficient to induce similar inflammatory responses to footshock.

Hippocampus particularly vulnerable to impact of short-term HFD

We observed no alterations of HFD within frontal cortex. While the inflammatory effects of LPS were widely observed on inducing inflammation in all regions, both hypothalamus and frontal cortex failed to mirror the hippocampal HFD potentiation of IL-1β or NLRP3 in response to LPS. Hypothalamic gene expression of IκBα was potentiated from the interaction on HFD and LPS, but that was the only potentiated effect of HFD and LPS observed in non-hippocampal brain tissue. Therefore, while evidence of inflammatory priming and potentiated inflammation was not isolated to the hippocampus, most of the significant elevations from HFD and potentiated responses to LPS were exclusively observed in hippocampus.

As discussed in Chapter 1, there is a body of literature supporting the theory that HFD-induced alterations in hippocampal function mediate further increased HFD consumption leading to obesity development (Davidson et al., 2007, Francis and Stevenson, 2013). One possible mediator of this process is brain derived neurotropic factor (BDNF). Decreased hippocampal BDNF is well documented in models of HFD or obesity (Molteni et al., 2002, Park et al., 2010, Dinel et al., 2011),

and BDNF maintains important regulatory control over food intake and body weight regulation (Lebrun et al., 2006), suggesting HFD may inhibit regulatory control over further HFD consumption. Another potential mechanism mediating HFD-regulation of food consumption, which will be discussed in more detail later, is CORT.

5.1.4. Mechanisms of HFD-induced neuroinflammatory priming

In addition to the observations that 3 days of HFD induce phenotypic alterations indicative of inflammatory priming, we also observed that HFD induced increased levels of both CORT and HMGB1. As discussed previously, both of these molecules are implicated in mediating stress-induced neuroinflammatory primed alterations in microglia (Frank et al., 2012, Weber et al., 2015). As evidence of a primed phenotype was evident by 3 days HFD, it is likely priming processes had been initiated prior to tissue collection on day 3. Therefore, we sought to block action of either CORT or HMGB1 during the 3 days HFD consumption. As discussed previously, the impacts of HFD appear most evident in hippocampus, so for simplicity, evaluations presented in Chapter 4 focus on effects in hippocampus.

Blocking GR with RU486

To block action of CORT signaling during HFD, the GR antagonist RU486 (Schreiber et al., 1983) was used. RU486 has previously demonstrated to effectively block GR,: to prevent stress-induced priming (Frank et al., 2012), and to prevent the impact of age on a primed phenotype (Barrientos et al., 2015). Furthermore, RU486 has shown to be an effective therapeutic agent long-term to alleviate metabolic disruption in DIO (Hashimoto et al., 2013). Reg or HFD rats were given dual s.c. injections of RU486 (50mg/kg) or vehicle (propylene glycol) 24 and 48 hours after

start of HFD, with LPS or saline on day 3, and hippocampal tissue collected 2 hours later.

Evaluating the impact of RU486 on HFD revealed that GR blockade prevented HFD increased IκBα mRNA, and decreased, but not significantly, the impact of HFD on CD11b mRNA and HMGB1 protein. In addition, RU486 during HFD negated the impact of HFD on hippocampal CORT increases, and in regular diet animals induced an increase in GILZ mRNA, which is a marker of GR activation. Observations demonstrating prior GR blockade inhibited CORT while also inducing evidence of GR signaling (ie GILZ) might appear inconsistent, but could be understandable when evaluated in terms of CORT feedback regulation. CORT levels are tightly controlled through a number of feedback mechanisms which influence HPA axis activity (Carrasco and Van de Kar, 2003). In addition, CORT levels follow a daily circadian rhythm, but levels do not consistently rise and fall; rather CORT levels demonstrate small pulses within the overall rhythmicity (Lightman et al., 2008). These pulses occur from alternating activation and inhibition of the HPA axis, as regulated by GR and MR signaling (Lightman et al., 2008). Absence of CORT signaling in adrenalectomized rats induces increased expression of GR in hippocampus (Reul et al., 1987), so it is likely that GR blockade with RU486 may have also done the same, but this is not clear. Furthermore, the half-life of RU486 is around 18 hours, and while the animals received dual injections of the drug, the second administration was approximately 26 hours prior to tissue collection. Thus, if RU486 was being metabolized and the effects were wearing off, it would not be surprising that we observed evidence of GR activation. GILZ does not appear to be increased by RU486

in cell culture (Aguilar et al., 2013), however the subtle regulation of GR activity and CORT levels are much more complex *in vivo*. The effect of prior RU486 appears to have normalized measured hippocampal CORT in HFD to levels comparable to regular diet controls.

RU486 blockade prevented the HFD-induced potentiated response to LPS, as seen by lack of exaggerated levels of IL-1β protein and mRNA, as well as NLRP3 levels comparable to Reg diet controls. In addition, RU486 prevented HFD potentiated LPS-increased steroid CORT and IκBα mRNA. These data strongly implicate HFD elevations in CORT as mediating the primed and potentiated neuroinflammatory effects of HFD consumption.

Inhibition of HMGB1 with BoxA

A similar protocol as was used with RU486 was implemented to evaluate whether blockade of HMGB1 with BoxA (10 μg/5 μl/per injection, ICM) would produce similar inhibitions of the influence of HFD on neuroinflammatory function. We administered BoxA directly into the CNS via ICM injections that occurred 24 and 48 hours after start of HFD. This study was designed as an exploratory evaluation of use of BoxA as the half-life of BoxA is not known, and it was not clear what dosing procedure would adequately inhibit HMGB1 throughout the 3 days of HFD consumption. Prior use of BoxA in our laboratory has used ICM administration, but previously rats have only received single administrations. ICM injection of BoxA is ideal to target the CNS and it would not be cost-effective to inject BoxA peripherally. As ICM administration requires anesthesia and involves direct injection into the CNS, the risks are higher associated with drug administration in this manner,

particularly with multiple injections. However, a dual ICM injection protocol has been used previously (Barrientos et al., 2015), which did not appear to result in harmful effects. Therefore, we proposed 2 daily injections, at 24 and 48 hours of HFD, would be a sufficient starting dosing protocol, to both maximize duration of BoxA action, yet minimize risks associated with repeated injections.

In general, the results from the BoxA study were encouraging, but inconclusive. We observed that prior BoxA prevented the impact of HFD on increased hippocampal HMGB1 levels, and negated the HFD-potentiated response of IL-1β to LPS. While HMGB1 is associated with peripheral inflammation in obesity (Gunasekaran et al., 2013), HMGB1 has only recently been implicated in mediating neuroinflammation (Weber et al., 2015). The results presented in this dissertation are the first to implicate diet in mediating HMGB1 centrally. While this study was carefully designed and carried out, the objective was primarily exploratory, so data that is suggestive, but not conclusive, is still valuable here. Therefore, results from the BoxA study provide strong evidence to support the role of HMGB1 in mediating the neuroinflammatory environment with HFD, and provide justification for further study into this phenomenon.

5.2. Inflammatory influence by different fat types

While the nature of the diet used in the studies presented here was initially discussed in detail in Chapter 1, it is worth noting again that not all fat types produce the same effects on inflammation. As ketogenic diets, which can contain up to 85-90% fat (Mobbs et al., 2013), are known to reverse cognitive effects in aging

(Krikorian et al., 2012), improve metabolic complications from obesity (Badman et al., 2009), and can reduce pain and inflammation (Ruskin et al., 2009), it may not be accurate to universally accuse any 'high-fat' food as inflammatory. Throughout this dissertation, the diet used is referred to as a 'high-fat diet'. While this is arguably not a comprehensive description of what the diet actually entails, referencing the diet this way is consistent with literature on the subject.

The HFD (TD.06414, Harlan), consists of 60% fat, and while the exact components of the diet are not revealed due to proprietary reasons, the diet is supplemented with essential vitamins, minerals and amino acids. However, most of the 60% fat listed consists of saturated fat (41.4%), primarily palmitate (palmitic acid, PA), which can directly activate inflammation by targeting TLR4 (Milanski et al., 2009), induce microglial NFκB activation (Wang et al., 2012) and has demonstrated to prime NLRP3 in dendritic cells (Reynolds et al., 2012). In addition, HPA activation and alterations in hypothalamic gene expression appear to occur selectively in response to saturated fatty acids, but not other types of fat (Dziedzic et al., 2007, Oh et al., 2014).

Conversely, long-chain polyunsaturated fatty acids (LCPUFAs), omega 3s, in particular, are demonstrated to be anti-inflammatory and can inhibit NFκB activity (Kang and Weylandt, 2008). LCPUFAs are protective against neuroinflammation in models of sickness, aging and depression (Orr et al., 2013), and LCPUFA deficiency may amplify neurodegeneration in Alzheimer's and Parkinson's disease (Janssen and Kiliaan, 2014). Harlan reports the HFD used here contains 1.5% omega 3 PUFAs, *of the 60% fat* content, which is quite low. Comparatively, the Reg chow (Teklad

8640, Harlan) delivers 17% of total calories from fat (total fat: 5.5%), which is composed of mostly LCPUSFAs, and very little saturated fat. Therefore, the HFD might be inducing inflammation directly through saturated fatty acid signaling, and effects could be amplified by a relative LCPUFA deficiency. Examination of these factors is beyond the scope of the present paper, and while actual content of the diet is not the primary focus, it is important to note that dietary components may directly influence neuroinflammation.

5.3. Reciprocal influence of HFD and CORT

Long-term consumption of HFD increases levels of basal and stress-induced CORT peripherally (Tannenbaum et al., 1997, Buchenauer et al., 2009), and HFD increases GR immunoreactivity in the brain (McNeilly et al., 2015). HFD fosters an exaggerated HPA axis induction of CORT in response to stress (Tannenbaum et al., 1997, Park et al., 2014), indicating that long-term consumption of HFD fosters a hyper-stress responsive state, which is particularly detrimental to the hippocampus (Yehuda et al., 2005), which our results confirm. Interestingly, stress also exerts a reciprocal influence on feeding behavior, which is likely due to shared anatomy of the systems that regulate food intake and stress responsivity within the hypothalamus (Maniam and Morris, 2012). While the initial short-term impact of elevated CORT on feeding is inhibitory, the overall, long-term impact of CORT is to stimulate feeding (Sapolsky et al., 2000). Stressful events induce preference for nutrient-dense, fatty or sugary food, which is a phenomenon that is well established in human and animal models (Torres and Nowson, 2007, Hanamsagar et al., 2011).

In the very short-term, this may be advantageous as stress-induced consumption of high-fat 'comfort food' may actually reduce CORT levels during a stressful event (Pecoraro et al., 2004, South et al., 2012). However, HFD consumption can very quickly become maladaptive, as peripherally elevated CORT has been observed as soon as 7 day after initiation of HFD (Tannenbaum et al., 1997), and here we establish 3 days of HFD is sufficient to elevate CORT centrally.

The reciprocal nature of HFD consumption and CORT may induce a positive feedback loop that can quickly spiral into obesity development. For instance, a single meal can induce a postprandial increase in plasma CORT (Hansen et al., 1997), which can facilitate increased caloric intake during a subsequent meal (Gaysinskaya et al., 2007). Increased calorie consumption can amplify HPA axis activity (Oh et al., 2014) thus facilitating the further selection of high-fat or high-sugar food (Zellner et al., 2006). Additionally, when animals that had been consuming high-caloric food previously, were switched back to standard chow, they exhibited increased HPA activity, (South et al., 2012, Martire et al., 2014) which indicates breaking the cycle of HFD consumption may act as an additional stressor.

In terms of obesity development, it appears that CORT mediates the process of fat storage leading to increased weight gain. Cushing's syndrome, a disorder that is diagnosed from elevated CORT, in response to a number of possible etiologies, manifests with obesity and cognitive disturbance. This strongly indicates obesity development is a side effect of CORT elevations. Furthermore, CORT signaling may also be required for obesity development to occur, as obesity doesn't progress when GR levels are genetically reduced (de Kloet et al., 2015) or blocked with RU486

(Okada et al., 1992), and strain differences in susceptibility to obesity development can be explained by differences in GR affinity (Marissal-Arvy et al., 2011). This effect appears to be selective to CORT signaling during HFD consumption, as CORT administration amplifies fat mass development in HFD animals, but does not impact weight gain in regular diet controls (Auvinen et al., 2013).

Once obesity develops, increased signaling of CORT mediates obesity-associated peripheral inflammation (Staab and Maser, 2010), which facilitates the negative metabolic consequences of obesity (Donath and Shoelson, 2011). While obesity is linked with decreased efficiency of inhibitory feedback regulation of the HPA axis, peripherally elevated CORT levels are not a hallmark characteristic of obesity (Morton, 2010). Rather, CORT signaling with obesity appears to be mediated by tissue-specific increases in activity of the CORT converting enzyme 11βHSD1 (discussed in detail in Chapter 1). Elevated levels of 11βHSD1 in adipose tissue are implicated in mediating obesity development and resulting metabolic alterations (Paulsen et al., 2008, Tagawa et al., 2009). It is possible that cognitive alterations observed with obesity are mediated through increased 11βHSD1 activity, as concentrations in the brain are region specific, tend to localize with GR distribution (Chapman and Seckl, 2008) and are found highest in the hippocampus (Seckl, 1997). Furthermore, increased hippocampal 11βHSD1 has been implicated in mediating hippocampal memory deficits in aging (Holmes et al., 2010, Sooy et al., 2010), and circadian fluctuations in 11βHSD1 observed in aging may mediate age-induced alterations in circadian CORT (Barrientos et al., 2015). Taken together, the evidence presented here heavily implicates action of CORT in mediating neuroinflammatory

processes from HFD consumption. Furthermore, CORT signaling likely facilitates increased HFD consumption, the resulting obesity progression, as well as the negative metabolic health and cognitive consequences associated with obesity.

5.4. Exercise as a therapeutic intervention

The studies presented here do not examine the impact of exercise on preventing the effects of HFD. However, there is overwhelming evidence to support regular exercise as anti-inflammatory (Gleeson et al., 2011) and stress-protective (Fleshner, 2005), and would be a logical approach to alleviate the impact of HFD on neuroinflammation. Not surprisingly, exercise intervention in rats fed HFD reduces body fat (Zoth et al., 2010), improves peripheral metabolic health markers (Beaudry et al., 2015), and reduces HFD-induced inflammation in hypothalamus (Yi et al., 2012) and aging-induced neuroinflammatory priming in hippocampus (Barrientos et al., 2011). Short-term exercise interventions reduce peripheral inflammatory markers in humans; an effect that is unrelated to body weight (Izadpanah et al., 2012), indicating weight reduction is not the only benefit of exercise.

Exercise appears to mediate anti-inflammatory processes through a number of mechanisms. Due to the inflammatory nature of adipose tissue, exercise-induced weight reduction reduces inflammatory signaling by adipokines, but exercise can also reduce macrophage infiltration into adipose tissue (Vieira et al., 2009) and improve the adipose ratio of anti-inflammatory signaling factors (Speaker et al., 2013). In addition, contracting skeletal muscle fibers can release IL-6, which serves as a ‘myokine’ to promote beneficial impacts of exercise by inducing release of the

anti-inflammatory mediators IL-1RA and IL-10 (Petersen and Pedersen, 2005). Exercise has demonstrated to downregulate TLRs (Francaux, 2009) and can reverse decreases in hippocampal BDNF that occur as a result of HFD (Molteni et al., 2004), early life stress (Maniam and Morris, 2010) and aging (Barrientos et al., 2011). Recall that, in addition to mediating food intake and body weight, BDNF is critical for hippocampal learning and memory functions, and signals through the TrkB receptor to promote cell survival and LTP, indicating exercise serves a neuroprotective role for cognitive function. Interestingly, genetic variants in BDNF correlate with reported affective responses to exercise (Bryan et al., 2007), which predict exercise motivation (Kwan and Bryan, 2010), indicating alterations in BDNF may help regulate motivation to exercise. As inhibition of BDNF signaling in genetically TrkB-deficient mice develop obesity and impaired cognition (Yeo et al., 2004), BDNF may further link processes involved in obesity, memory and exercise.

Exercise also appears to exert stress-protective effects in a number of ways. Wheel running facilitates HPA and CORT response habituation to repeated mild stress (Sasse et al., 2008, Nyhuis et al., 2010), but not intense stress (Campeau et al., 2010), and exercise reduces concentrations of hippocampal glucocorticoid receptor in aged animals (Barrientos et al., 2015). Furthermore, symptoms of depression from HFD are blunted with exercise (Liu et al., 2014b), and exercise reduces negative consequences of intense stress by attenuating serotonin activity during and after a stressful event (Greenwood et al., 2003, Greenwood and Fleshner, 2011). Serotonergic sensitization, which occurs in response to an uncontrollable, but not controllable, stress, mediates negative behavioral effects of uncontrollable stress

(Maier and Watkins, 2005). Therefore, as we encounter mild stress in our everyday lives, it appears that exercise may buffer the effects of daily mild stress by blunting stress-induced CORT signaling and by conferring perceived control over the stressors we encounter, yet does not inhibit the adaptive stress response that is required in dangerous or life-threatening situations. Recall that animals fed HFD for a prolonged (5 month) period, demonstrated stress sensitization as evidenced by a neuroinflammatory response to a single footshock, a stimulus that doesn't induce an inflammatory response in Reg diet animals. It appears that HFD and exercise may exert opposite influences on neuroinflammation and stress responsivity, which further illustrates the potential for exercise to counteract the negative impact of HFD consumption.

5.5. Clinical impacts

Dietary intervention as a tool to promote recovery

The data presented in this dissertation indicates short-term (3 days) consumption of HFD is sufficient to foster a pro-neuroinflammatory environment. Conversely, the negative effects of prior long-term HFD consumption, notably elevated neuroinflammatory processes and memory disruption, were abolished with 4 weeks dietary reversal. Therefore, while the negative effects of HFD may appear quickly, they likely do not linger long following a dietary intervention. Due to the nature of a primed neuroinflammatory environment, exaggerated inflammation occurs with a subsequent injury or inflammatory challenge, but only if a subsequent challenge occurs while the phenotype resembles a primed state. Therefore, if such a

subsequent secondary challenge is anticipated, such as a scheduled surgery, a dietary intervention prior to the event would likely support positive outcomes.

Research examining impact of preoperative diet on surgery outcomes indicate that DIO in mice potentiates an inflammatory response in adipose tissue following surgical trauma, which preoperative diet restriction preventes (Nguyen et al., 2013). These effects were also observed in the human clinical context as 1 week of preoperative calorie and fat restriction produced improved outcomes in patients undergoing scheduled liver resection surgery, as observed by less blood loss from surgery and reduced evidence of moderate and severe inflammation-associated fatty liver disease (termed steatosis and steatohepatitis, respectively)(Reeves et al., 2013). Therefore, preoperative dietary intervention is likely to improve surgery outcomes, particularly for surgery types that are prevalent with obesity. However, other than short-term (2-6 hour) preoperative fasting to prevent anesthesia-induced vomiting, pre-surgery dietary intervention does not appear to be common practice.

The addictive properties of HFD

Drugs of abuse are thought to induce rewarding and reinforcing properties through activity of the mesolimbic dopamine system, which stimulates dopamine cells of the ventral tegmental area (VTA) to release dopamine in the nucleus accumbens (NAc) (Koob and Bloom, 1988). Acute (2 hours) HFD activates this pathway (Valdivia et al., 2014), indicating the immediate response to HFD is one of pleasure and reward. Similar activation of the mesolimbic dopamine system is observed after 9 days, but the effect is gone after 16 weeks, of high sugar diet (South

et al., 2012), which indicates decreased rewarding properties of the diet with prolonged consumption, and continued increased feeding may be a resulting effect of a reward-deficient state (Martire et al., 2014). There is a growing trend to discuss the nature of HFD consumption in terms of drug use. Recent advertisements indicate that 'binge-eating disorder' is something you should speak with your doctor about, and high-fat and high-sugar foods are thought to possess addictive-like properties (Morris et al., 2014). However, due to the homeostatic requirement for caloric consumption, the pathways involved in mediating the rewarding impact of food is much more complex and extensive than the mesolimbic system (Baldo and Kelley, 2007). A neurobiological distinction is starting to be made between addictive eating behavior and classic observations of substance abuse, indicating the phenomenon of over-consumption that has become common in western societies, may be due more to an addiction to the behavior of eating, rather than to the food itself (Hebebrand et al., 2014).

While this area of study is still in an early stage, it appears that the mesolimbic circuit does not adequately explain the nature of addictive-like behavior to HFD and high-sugar foods. A recent, rather groundbreaking, paper has demonstrated that neuroinflammation-activation of TLR4 signaling might be the primary regulator of the rewarding effects of cocaine (Northcutt et al., 2015). As discussed extensively, the nature of HFD on mediating neuroinflammation likely involves direct activation of TLR4, such as through signaling by FFA or HMGB1. The resulting impact of HFD-induced TLR4 activation would foster rewarding effects of

consuming HFD, and therefore, might be a primary mechanism facilitating further HFD consumption and obesity development. However this is not yet known.

5.6. Summary

The studies presented here serve to establish that high-fat diet consumption mediates a primed neuroinflammatory environment, which leads to potentiated neuroinflammatory responding to a secondary challenge. Such potentiated neuroinflammation mediates hippocampal memory disruption in a model of HFD-induced obesity, and the same processes involved in mediating a primed neuroinflammatory state may occur upon the onset of HFD consumption. Obesity development from HFD is not required, as an altered neuroimmune phenotype is observed from just 3 days of HFD consumption. The results here serve to elucidate the impact of HFD in mediating neuroinflammation, and are the first to demonstrate a link between diet and HMGB1 in the brain. Furthermore, observations that CORT signaling mediates the pro-inflammatory effects of HFD serve to further support the growing body of literature suggesting CORT regulates pro-inflammatory processes.

6. References

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