Does a High-Fat Diet Cause Inflammation in Female Rat Brain?

by
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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Obesity results from a disruption of normal energy homeostasis, and leads to a state of chronic low-grade inflammation. Given that inflammatory cytokines can disrupt synaptic activity and that obesity has been shown to cause cognitive impairment, I hypothesized that diet-induced obesity may cause an inflammatory response in regions of the brain important for learning and memory. Notably, previous diet-induced obesity studies have tended to focus on male animals, but sexual dimorphism in response to a high-fat diet (HFD) has been suggested; as a result, female rats were randomly assigned to either a control diet (CD; 10% kcal from fat), or a HFD (45% kcal from fat) at post-natal day 28. After 10 weeks, the hippocampus and pre-frontal cortex were extracted from each animal and homogenized. Immunoblotting was used to determine if a HFD affected levels of pro/anti-inflammatory cytokines, glial cells, and proteins involved in leptin signal transduction. A HFD was found to cause enhanced expression of anti-inflammatory IL-1ra in the prefrontal cortex, but no cytokine-related changes in the hippocampus (although levels of the leptin receptor and AKT/PKB were reduced in the hippocampus, but not the pre-frontal cortex). Although the data demonstrate that our protocol did not induce obvious proinflammatory changes, the possibility exists that the elevated IL-1ra levels reflect a compensatory response to an earlier period of inflammation caused by the HFD. Future work should expand on my current findings with the inclusion of male animals to allow for a direct sex comparison of the effects of the HFD. Our model could also be expanded with an immune challenge via lipopolysaccharide injection, or leptin stimulation in order to tease out potential differences not observed under basal conditions. Transgenerational effects should also be explored by examining the effects a maternal HFD may have on the expression of similar proteins in the offspring.

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List of Abbreviations

AKT/PKB = protein kinase B

AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB = blood brain barrier

BDNF = brain derived neurotrophic factor

BMI = body mass index

CD = control diet

CNS = central nervous system

CRP = C-reactive protein

CX3CL1 = fractalkine

ECL = enhanced chemiluminescence

ELISA = enzyme-linked immunosorbent assay

ERα = estrogen receptor alpha

FOXO1 = Forkhead box protein O1

GFAP = glial fibrillary acidic protein

HFD = high-fat diet

HPA = hypothalamic-pituitary-adrenal

Iba-1 = ionized calcium binding adaptor molecule 1

IC = internal control

IGF-1 = insulin-like growth factor 1

IL = interleukin

IRS/PI3K = insulin receptor substrate/phosphoinositide-3-kinase

JAK/ STAT = Janus-kinase/signal transducer and activator of transcription

LTP = long term potentiation

LPS = lipopolysaccharide

MAPK = mitogen-activated protein kinase

MCP-1 = monocyte attractant protein 1

NMDA = N-methyl-D-aspartate

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOCS3 = suppressor-of-cytokine-signaling-3

Ob-R = leptin receptor

OGTT = oral glucose tolerance test

PIC = polyinosinic-polycytidylic acid

PSD-95 = post-synaptic density-95

PVDF = polyvinylidene fluoride

qRT-PCR = quantitative reverse-transcription polymerase chain reaction

TBS-T = tris-base saline with tween-20

TNFα = tumor necrosis factor alpha

TLR = toll-like receptor

TrkB = tropomyosin receptor B

1.0 Introduction

Although the true definition of obesity is often not clear, obesity may be broadly defined as the accumulation of a level of body fat sufficient to cause negative health outcomes (Heshka & Allison, 2001; Allison et al., 2008). Obesity can be operationally defined by utilizing a simple ratio of weight to height, where individuals are considered overweight if they attain a body-mass index (BMI) between 25.0 – 29.9 kg/m^2, and obese if their BMI is 30 kg/m^2, or more (Heshka & Allison, 2001; Allison et al., 2008). Factors such as genetic predisposition play a role in the rise of individuals diagnosed with obesity, but one of the largest environmental factors is likely the chronic overconsumption of the Western diet (foods consisting of high levels of sugar and fat), and reduced physical activity (Kanoski & Davidson, 2011). Collectively, these factors lead to an alteration in normal metabolism and hormonal function, resulting in the early onset of obesity, which then increases one's risk for the development of related co-morbidities, such as type II diabetes mellitus, cardiovascular disease, and cancer (Heshka & Allison, 2001; Allison et al., 2008; Kanoski & Davidson, 2011).

Another important aspect of obesity that is starting to gain attention is its effect upon cognition (Kanoski & Davidson, 2011). In a longitudinal study by Whitmer et al. (2005), overweight middle aged adults were 1.35 times more likely to be diagnosed with dementia compared to their normal weight counterparts, while age matched obese individuals were 1.74 times more likely to be diagnosed. Similarly, Gunstad et al. (2006) found an inverse relationship between BMI and verbal memory performance across the adult lifespan. Obesity-induced cognitive impairments can also be recreated experimentally using animal models, where animals fed a high-fat and/or high-sucrose diet show impairments in a variety of memory related behavioural tasks (Pistell et al., 2010; Beiharz et al, 2014; Boitard et al., 2014). Despite the

significance of diet-induced cognitive impairment, the underlying neurophysiological mechanisms are still poorly understood. The hippocampus and the pre-frontal cortex have been the target of much diet related memory research due to their involvement with long-term/spatial memory formation and cognitive flexibility, as well as their susceptibility to environmental insults (Kanoski & Davidson, 2011; Preston & Eichenbarum, 2013). This thesis document will review the literature regarding how obesity, particularly when caused by a high-fat diet, can affect hippocampal and pre-frontal cortical function. After a description and discussion of the connections between diet-induced obesity and behaviour, experiments were completed to expand our current knowledge about how obesity may affect neuronal function will be provided.

2.0 Literature Review

2.1 Obesity and Brain Function

2.1.1 The Hippocampus and the Pre-frontal Cortex: Structure and Function

The hippocampus is a bilateral limbic structure located in the medial temporal lobe, and can be divided into two major regions: the dentate gyrus, consisting primarily of granule cells, and the *Cornu Ammonis* (CA), consisting primarily of pyramidal cells (Neves et al., 2012). The flow of information within the hippocampus is typically described as a tri-synaptic circuit (Neves et al., 2012). Information travels from the entorhinal cortex into the dentate gyrus through the perforant path. Granule cells in the dentate gyrus project their axons toward the pyramidal neurons in the CA3 region through the mossy fibers, which, in turn, project onto the ipsilateral CA1 pyramidal cells through the Schaffer collaterals (Neves et al., 2012). Afterwards, signals are directed back to the entorhinal cortex, and then to other brain areas (Preston & Eichenbarum, 2013).

The pre-frontal cortex is the region of the cerebral cortex that covers the anterior part of the frontal lobe and receives projections from the hippocampus into its rostroventral region (Preston & Eichenbarum, 2013). Without proper maintenance of the hippocampal-prefrontal cortical neuronal network, dysfunction of either brain region can negatively impact the function of the other (Preston & Eichenbarum, 2013). Therefore, while the hippocampus is primarily responsible for consolidation of information from short term into long term memory and spatial navigation, it also has an indirect role in mediating pre-frontal cortical behaviours, such as executive function, working memory, attentional control, cognitive flexibility, and behavioural inhibition (especially of those related to the reward system) (Neves et al., 2012; Preston & Eichenbarum, 2013). Other brain regions that project to the hippocampus and the pre-frontal cortex include the amygdala and the hypothalamus, suggesting that the hippocampus and the pre-frontal

cortex also serve a role in mediating emotional regulation and the stress response (Kanoski & Davidson, 2011; Neves et al., 2012). See Figure 1 for a schematic map of the connected brain areas.

2.1.2 High-Fat Diet and Animal Behaviour

In order to closely study the effects of obesity on animal cognition, a high-fat diet (HFD) feeding protocol is often used (Kanoski & Davidson, 2011). Animal models have provided evidence of the effects of a HFD on hippocampal-dependent behavioural tasks, most commonly tested through spatial memory performance on the Morris water maze, or a novel place recognition task (Mielke et al., 2006; Pistell et al., 2010; Beiharz et al., 2014; Boitard et al., 2014). Beilharz et al. (2014), for example, were able to show that short term exposure to a HFD affects mainly the hippocampus in male Sprague-Dawley rats, based on behavioural impairment being observed in a place recognition task, where time spent interacting with a familiar object placed in a novel location was measured. On the other hand, diet had no effect on performance in the novel object recognition task, where animals were measured on time spent interacting with a novel object in a familiar location (Beilharz et al., 2014). Further evidence for the effects of a HFD was found by Boitard et al. (2014), who demonstrated that HFD impaired long term memory in male Wistar rats after 1-2 months of exposure when tested via the probe trials of the Morris water maze, indicating impairments in the ability to recall a previously acquired spatial learning memory.

Similar deficits were found with HFD-fed (60% of calories from fat) mice used in a study conducted by Pistell et al. (2010), where significantly more errors were observed in male mice in the Stone T-maze, another test for spatial ability and cognition. However, no effect was seen when this group used a less intensive HFD (41% of calories from fat with an additional 29% of calories from sucrose) (Pistell et al. 2010). Male C57BL/6 mice used by Mielke et al (2006) showed no

significant differences in performance in the water maze during the memory acquisition phase (indicated by the animal's ability to learn where a hidden platform is in the Morris water maze) after 5 months and 10 months on the diet, suggesting that the hippocampus was not affected. On the other hand, mice fed the HFD were unable to complete an operant-learning task, which was as an alternate measure of hippocampal memory acquisition where animals needed to learn to press a bar to gain access to their food (Mielke et al., 2006).

Pre-frontal cortical-dependent behaviours are most commonly tested through reversal training, where animals must learn to extinguish a previously learnt behaviour in order to acquire a new one (Kanoski et al., 2007). Evidence for pre-frontal cortical dependent behaviours can be seen in the results from Kanoski et al. (2007) and their discrimination reversal task where trained male Sprague-Dawley rats had to learn to respond to a new conditioned stimulus. In their study, animals fed a HFD supplemented with dextrose over a 90 day period performed significantly worse compared to animals on the control diet (CD). Interestingly, these deficits were abolished when animals had restricted access to their food, instead of *ad libitum* access (Kanoski et al., 2007). Although, in general, animals given a HFD perform worse in behavioural tasks than CD animals, the variability between feeding protocols, makes the results difficult to compare. See Table 1 for a summary of several different protocols used by different authors.

In order to gain a better understanding of the potential mechanisms underlying the effect obesity has on hippocampal and pre-frontal cortical function, researchers often accompany behavioural experiments with various biochemical tests to probe for changes in factors that can directly, or indirectly, impact these brain regions. However, the link between diet-induced cognitive deficits and specific biomarkers is weak, since obesity has been connected to many physiological conditions that can have a negative impact on the brain (Kanoski & Davidson, 2011;

Neves et al., 2012). The majority of the literature has focused on processes that impair synaptic plasticity, such as reduced neurogenesis, neuroinflammation, and altered hormonal signaling in the form of insulin and leptin resistance (Kanoski & Davidson, 2011; Miller & Spencer, 2014). Together, these cognitive impairments can act as both a cause and a consequence of obesity; that is, with reduced hippocampal and pre-frontal cortical function, individuals may become more prone to obesity-related behaviours, such as greater food intake, overeating, and less physical activity (Myers et al, 2006; Kanoski & Davidson, 2011).

2.2 Obesity and Inflammation

2.2.1 Obesity and Chronic Inflammation

Obesity has been characterized as a state of low-grade chronic inflammation (Monteiro & Azevedo, 2010), which, in turn, can have a number of effects on cognitive function (Lumeng & Saltiel, 2011; Miller & Spencer, 2014). There are a variety of factors that can contribute to obesity-induced inflammation, one of which is the accumulation of saturated fatty acids leading to greater activation of Toll-like receptors 2 and 4 (TLR2; TLR4), which normally detect pathogens and activate the innate immune system upon detecting foreign substances, such as lipopolysaccharides (LPS) from Gram-negative bacteria (Lumeng & Saltiel, 2011; Miller & Spencer, 2014). Saturated fatty acids appear to act on TLR2 and TLR4 in a manner similar to LPS (Lumeng & Saltiel, 2011; Miller & Spencer, 2014). Upon activation, TLRs can lead to greater metabolic stress, especially in the endoplasmic reticulum, which results in higher production of unfolded proteins and mitochondrial dysfunction (Lumeng & Saltiel, 2011; Miller & Spencer, 2014). With the accumulation of reactive oxygen species and inflammation, cells become damaged and are lost from excessive TLR stimulation (Monteiro & Azevedo, 2010; Kanoski & Davidson, 2011; Lumeng & Saltiel, 2011; Miller & Spencer, 2014).

2.2.2 Inflammatory Cytokines

TLR activation can also initiate the production of inflammatory cytokines. Cytokines are a group of low-molecular weight, non-antibody polypeptides that mediate cellular intercommunication via autocrine, paracrine, or endocrine mechanisms (Wilson et al, 2002; Lyman et al. 2014). Various cells can secrete cytokines, but their transcription is highly controlled and they bind only to specific receptors to form complex networks with a high degree of pleiotropism (Wilson et al., 2002; Lyman et al., 2014). Cytokines may be classified based on their role in the body: proinflammatory, anti-inflammatory, or involved in hematopoiesis (Wilson et al., 2002; Lyman et al., 2014). Chemokines, such as monocyte attractant protein 1 (MCP-1), are a subset of cytokines with chemotactic properties responsible for recruiting immune cells to the site of inflammation (Wilson et al., 2002; Lyman et al., 2014). Through the communication of various cytokines, a number physiological changes, both locally and systemically, can be induced by mediating the innate and adaptive arms of the immune system to produce a suitable response to the threat at hand (Wilson et al., 2002; Lyman et al., 2014).

Expression of proinflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα), are usually initiated by detection and recognition of a foreign particulate, such as LPS, or tissue damage (Wilson et al., 2002; Lyman et al., 2014). Notably, although IL-6 is mostly regarded as a proinflammatory cytokine, alternate signaling properties of IL-6 can also induce anti-inflammatory effects (classical signaling rather than trans-signaling of IL-6), making IL-6 a pleiotropic cytokine (Scheller et al., 2011). Stimulation induces the rapid synthesis of cytokines, which are packaged into secretory vesicle or endosomes by the endoplasmic reticulum-Golgi complex, and then released by means of conventional secretion, shedding of plasma microvesicles, or cell lysis (Stanley & Lacy, 2010; Lopez-Castejon & Brough,

2011). However, other cytokines like IL-1, which are initially synthesized in an inert pro-form, may require further processing, like proteolytic cleavage to its mature active form, before being secreted (Stanley & Lacy, 2010; Lopez-Castejon & Brough, 2011). Collectively, the proinflammatory cytokines secreted result in physiological changes, such as vasodilation, swelling, clotting to contain the particulate, recruitment of additional immune cells, and activation of immune cells to clear the foreign particulate (Wilson et al., 2002; Lyman et al., 2014). Upon clearing the foreign substance, inflammation is then diminished by anti-inflammatory cytokines, such as interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra), which inactivate the immune cells to return the body to a state of homeostasis and reduce damage to nearby cells that may occur through excessive immune activity and reactive oxygen species production (Wilson et al., 2002; Lyman et al., 2014). Since the inflammatory process is highly regulated, subtle shifts in cytokine expression can have dramatic effects on the body (Wilson et al., 2002; Lyman et al., 2014). Obese individuals, for example, typically display a greater presence of basal proinflammatory cytokines, such as IL-6, TNFα, and MCP-1, and elevated levels of acute phase proteins, such as C-reactive protein (CRP), indicating a tonic-grade activation of the immune system (Martin et al., 2008; Monteiro & Azevedo, 2010; Lyman et al., 2014).

During the progression of obesity, enlarged adipocytes become another contributing factor to the state of inflammation. In addition to their role following infection and injury, IL-1, IL-6, and TNFα are also common adipokines; cytokines which are secreted by adipocytes and not solely by immune cells (Myers et al., 2007; Martin et al., 2008). As the level of adipose tissue grows, due to the overconsumption of a HFD, so too do the levels of proinflammatory cytokines secreted into the circulation (Monterio & Azevedo, 2010; Lumeng & Saltiel, 2011). The proinflammatory state is accompanied by an enhanced migration of macrophages and monocytes into the white adipose

tissue, and leads to greater communication between immune cells and metabolic cells (Lumeng & Saltiel, 2011). In fact, an estimated 20-30 million macrophages accumulate in each kilogram of excess fat in humans, and these can lead to downstream effects, such as dysfunction in normal insulin and leptin signaling, which can promote lipid storage and suppress lipid metabolism (Lumeng & Saltiel, 2011). Expanding adipocytes are also more prone to rupture, leading to the release of reactive oxygen species, resulting in even greater production of IL-6, TNFα, and CRP, thus perpetuating the state of inflammation (Monteiro & Azevdeo, 2009). As a result, in response to a HFD, the immune system will become chronically stimulated, resulting in systemic inflammation that can lead to disruption and damage to nearby cells that further deteriorates the health of the obese individual (Monteiro & Azevdeo, 2009; Lumeng & Sailtiel, 2014).

2.2.3 Obesity and Neuroinflammation

Considering the link between obesity and systemic inflammation, it is not surprising that inflammation can also affect the central nervous system (CNS) in obese individuals (Cai, 2013; Miller & Spencer, 2014). Normally, the blood-brain barrier (BBB), a specialized microvascular endothelium, limits the entry of many blood components in the periphery from entering the CNS (Cai, 2013; Miller & Spencer, 2014). However, the integrity of the BBB has been shown to be compromised during intake of high-energy diets, especially in aged obese animals (Kanoski et al., 2010; Tucsek et al., 2014). Immune factors derived from diet-induced systemic inflammation, such as inflammatory cytokines, prostaglandin, and CRP, can contribute to increased permeability of the BBB by altering tight junctions in endothelial cells in the brain vasculature (Lyman et al., 2014; Miller & Spencer, 2014; Hsuchou et al., 2012). Inflammatory cytokines from the periphery can also be transported into the brain through circumventricular organs, regions of the brain that are highly vascularized and lack a complete BBB (Lyman et al., 2014; Miller & Spencer, 2014;

Hsuchou et al., 2012). With the BBB compromised, leukocytes and inflammatory cytokines from the periphery can readily enter the brain to initiate a proinflammatory cascade (Lyman et al. 2014; Miller & Spencer, 2014). As well, saturated fatty acids can act on the TLR4 of microglia and astrocytes to elicit an immune response similar to that caused by LPS interaction with TLR4s in the periphery (Lyman et al. 2014; Miller & Spencer, 2014). Activated glial cells, primarily stimulated microglia, secrete inflammatory cytokines, nitric oxide, and prostaglandin converted from arachidonic acid to further promote the state of inflammation and endoplasmic reticulum stress, even at the neuronal level (Lyman et al. 2014; Miller & Spencer, 2014).

Neuroinflammation due to cytokine production and recruitment of peripheral macrophages can disrupt normal physiological processes in the CNS (Lyman et al. 2014; Miller & Spencer, 2014). Neurons in the midst of a proinflammatory milieu can experience unregulated and excessive activity, resulting in excitoxicity and neurodegeneration (Yirmiya & Goshen, 2011). For example, as a result of HFD, inflammation of the hypothalamus is linked to hypothalamic-pituitary-adrenal (HPA) axis dysfunction, which, in turn, disrupts normal feeding and metabolic regulation via disruption of insulin and leptin signaling in the periphery (Cai, 2013; Lyman et al., 2014; Miller & Spencer, 2014). Neuroinflammation can also extend into the hippocampus and pre-frontal cortex, causing gliosis and neuronal damage (Miller & Spencer, 2014). For example, ionized calcium-binding adaptor molecule 1 (Iba-1) is commonly used as a marker for microgliosis and is involved with the remodeling of the actin cytoskeleton that allows for motility, proliferation, and phagocytosis in activated microglia (Imai & Kohsaka, 2002). Glial fibrillary acidic protein (GFAP), on the other hand, is used as a marker for astrogliosis as it is the main intermediate filament protein expressed in mature astrocytes and is involved in cell motility, proliferation, vesicle trafficking, and controlling neuronal outgrowth (Middledorp & Hol, 2011). Over time,

chronic activation of microglia can lead to abnormal synaptic remodeling as microglia serve a role in synaptic pruning via fractalkine (CX3CL1) mediated phagocytosis while hypertrophy of reactive astrocytes can disrupt neuronal networks through the formation of compact glial scar tissues (Palicelli et al. 2011; Sofroniew & Vinter, 2010).

Inflammation mediated by cytokines is also a hallmark of cognitive dysfunction that negatively impacts neuronal plasticity (McAfoose & Baune, 2009). For example, proinflammatory IL-1β has been shown to have an inverted "U" relationship with hippocampal dependent memory and synaptic plasticity. At low physiological levels, IL-1 expression can assist with the maintenance of long term potentiation (LTP), an example of synaptic plasticity in neurons, and a cellular model for learning and memory (Goshen et al., 2007; McAfoose & Baune, 2009). However, both insufficient IL-1 signaling and high concentrations of IL-1, such as those seen with aging, stress, and disease, can cause inhibition of both LTP and fear conditioning memory (Goshen et al., 2007; McAfoose & Baune, 2009). Similarly, pleiotropic IL-6 appears to have a role governing LTP by opposing the action of IL-1 (Balschun et al., 2003; McAfoose & Baune, 2009). Under physiological conditions, IL-1 and IL-6 oppose one another in terms of LTP maintenance, as a means to fine-tune long-term plasticity and hippocampal dependent learning (Balschun et al., 2003; McAfoose & Baune, 2009). However, an elevation of IL-6 is highly correlated with age-related memory disturbance (Yirmiya & Goshen, 2011).

Gene expression data taken from the hippocampus and the pre-frontal cortex collected by Boitard et al (2014), revealed no differences between IL-1β, IL-6, or TNFα expression at a basal level between CD and HFD animals. However, when given an immune challenge (intraperitoneal LPS injections), animals on the HFD were more sensitive and displayed enhanced production of IL-1β and TNFα relative to CD rats; notably, this enhanced immune response was not observed in

adult animals, suggesting that there is a window of vulnerability to the adverse effects of HFD on hippocampal function (Boitard et al., 2014). Baumgarner et al. (2014) revealed the opposite trend where HFD mice were less sensitive to LPS induced expression of IL-1β and IL-6 mRNA in the hippocampus, suggesting that the impairments in spatial memory they also observed in these animals were likely not due to alterations in the immune system. Instead, they found that HFD mice showed significantly reduced brain derived neurotrophic factor (BDNF), synaptophysin, and post-synaptic density-95 (PSD-95) mRNA expression, indicating a reduction in neurotrophic support and impaired synaptic activity (Baumgarner et al., 2014). Even with the absence of an immune challenge, Pistell et al. (2010) showed that as one raises the fat content from 41% of calories to 60% of calories, basal protein levels of GFAP, Iba-1, TNFα, IL-6, and MCP-1 become elevated in the whole brain, accompanied by reduced BDNF protein expression in HFD mice relative to their CD counterparts. While the study conducted by Kanoski et al. (2007) did not assess cytokines, they found evidence of how an energy dense diet can negatively impact the hippocampal-prefrontal cortical circuitry, which they attribute to a neuroinflammatory mechanism after finding reduced protein expression of BDNF in the ventral hippocampus and the pre-frontal cortex.

2.3 Obesity and Leptin Resistance

2.3.1 Leptin and its Receptor (Ob-R)

Concurrent with the onset of inflammation in obese individuals is the development of leptin resistance (Otero et al., 2005; Martin et al., 2008; Irving & Harvey, 2014). Leptin is a 16-kDa polypeptide hormone primarily produced by white adipose tissue (Otero et al., 2005; Martin et al., 2008). Leptin and its receptor, Ob-R, are encoded by the *ob* gene and they serve a role in mediating energy homeostasis (Otero et al., 2005; Martin et al., 2008; Irving & Harvey, 2014). As one eats,

the accumulation of excessive calories and fatty acids in adipose tissue releases adipokines, such as leptin, to regulate and maintain a normal body weight (Otero et al., 2005; Martin et al., 2008; Irving & Harvey, 2014).

The Ob-R is comprised of six different isoforms (Ob-Ra-f), each derived from alternative mRNA splicing, resulting in identical leptin binding domains in the N-terminus, but different intracellular C-terminal domains (Myers et al, 2007; Zhou & Rui, 2013; Irving & Harvey, 2014). The isoforms of Ob-R can be further divided into three categories: long form (Ob-Rb), short form (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf), and secretory form (Ob-Re). Signal transduction is primarily mediated by Ob-Rb since it is the only isoform with the full-length intracellular domain required to mediate downstream signaling (Zhou & Rui, 2013; Irving & Harvey, 2014). Two major signaling cascades originating from Ob-Rb are the Janus kinase-2 (JAK2)/Signal Transducer and Activator of Transcription (STAT3) pathway and the insulin receptor substrate (IRS)/phosphoinositide-3-kinase (PI3K) pathway. In response to leptin, proximal membrane associated JAK2 phosphorylates tyrosine residue 1138 on the C-terminus of the Ob-Rb to cause the subsequent phosphorylation of STAT3 (Zhou & Rui, 2013; Irving & Harvey, 2014). Phosphorylated-STAT3 (p-STAT3) then influences the expression of genes, which alters energy expenditure, food intake and other related leptin-induced anti-obesity effects (Zhou & Rui, 2013; Irving & Harvey, 2014). Alternatively, in the IRS/PI3K signaling pathway, leptin induced phosphorylation of IRS results in phosphorylation of PI3K, which then acts on protein-kinase B (AKT/PKB) (Zhou & Rui, 2013; Irving & Harvey, 2014). AKT/PKB can then phosphorylate the Forkhead box protein O1 (FOXO1) transcription factor that acts to maintain nutritional homeostasis (Zhou & Rui, 2013; Irving & Harvey, 2014). See Figure 2 for a schematic of the aforementioned Ob-Rb signaling pathways.

Although the short forms of Ob-R are also involved with JAK/STAT signaling pathways, the majority of action from these receptors is primarily involved with leptin internalization and degradation (Gorska et al., 2010). Secretory Ob-Re acts as a buffering system for free circulating leptin in the blood, where it can either inhibit or enhance leptin signaling by delivering the bound leptin to the membrane bound isoforms to facilitate signaling (Gorska et al., 2010). At basal conditions, only about 5-25% of all isoforms of Ob-Rs are on the cell surface while the rest are within the cells (Gorska et al., 2010). The Ob-Rb and Ob-Ra isoforms, in addition to facilitating signal transduction, are internalized where they are degraded or recycled and returned to the cell surface after ligand binding (Gorska et al., 2010).

2.3.2 Chronic Inflammation and Leptin Resistance

During the onset of obesity, there are a number of factors that can contribute to the impairment of normal leptin signaling (Li et al, 2006; Martin et al., 2008). Firstly, proinflammatory cytokines produced from the accumulated adipocytes stimulate the liver to synthesize CRP, which can act as a serum-leptin-interacting protein that can bind to serum leptin (Li et al, 2006; Martin et al., 2008). Interestingly, leptin itself also promotes MCP-1 and CRP production in the liver by Kupffer and hepatic satellite cells, leading to even further impairments in leptin signaling (Li et al, 2006; Martin et al., 2008). Leptin bound to CRP has reduced bioavailability and bioreactivity with Ob-Rb, ultimately resulting in reduced leptin receptor signaling, despite higher concentrations of circulating leptin (Li et al, 2006; Martin et al., 2008). Leptin could also be restricted from accessing Ob-Rb in the brain due to reduced expression of Ob-Ra, which is known to support the movement of leptin across the BBB (Li et al, 2006; Martin et al., 2008). As the body is exposed to chronic levels of high caloric intake and leptin production, Ob-Rb signaling may also be diminished through reduced trafficking of Ob-Rb as a compensatory mechanism (Li et al, 2006; Martin et al.,

2008). Lastly, production of adipocyte-secreted inflammatory cytokines, such as IL-1 and IL-6, can also exacerbate the state of leptin resistance through interruption of downstream receptor signaling mediated by the JAK/STAT pathway with the expression of intracellular suppressor-of-cytokine-signaling-3 (SOCS3) (Li et al, 2006; Martin et al., 2008). SOCS3 is normally produced as a negative feedback system in response to activation of the JAK/STAT pathway, but, in the case of inflammation, SOCS3 may be abnormally upregulated, resulting in an impaired leptin signaling process (Li et al, 2006; Martin et al., 2008). As a result, leptin resistance and inflammation continue to feed off of one another, resulting in many of the metabolic deficits that occur during obesity (Li et al, 2006; Martin et al., 2008).

2.3.3 Leptin Resistance and Synaptic Plasticity

The expression of the Ob-R is not limited to the hypothalamus; the Ob-R is also abundantly expressed on hippocampal and pre-frontal cortical neurons, and, as such, leptin resistance caused by obesity may modulate synaptic activity (Harvey et al, 2006; Irving & Harvey 2014). More specifically, the Ob-R has been shown to be expressed at the pre- and post-synaptic sites in the CA1 region of the hippocampus (Harvey et al., 2006). Excitatory signals among neurons are regulated primarily by glutamatergic signaling, especially through the ionotropic glutamate receptor subtype known as the N-methyl-D-aspartate (NMDA) receptor (Neves et al., 2012). Activation of NMDA receptors is critical for the induction of LTP (Neves et al., 2012). In genetic models, animals lacking functional Ob-Rs have impaired LTP in the CA1 region of the hippocampus, as well as impairments in spatial memory (Harvey et al, 2006; Irving & Harvey 2014). As well, leptin administered to the hippocampus of rats resulted in improved memory performance, and promoted the conversion of short lasting potentiation into LTP (Oomura et al, 2006; Harvey et al, 2006; Irving & Harvey 2014).

As noted, leptin activity is facilitated by PI3K, as well as the mitogen-activated-protein kinase (MAPK) signaling (Oomura et al, 2006; Harvey et al, 2006; Harvey, 2013; Irving & Harvey 2014). In adults, the PI3K signaling pathway enhances the activity of GluN2A subunits of the NMDA receptor to promote rapid intracellular calcium cation influx (Harvey, 2013). In turn, this cation influx promotes the trafficking of another glutamate receptor, the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, to the synapse to promote long-lasting synaptic efficacy and enhanced LTP (Moult & Harvey, 2011). Moult & Harvey (2011) showed that bath application of leptin to adult rodent hippocampal slices resulted in increased cell surface expression of AMPA receptor GluA1 subunits. Surface expression of AMPA receptor GluA2 subunits is also enhanced upon leptin application in the hippocampus, but a much higher concentration is required (Moult & Harvey, 2011). Together, these events also resulted in greater excitatory post-synaptic currents upon high frequency stimulation (Moult & Harvey, 2011). In addition, through the MAPK pathway, the growth of dendritic spines is stimulated by the rapid alteration in actin filaments in hippocampal neurons, which, in turn, lays the foundation to support continued LTP (Harvey et al, 2006; Irving & Harvey 2014). As such, with the development of leptin resistance, maintenance of synaptic plasticity may be impaired, which could lead to cognitive impairment (Oomura et al, 2006; Harvey et al, 2006; Harvey, 2013; Irving & Harvey 2014).

In relation to the effects of leptin resistance on the hippocampus as a result of diet-induced obesity, Valladolid-Acebes et al. (2013) found that 5 week old male C57BL/6L mice subjected to 8 weeks of HFD experienced impaired hippocampal p-AKT/PKB signaling, but no difference in p-STAT3 signaling, relative to the CD animals, when given an acute intraperitoneal leptin administration. However no detriments were found between the groups when 8 week old male

mice were subjected to 8 weeks of the feeding protocol (Valladolid-Acebes et al., 2013). The age related difference was attributed to the varying stage of neuronal development and neurogenesis, suggesting that younger mice were more vulnerable to the effects of a HFD, while more drastic conditions may be required to develop the same degree of impairment in adults (Valladolid-Acebes et al., 2013).

The results were comparable to Lu et al. (2011), who were able to show that a feeding protocol of 20 weeks with male mice resulted in a decrease in basal p-AKT/PKT protein expression and increased basal p-STAT3 protein expression relative to CD animals in the hippocampus even without leptin stimulation. Interestingly, the effects of a HFD in this study were mediated by a 20 week oral administration of ursolic acid, an antioxidant with anti-inflammatory properties (Lu et al., 2011). Similarly, Camer et al. (2015) were also able to show a basal reduction in p-AKT/PKB protein expression along with reduced expression of BDNF and its related signaling components in both the hippocampus and the pre-frontal cortex in 12 week old male mice fed a HFD, providing further evidence that impaired leptin signaling can negatively impact synaptic plasticity. Like the previous study, the deficits they observed in their HFD feeding protocol were attenuated by co-administration of another antioxidant with anti-inflammatory properties (bardoxoline methyl), indicating the important role of neuroinflammatory components earlier described (Camer et al., 2015).

2.4 Sexual Dimorphism in Response to a High-Fat Diet

Neurophysiological studies that focus on biochemical mechanisms often examine phenomena in male animals only, creating a sex bias in the field (Beery & Zucker, 2011). While the characterization of diet-induced obesity on cognitive processes is still developing, this area of research is no exception (refer to Table 1). Importantly, sexual dimorphism exists in terms of the

development of obesity (Lovejoy et al., 2008; Palmer & Clegg, 2015). Men and post-menopausal women tend to accumulate fat in their abdominal depots, while pre-menopausal women tend to accumulate fat in their gluteal-femoral and subcutaneous depots (Lovejoy et al., 2008; Palmer & Clegg, 2015). In addition to differences in fat distribution, the fat mass developed in these different areas also exhibit different characteristics (Lovejoy et al., 2008; Palmer & Clegg, 2015). Visceral adipose tissue typically puts individuals more at risk of metabolic dysregulation and cardiovascular disease, as the hypertrophy of fat cells displays impaired leptin sensitivity and greater immune activation via elevated proinflammatory cytokine production (Lovejoy et al., 2008; Palmer & Clegg, 2015). In contrast, the expansion of subcutaneous adipose tissue via hyperplasia of adipocyte progenitor cells results in smaller adipocytes with greater sensitivity to insulin and leptin (Lovejoy et al., 2008; Palmer & Clegg, 2015). As a result, accumulation of subcutaneous adipose tissue actually exerts a protective effect against the metabolic irregularities that often result from diet-induced obesity (Lovejoy et al., 2008; Palmer & Clegg, 2015).

Differences between men and women in their fat development is mainly mediated by the gonadal hormone estrogen (Monteiro et al., 2014; Palmer & Clegg, 2015). Estrogen attenuates the accumulation of fat primarily through estrogen receptor alpha (ERα) by promoting lipolysis and enhancing adipose tissue sympathetic nerve activity (Monteiro et al., 2014; Palmer & Clegg, 2015). As a result, differential distribution of ERα from neurons projecting into different fat depots results in the differences in fat build-up in different areas of the body between men and women (Monteiro et al., 2014; Palmer & Clegg, 2015). Estrogen also enhances energy expenditure by converting white adipose tissue to beige brown fat through the production of natriuretic peptides, which, in turn, results in adipose tissue that is more metabolically active (Monteiro et al., 2014; Palmer & Clegg, 2015). Lastly, estrogen deters the development of the low-grade inflammation

typically seen in obese individuals by mediating the production of hypoxia-inducible factor and preventing macrophage recruitment in the adipose tissue (Monteiro et al., 2014; Palmer & Clegg, 2015).

Due to the accumulated evidence for the protective effects of estrogen against obesity, it is therefore possible to assume that a HFD would affect male and female brains differently. Notably, Hwang et al. (2010) were able to demonstrate male animals fed a HFD were more susceptible to metabolic alterations, deficits in hippocampal-dependent learning, and impairments in the strength of LTP generated in the CA1 region of their hippocampus, relative to female animals. Sexual dimorphism in the response to HFD on synaptic plasticity was attributed to the lack of estrogen and ERα activity in the male animals providing protection against the effects of diet-induced obesity (Hwang et al., 2010). Jayarman et al. (2014) also highlighted the role of testosterone in neuroinflammation and neuronal viability as glial cultures from gonadectomized male mice displayed the greatest expression of TNFα and IL-1β mRNA expression when given a HFD. However, whether the neuroprotective effect of testosterone observed in this study was due to activity on its androgen receptor, or conversion into estradiol through aromatases as a means to mediate the inflammation remains unclear (Jayarman et al., 2014; Monterio et al., 2014). Taken together, results from these studies highlight the importance of gonadal hormone differences in response to a HFD, and how the brains of female and male animals are clearly affected differently by a HFD (Jayarman et al., 2014; Monterio et al., 2014).

Table 1: A Summary of High-Fat Diet Feeding Protocols

Authors	Species	Sex	Age of Animals (start of diet)	High Energy Dietary Intervention	Duration of Feeding
Baumgarner et al. (2014)	C57BL/6J mice	Male	14 weeks	60% calories from fat	2-5.5 months
Beilharz et al. (2014)	Sprague-Dawley rats	Male	Not specified	45% calories from fat with a 10% sucrose solution	20-29 days
Boitard et al. (2014)	Wistar rats	Male	3-12 weeks	45% calories from fat	2-4 months
Camer et al. (2015)	C57BL/6J mice	Male	12 weeks	Half lard, half sunflower oil	21 weeks
Hwang et al. (2010)	C57BL/6J mice	Both	3 weeks	Not specified	8-11 months
Kanoski et al. (2007)	Sprague-Dawley rats	Male	3 months	40% calories from fat with either sucrose and dextrose supplement	3 months
Kanoski et al. (2010)	Sprague-Dawley rats	Male	2 months	40% calories from fat	3-4 months
Lu et al. (2011)	C57BL/6J mice	Male	1 month	60% calories from fat	20 weeks
Mielke et al. (2006)	C57BL/6J mice	Male	Not specified	45% calories from fat	12 months
Pistell et al. (2010)	C57BL/6J mice	Male	12 months	41-60% calories from fat	12-21 weeks
Tucsek et al. (2014)	C57BL/6J mice	Male	7-24 months	60% calories from fat	5 months
Valladolid-Acebes et al. (2013)	C57BL/6J mice	Male	5-8 weeks	45% calories from fat	2 months
Average Age of Animals (start of diet)			**5.42 months ± 7.26**	**Average Duration of Feeding**	**5.25 months ± 3.24**

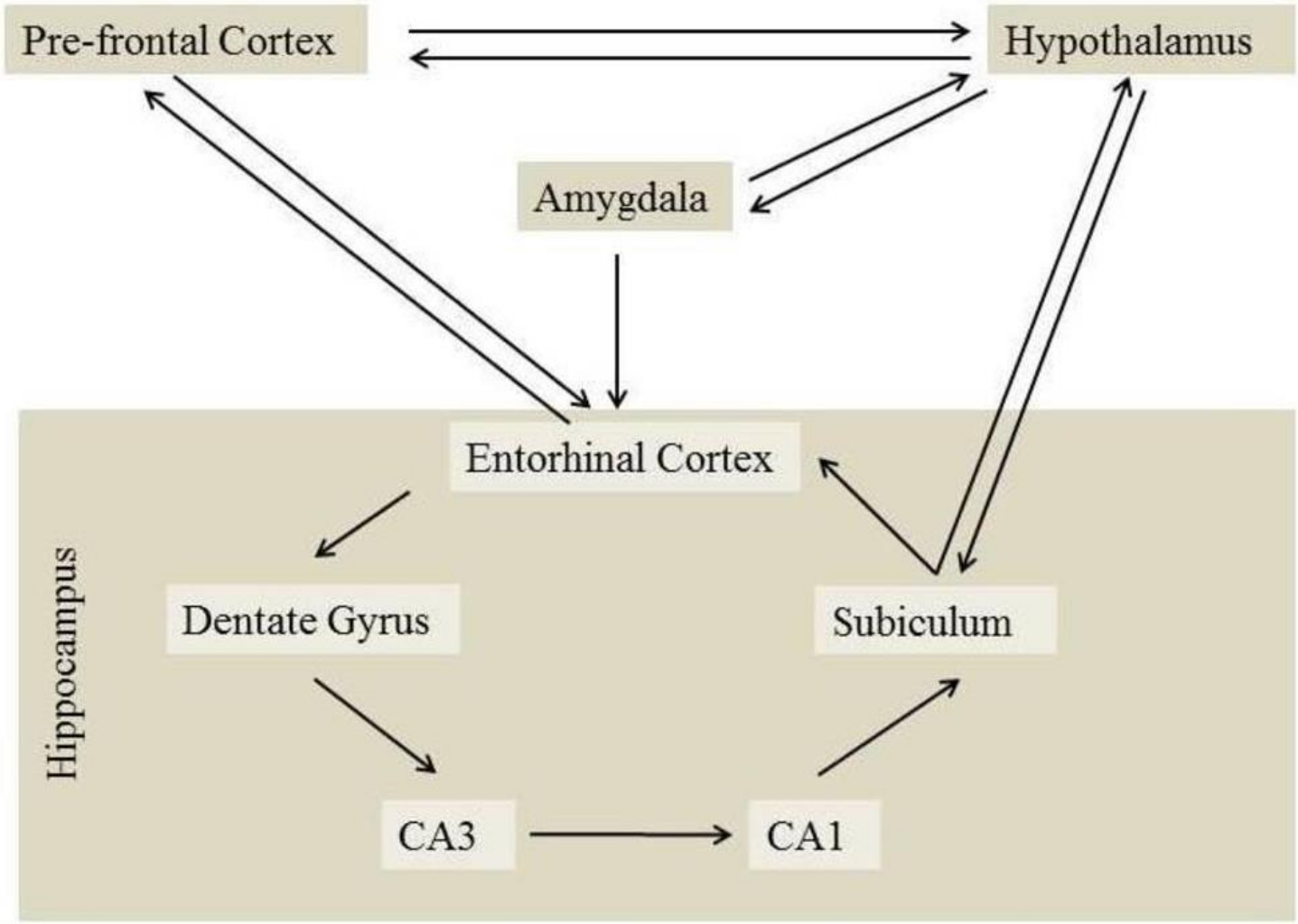

Figure 1: Map of Brain Areas Connected with the Hippocampus.

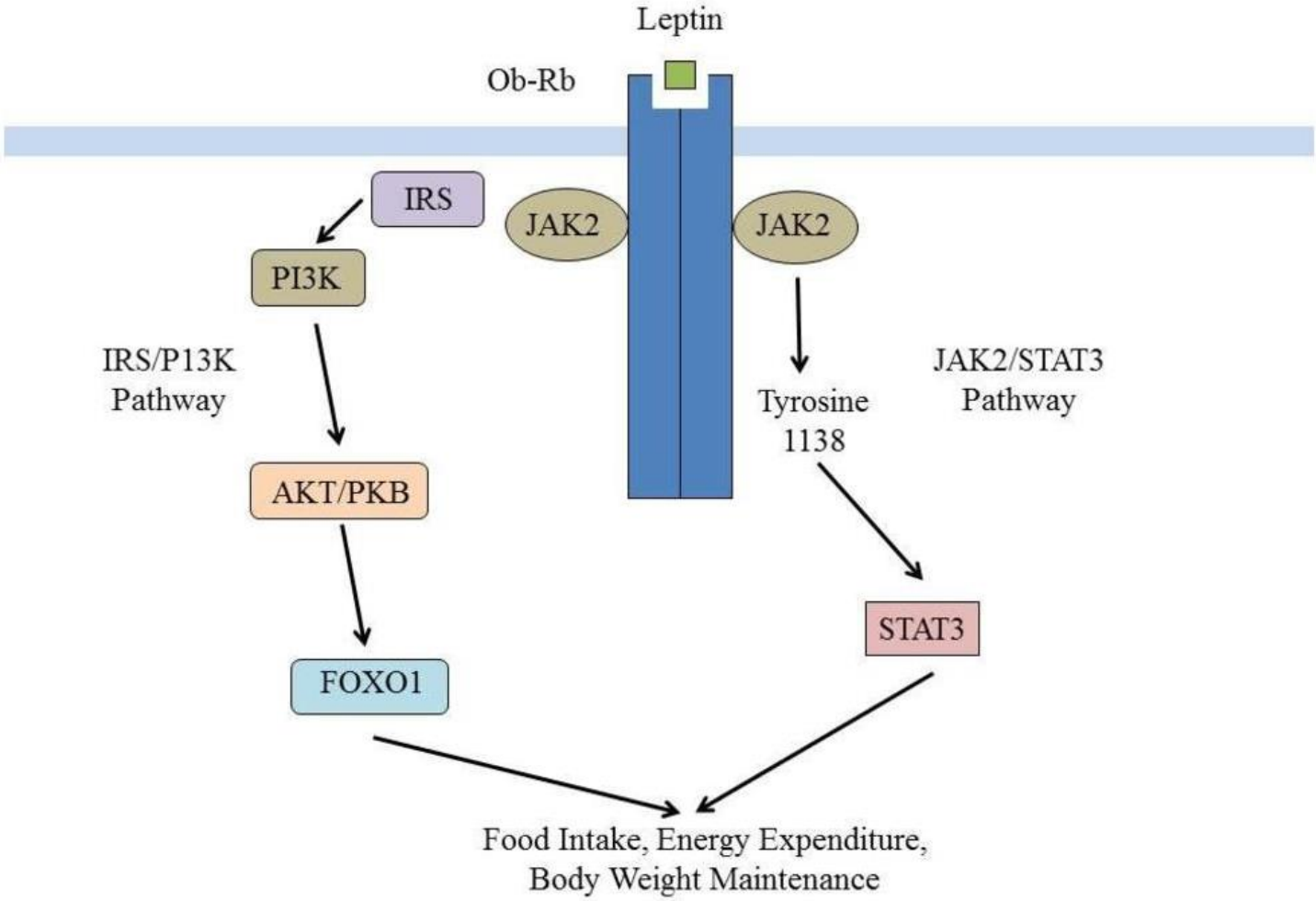

Figure 2: Ob-Rb Signal Transduction.

3.0 Study Rationale:

In light of the molecular data illuminating the effects of an obesogenic diet on hippocampal and pre-frontal cortical function (Pistell et al., 2010; Valladolid-Acebes et al., 2013; Baumgarner et al., 2014; Boitard et al., 2014), I hoped to expand on what is known by examining the effects of a high-fat diet on markers of neuroinflammation and leptin signaling. In order to explore the sexual dimorphism in response to diet that is often neglected in neurophysiological studies, female animals were the focus of my experiments.

I set the following objectives:

1) Determine the effects of a high-fat diet on gliosis in female rat hippocampus and pre-frontal cortex. GFAP acted as a marker of reactive astrocytes, while Iba-1 acted as a marker for activated microglia.
2) Determine the effects of a high-fat diet on neuroinflammation in female rat hippocampus and pre-frontal cortex. In order to accurately survey the inflammatory milieu in the brain, a wide selection of cytokines was chosen: IL-1β was selected as a proinflammatory cytokine, IL-6 was selected as a pleiotropic cytokine, and IL-1ra and IL-10 were used as anti-inflammatory cytokines.
3) Determine the effects of a high-fat diet on cellular expression of proteins involved with leptin signal transduction (Ob-R, AKT/PKB, p-AKT/PKB, STAT3) in female rat hippocampus and pre-frontal cortex. Since impaired leptin signaling may be due to either the reduced expression of Ob-R, or impaired transduction via the IRS/PI3K, or JAK/STAT pathways, levels of p-AKT/PKB, AKT/PKB and STAT3 were used as downstream markers of Ob-R signaling.

Based on the reviewed literature, I hypothesized that hippocampi and pre-frontal cortices from obese female rats would display elevated markers of neuroinflammation and gliosis. As a result, our animals would also exhibit reduced phosphorylation, or expression of signaling proteins involved with leptin signal transduction.

4.0 Materials and Methods

4.1 Animals and Diets

Experiments with animals were carried out in accordance with the guidelines for animal use issued by the University of Waterloo Animal Care Committee according to the protocols outlined by the Canadian Council of Animal Care. Sixty non-sibling female Sprague-Dawley rat pups were delivered at post-natal day 21 with foster mothers. On post-natal day 28, animals were randomly allocated to one of two feeding protocols for 10 weeks: a high-fat diet (HFD: n = 30; Catalogue #D12451, Research Diets Inc., New Brunswick, NJ, USA) consisting of 45% calories from saturated fat, or a control diet (CD: n = 30; Catalogue #D12450K, Research Diets Inc., New Brunswick, NJ, USA). See Table 2 for the full composition of the two diets. Animals were group housed (3 animals per cage), given *ad libitum* access to their respective diets and water, and were housed under a standard 12 hour light/dark cycle at a constant room temperature (23°C). Over the course of the experiments, body weights and food weights were recorded on a weekly basis. See Figure 3 for a summary of the feeding and experimental protocols.

4.2 Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed on a subset of animals (CD: n = 20; HFD: n = 20) after the first and second months of the diet by tracking the blood glucose levels from the tail vein over a course of 2 hours at 30 minute intervals following an oral gavage of a glucose bolus (2 g/kg of body weight).

4.3 Sacrifice and Terminal Biometrics

Around post-natal day 98, half of the experimental animals (CD: n = 15; HFD: n = 15) were anesthetized with CO_2 and terminal body weights and naso-anal lengths were recorded. After euthanizing animals via decapitation, additional terminal biometric measurements (liver weight,

adrenal gland weight, spleen weight, and retroperitoneal fat pad weight) were recorded. Brain tissues were quickly extracted in artificial cerebrospinal fluid [124.0 mM NaCl, 3.0 mM KCl, 1.2 mM NaH_2PO_4/H_2O, 1.0 mM $MgSO_4/7H_2O$, 2.0 mM $CaCl_2/2H_2O$, 26 mM $NaHCO_3$, 10 mM D-Glucose, and 10 mM HEPES, equilibrated with carbogen (95% O2/5% CO2), pH 7.37-7.43]. Hippocampal and pre-frontal cortical tissues were then manually homogenized in non-ionizing lysis buffer [10 mM NaCl, 25 mM EDTA, 10 mM Tris, 1% (v/v) Triton X-100, 1% (v/v) NP-40] supplemented with a protease inhibitor cocktail [AEBSF, aprotinin, bestatin hydrochloride, E-64, leupeptin hemisulfate salt, and pepstatin A] and then centrifuged at 1000 x g for 10 minutes. Supernatants were then kept at -80°C until the point of analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration of homogenates was determined using a BioRad DC protein assay kit and comparing the optical density of homogenates with a known concentration of bovine serum albumin.

4.4 Immunoblotting Analysis

Frozen hippocampal and pre-frontal cortical homogenates were thawed, and a total of 40 µg of each homogenate was used for SDS-PAGE. Samples were denatured in an equal volume of sample buffer [125 mM Tris, 10% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) beta-mercaptoethanol, 0.002% bromophenol blue, pH 6.8] at 95°C for 5 minutes before being separated electrophoretically (200 V at room temperature for 1 hour) with either a 10%, or 15% SDS-polyacrylamide gel, depending on the molecular weight of the protein of interest. Afterwards, proteins were blotted onto polyvinylidene fluoride (PVDF) membranes via wet transfer (35 V at 4°C overnight). Each membrane was then stained with Ponceau S solution to confirm that the protein transfer was successful, and then blocked with 5% (w/v) non-fat milk/tris-buffered saline

with Tween-20 (TBS-T) [20 mM Tris, 138 mM NaCl, 1% (v/v) Tween-20, pH 7.60] for 1 hour at room temperature.

In order to probe for markers of gliosis, membranes were processed for GFAP (mouse monoclonal anti-GFAP, EMD Millipore, United States; 1:1000) and Iba-l (rabbit polyclonal anti-Iba-1, Abcam PLC, United Kingdom; 1:200). For markers of neuroinflammation, membranes were processed for the proinflammatory cytokine IL-1β (rabbit polyclonal anti-IL-1β, Santa Cruz Biotechnology, United States; 1:200), pleiotrophic cytokine IL-6 (mouse monoclonal anti-IL-6, Santa Cruz Biotechnology; 1:200), and anti-inflammatory cytokines IL-1ra (rabbit polyclonal anti-IL-1ra, Santa Cruz Biotechnology; 1:200) and IL-10 (mouse monoclonal anti-IL-10, Santa Cruz Biotechnology; 1:200). For markers of leptin signaling, membranes were probed for Ob-R (mouse monoclonal anti-Ob-R, Santa Cruz Biotechnology; 1:200), STAT3 (rabbit polyclonal anti-STAT3, Santa Cruz Biotechnology; 1:200), and p-AKT/PKB (mouse monoclonal anti-p-AKT/PKB (Ser 473) EMD Millipore; 1:1000), which was then reprobed for total AKT/PKB (mouse monoclonal anti-AKT/PKB, EMD Millipore; 1:1000). All blots were incubated with primary antibodies at 4°C overnight, followed by an incubation of 1 hour at room temperature with the appropriate enzyme-linked secondary antibody (goat anti-rabbit, or anti-mouse, EMD Millipore; 1:2000). All solutions were prepared in 5% non-fat milk/TBS-T. Immunoblots were then incubated with enhanced chemiluminescence (ECL) substrate to activate the secondary antibody. SYNGENE was used to detect the luminescence with exposure lengths ranging from 30 seconds to 10 minutes. Optical density measurements were quantified using Adobe Photoshop CS6. In the event where a second trial was required for a specific blot due to distortion of a protein band, the usable data were averaged across the trials.

4.5 Statistical Analysis

The data collected were stored in Microsoft Excel. Calculations were conducted in order to determine adjusted biometric measurements, such as BMI (body weight divided by naso-anal length^2), percent liver weight (liver weight/body weight x 100%), percent spleen weight (spleen weight/body weight x 100%), percent adrenal gland weight (adrenal gland/body weight x 100%), spleen weight (spleen weight/body weight x 100%) and percent retroperitoneal fat pad weight (percent retroperitoneal fat pad weight/body weight x 100%). Body weight, BMI, liver weight, spleen weight, adrenal gland weight, and retroperitoneal fat pad weight were compared across dietary conditions using a two way unpaired Student's *t* test in Graph Pad Prism.

Background-subtracted optical density measurements for each band of interest were normalized to the total protein expression in each respective lane of an immunoblot (viewed with Ponceau S straining) with the exception of p-AKT/PKB expression, which was normalized to total AKT/PKB. A ratio was then constructed for each band by normalizing to non-experimental cortical tissue taken from CD males (that is, the internal control; IC). The protein expression ratios were then used to compare the total hippocampal and pre-frontal cortical protein expression between the two dietary conditions using a Student's unpaired *t* test in Microsoft Excel. In the event that an extreme outlier was suspected, Grubb's test was applied. Differences with $p < 0.05$ were considered significant.

Table 2: Diet Composition

	CD (D12450K)		HFD (D12451)	
Composition	%gram	%kcal	%gram	%kcal
Protein	19.2	20	24	20
Carbohydrate	67.3	70	41	35
Fat	4.3	10	24	45
Total	-	100	-	100
kcal/gram	3.85	-	4.73	-
Ingredients	gram	kcal	gram	kcal
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	550	2200	72.8	291
Maltodextrin 10	150	600	100	400
Sucrose	0	0	172.8	691
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	177.5	1598
Mineral Mix	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2	0	2	0
Red Food Dye	0.025	0	0.05	0
Blue Food Dye	0.025	0	0	0
Total	1055.05	4057	858.15	4057

CD = control diet; HFD = high-fat diet

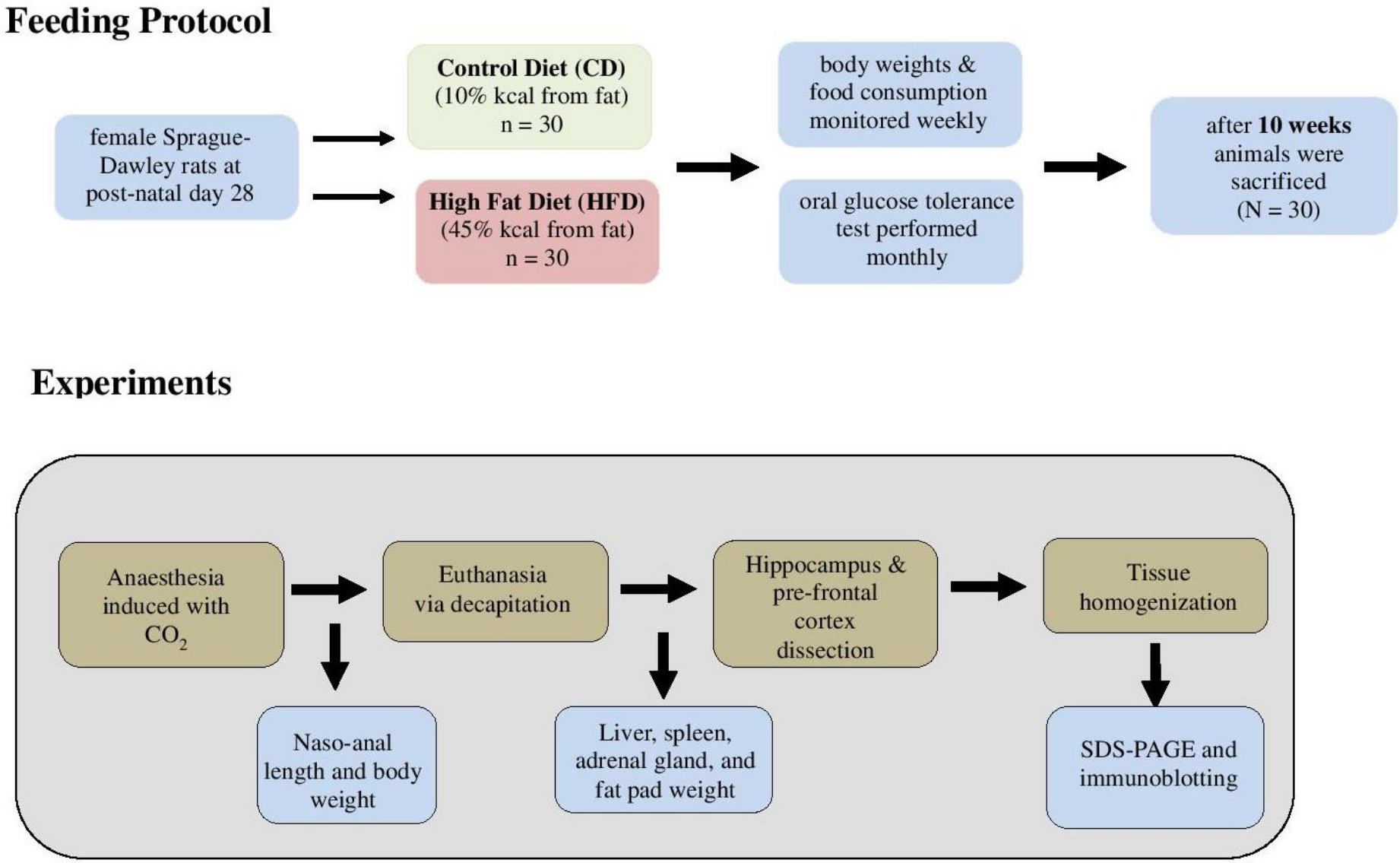

Figure 3: Summary of Experimental Protocol.

5.0 Results

5.1 Body Weights and Food Intake

Mean food weights and body weights were monitored for all animals (CD: n = 30 animals; HFD: n = 30 animals) weekly over the course of the 10 week feeding period. No significant differences were found at the beginning of the experiment between HFD and CD animals (Figure 4; $t_{(58)} = 0.54$, $p = 0.5945$). HFD animals had a significantly higher body weight than CD animals at week 1 ($t_{(58)} = 1.44$, $p < 0.01$), week 2 ($t_{(58)} = 3.00$, $p < 0.01$), week 3 ($t_{(58)} = 2.12$, $p < 0.05$), week 4 ($t_{(58)} = 2.07$, $p < 0.05$), week 6 ($t_{(58)} = 2.00$, $p < 0.05$), week 8 ($t_{(58)} = 2.06$, $p < 0.05$), and week 10 ($t_{(58)} = 2.11$, $p < 0.05$).

In terms of food consumption (CD: n = 10 cages; HFD: n = 10 cages; 3 animals/cage), relative to CD animals, the HFD animals ate significantly more food at week 1 (Figure 5A; $t_{(18)} = 3.53$, $p < 0.01$) and then ate significantly less food at week 3 ($t_{(18)} = 4.99$, $p < 0.01$), week 5 ($t_{(18)} = 3.92$, $p < 0.01$), week 6 ($t_{(18)} = 3.30$, $p < 0.01$), week 8 ($t_{(18)} = 3.35$, $p < 0.01$), and week 9 ($t_{(18)} = 4.94$, $p < 0.01$). In contrast, in terms of caloric intake, HFD animals consumed a significantly greater number of calories at week 1 (Figure 5B; $t_{(18)} = 7.76$, $p < 0.01$), week 3 ($t_{(18)} = 2.43$, $p < 0.05$), week 4 ($t_{(18)} = 3.47$, $p < 0.01$), week 5 ($t_{(18)} = 2.72$, $p < 0.05$), week 6 ($t_{(18)} = 2.14$, $p < 0.05$), week 7 ($t_{(18)} = 2.57$, $p < 0.05$), week 8 ($t_{(18)} = 3.90$, $p < 0.01$), and week 9 ($t_{(18)} = 3.41$, $p < 0.01$).

5.2 Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed on a subset of animals from each diet after the first (CD: n = 18; HFD: n = 19) and second months of the diet (CD: n = 18; HFD: n = 18). Although the intended sample size was 20 animals per condition, due to unforeseen complications, an OGTT was not performed on two CD animals in month 1. Of the remaining animals, one animal of the HFD was excluded as a significant outlier. Similarly, OGTT was not

performed on two CD animals and two HFD animals in month 2. No significant differences between blood glucose levels was seen prior to the glucose bolus (Figure 6A; $t_{(35)} = 1.36$, $p = 0.1777$). Thirty minutes after glucose gavage, the HFD animals displayed a significantly higher blood glucose relative to CD animals ($t_{(35)} = 3.50$, $p < 0.01$; Effect Size = 1.17), which normalized by 60 minutes ($t_{(35)} = 1.71$, $p = 0.0967$) and remained stable at 90 minutes ($t_{(35)} = 0.28$, $p = 0.7824$) and 120 minutes ($t_{(35)} = 0.59$, $p = 0.5599$). After the second month of the diet, no significant differences were seen prior to the glucose bolus (Figure 6B; $t_{(34)} = 0.11$, $p = 0.9132$). After glucose gavage, HFD animals displayed a significantly elevated blood glucose level after 30 ($t_{(34)} = 3.16$, $p < 0.01$, Effect Size = 1.05) and 60 ($t_{(34)} = 2.11$, $p < 0.05$, Effect Size = 0.71) minutes, which normalized by 90 minutes ($t_{(33)} = 0.30$, $p = 0.7642$) and remained stable at 120 minutes ($t_{(34)} = 1.92$, $p = 0.0637$).

5.3 Terminal Biometric Analysis

Aggregated data from terminal biometrics collected from a subset of animals (CD: n = 15; HFD: n = 15) showing body weights, BMI, liver weight, spleen weight, adrenal gland weight, and retroperitoneal fat pad weight can be found in Figure 7(A) to (F), respectively. No significant differences were found between CD and HFD animal body weight (Figure 7A; $t_{(28)} = 1.59$, $p = 0.1234$), BMI (Figure 7B; $t_{(28)} = 0.46$, $p = 0.6511$), liver weight (Figure 7C; $t_{(28)} = 0.14$, $p = 0.8945$), spleen weight (Figure 7D; $t_{(28)} = 1.55$, $p = 0.1320$), or adrenal gland weight (Figure 7E; $t_{(28)} = 0.37$, $p = 0.7162$). However, relative to CD animals, HFD animals had heavier retroperitoneal fat pads (Figure 7F; $t_{(28)} = 2.64$, $p < 0.05$, Effect Size = 0.97).

5.4 Immunoblotting Housekeeping Data

5.4.1 Transfer Efficiency

To confirm that my electroblotting conditions were sufficient to effectively transfer the proteins separated in the polyacrylamide gel onto the PVDF membranes, a Coomassie Brilliant Blue stain was applied onto the gels after the transfer. As seen in Figure 8, a wet transfer of 35 V for 16 hours at 4^{o}C revealed limited protein staining throughout the gel, indicating that most proteins (with the exception of extremely high molecular weight ones) were removed from the polyacrylamide gel. A Ponceau S stain was also applied to the PVDF membrane after transfer. As seen in Figure 8, the transfer conditions were determined to be suitable, for the majority of proteins were transferred onto the PVDF membrane.

5.4.2 Optimal Protein Concentration for Immunoblotting

In order to determine the amount of protein that would produce a clear signal without saturation, each antibody was characterized by probing against hippocampal tissue over a series of different protein concentrations. See Figure 9 for two sample protein loading characterization blots conducted for IL-1β and p-AKT/PKB. Based on the results from all the antibodies, 40 µg was determined to be generally optimal.

5.4.3 Iba-1 Troubleshooting

Although Iba-1 was intended as a marker for microgliosis, despite examining a number of antibodies provided by various companies, a reliable signal could not be determined. Mouse monoclonal antibodies for Iba-1 from EMD Millipore (Catalogue #MABN92) and Santa Cruz (Catalogue #sc-32725) failed to yield a detectable signal even after loading up to 50 µg of tissue homogenate. A mouse monoclonal antibody (Catalogue #ab15690) and a rabbit polyclonal antibody (Catalogue #ab108539) for Iba-1 from Abcam were able to yield a detectable signal upon

loading 40 µg of tissue homogenate. However, when tested along with BV2, a microglial cell line used as a positive control, and MCF-7, a breast cancer cell line used as a negative control, the antibodies showed an unclear banding pattern (Figure 10). As a result, the decision was made to forgo probing for Iba-1 in the tissue samples.

5.5 Hippocampal and Prefrontal Cortical Biochemical Analysis

5.5.1 Expression of Gliosis Markers

To assess astrogliosis, a ratio of hippocampal (CD: n = 14; HFD: n = 15) and pre-frontal cortical (CD: n = 10; HFD: n = 10) protein expression of GFAP relative to an internal control (IC; non-experimental cortical tissue) was constructed. No significant differences were found between CD and HFD animals in either the hippocampus (Figure 11A; $t_{(27)} = 0.61$, $p = 0.5465$), or the pre-frontal cortex (Figure 11B; $t_{(18)} = 0.75$, $p = 0.4631$).

5.5.2 Expression of Neuroinflammatory Markers

To assess neuroinflammation, a ratio of hippocampal (CD: n = 13-14; HFD: n = 15) and pre-frontal cortical (CD: n = 9-10; HFD: n = 10) protein expression of IL-1β, IL-6, IL-ra, and IL-10 relative to IC was constructed. In the hippocampus, there were no significant differences in the expression of IL-1β (Figure 12A; $t_{(26)} = 0.83$, $p = 0.4162$), IL-6 (Figure 12C; $t_{(27)} = 0.60$, $p = 0.5550$), IL-1ra (Figure 13A; $t_{(27)} = 1.73$, $p = 0.0945$), or IL-10 (Figure 13C; $t_{(27)} = 0.30$, $p = 0.7690$) between CD and HFD animals. In the pre-frontal cortex, there was a significant increase in expression of IL-1ra (Figure 13B; $t_{(17)} = 2.62$, $p < 0.05$, Effect Size = 1.31) in HFD animals relative to CD animals. However there were no significant differences in the expression of IL-1β (Figure 12B; $t_{(18)} = 0.44$, $p = 0.6668$), IL-6 (Figure 12D; $t_{(18)} = 0.76$ $p = 0.4596$), or IL-10 (Figure 13D; $t_{(18)} = 1.65$, $p = 0.1171$) between CD and HFD animals.

5.5.3 Expression of Leptin Signaling Markers

To assess components of the leptin signaling pathway, a ratio of hippocampal (CD: n = 14; HFD: n = 15) and pre-frontal cortical (CD: n = 9-10; HFD: n = 10) protein expression of Ob-R, AKT/PKB, p-AKT/PKB, and STAT3 relative to IC was constructed. In the hippocampus, HFD animals displayed a significant reduction in the expression of Ob-R (Figure 14A; $t_{(27)} = 2.26$, $p > 0.05$, Effect Size = 0.84) and AKT/PKB (Figure 15A; $t_{(28)} = 2.03$, $p < 0.05$, Effect Size = 1.00) relative to the CD animals. However, no significant differences were found in the expression of STAT3 (Figure 14C; $t_{(27)} = 0.74$, $p = 0.4638$) and p-AKT/PKB (Figure 15C; $t_{(27)} = 0.74$, $p = 0.4638$) between CD and HFD animals. In the pre-frontal cortex, no significant differences were found in the expression of Ob-R (Figure 14B; $t_{(17)} = 1.08$, $p = 0.2973$), STAT3 (Figure 14D; $t_{(18)} = 1.52$, $p = 0.1448$), AKT/PKB (Figure 15B; $t_{(18)} = 0.44$, $p = 0.9657$), and p-AKT/PKB (Figure 15D; $t_{(18)} = 0.45$, $p = 0.6572$) between CD and HFD animals.

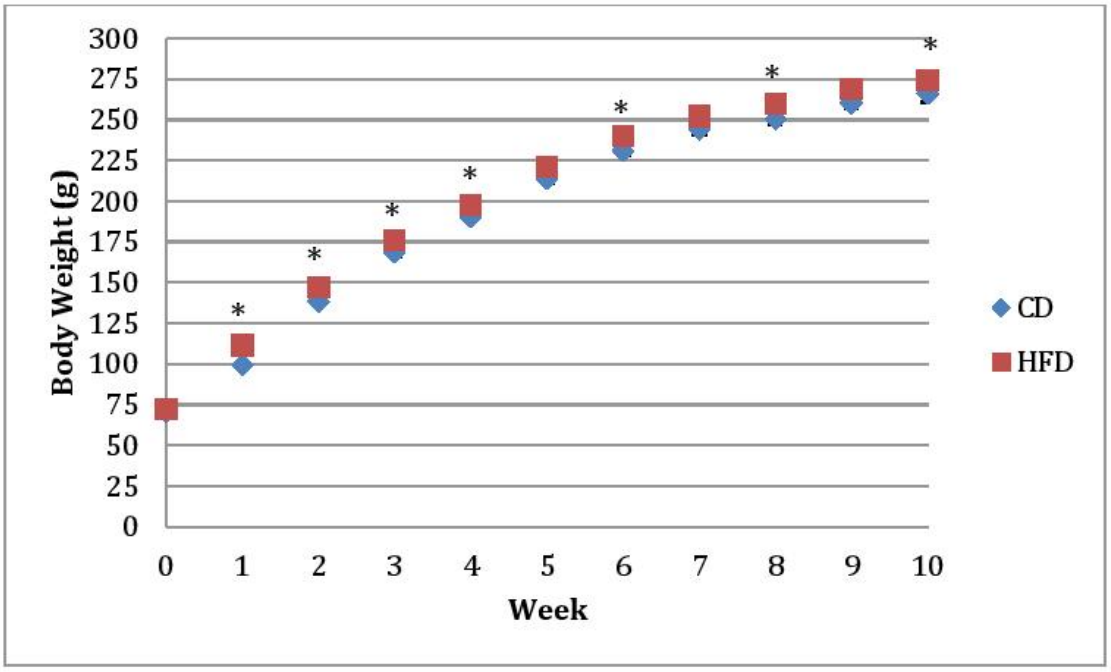

Figure 4: Longitudinal Body Weights. Data presented in graph are mean ± SEM for N = 30. CD = control diet; HFD = high-fat diet, *$p < 0.05$ using Student's *t* test.

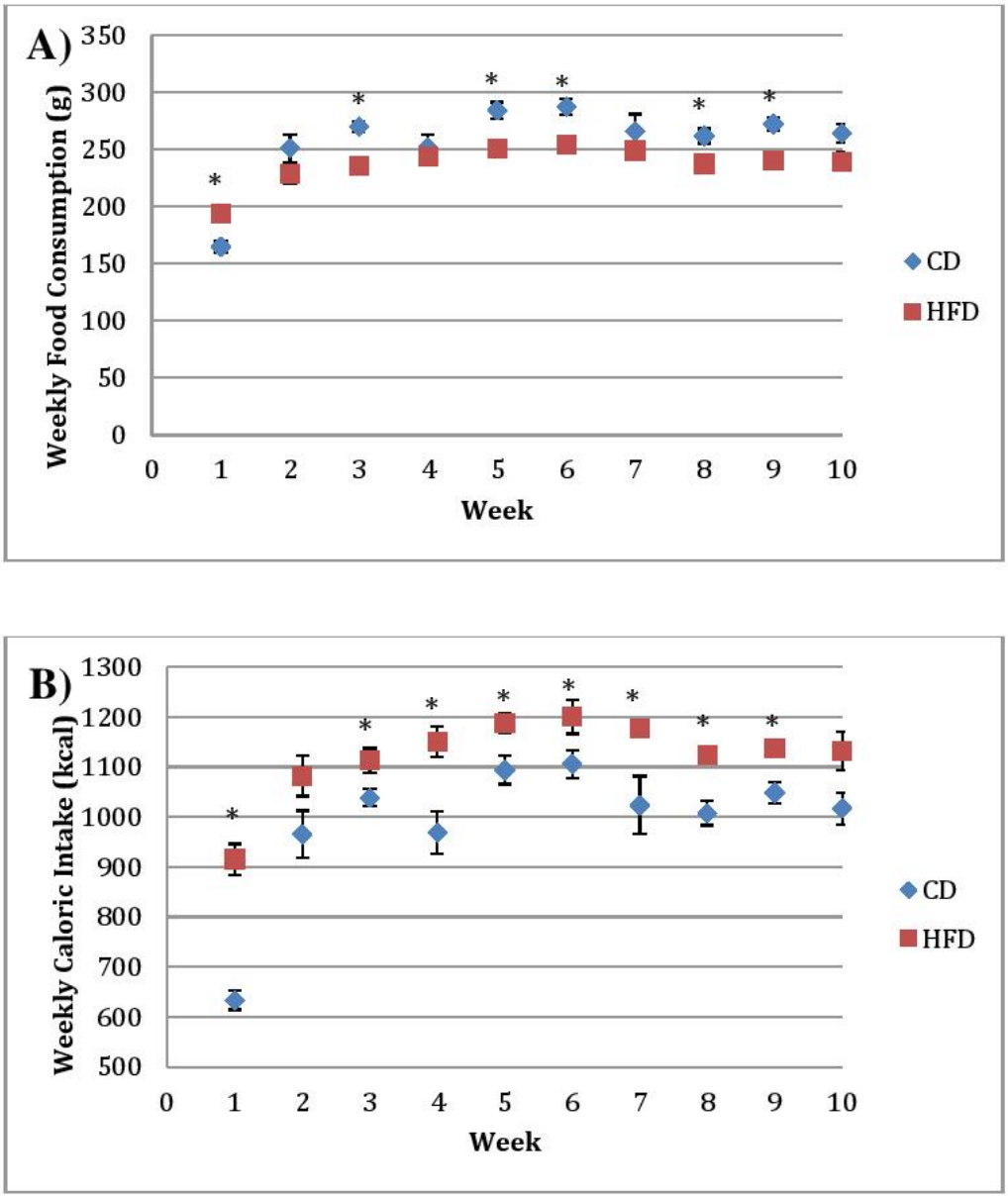

Figure 5: Weekly Food Consumption (A) and Weekly Caloric Intake (B). Data presented in each graph are mean ± SEM for N = 10 cages; 3 animals/cage. CD = control diet; HFD = high-fat diet, *$p < 0.05$ using Student's *t* test.

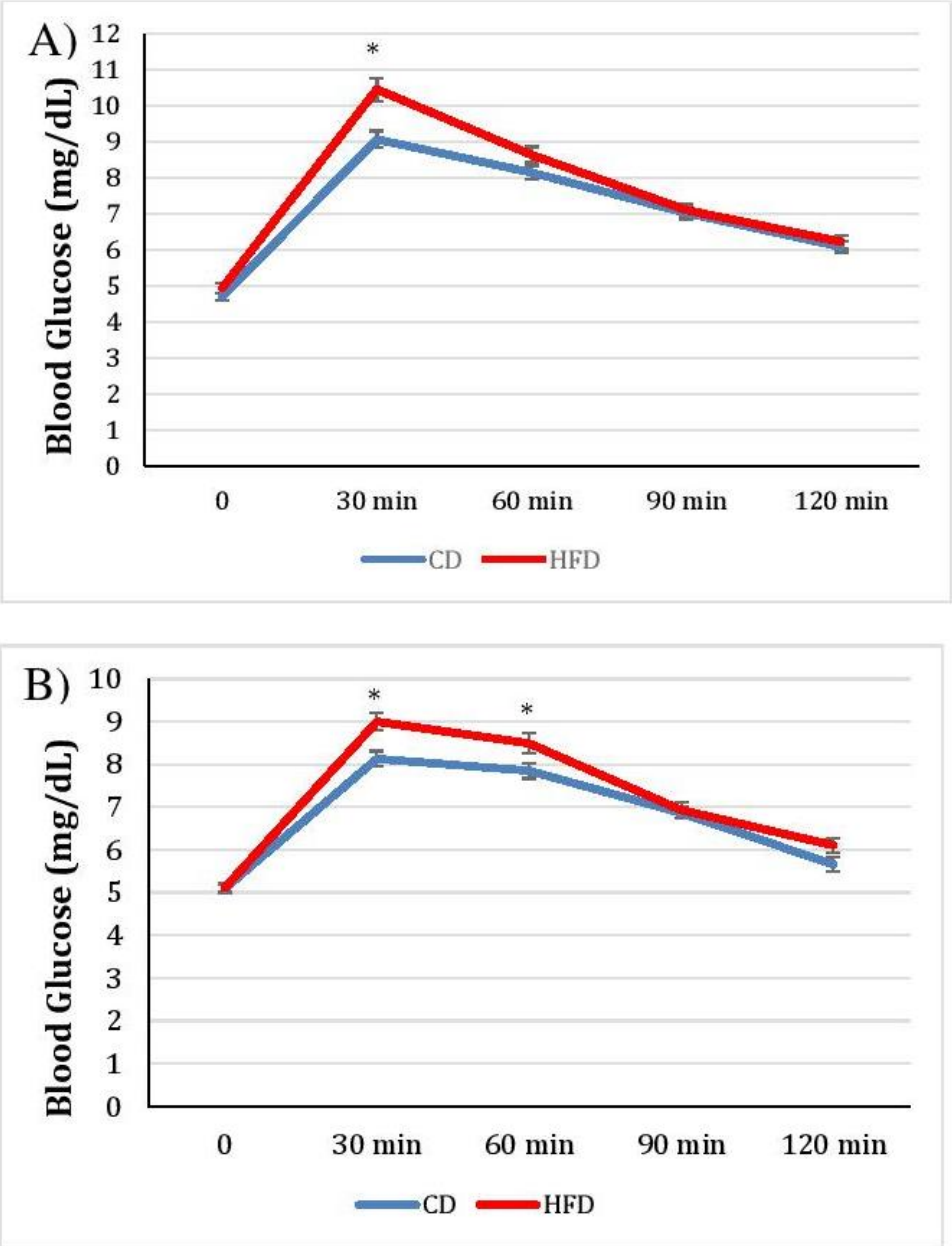

Figure 6: Oral Glucose Tolerance Test. After 1 month, animals fed the HFD displayed significantly elevated blood glucose after oral gavage of a glucose bolus (2 g/kg of body weight; A). By the second month of the diet, blood glucose over the two hour post-bolus period was significantly elevated after 30 minutes and 60 minutes (B). Data presented in each graph are mean ± SEM for N = 18 - 19. CD = control diet; HFD = high-fat diet, *$p<0.05$ using Student's *t* test.

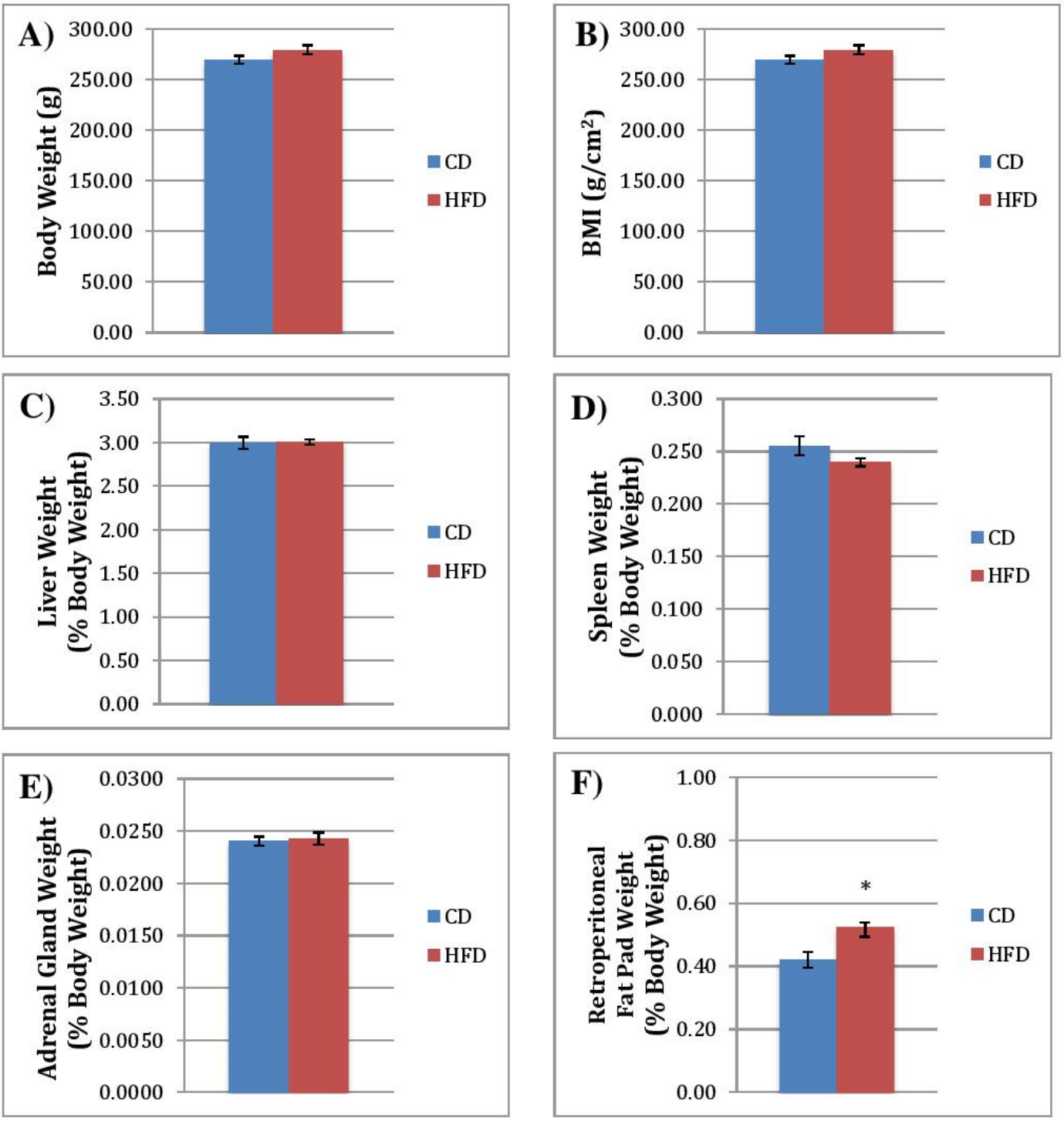

Figure 7: Terminal Biometric Analysis. No effect of dietary condition was observed on body weight (A), body-mass index (BMI; B) liver weight relative to body weight (C), spleen weight relative to body weight (D), or adrenal gland weight relative to body weight (E). HFD animals displayed a significant increase in retroperitoneal fat pad weight relative to CD animals (F). Data presented in each graph are mean ± SEM for N = 15 animals per condition. CD = control diet; HFD = high-fat diet, *$p<0.05$ using Student's *t* test.

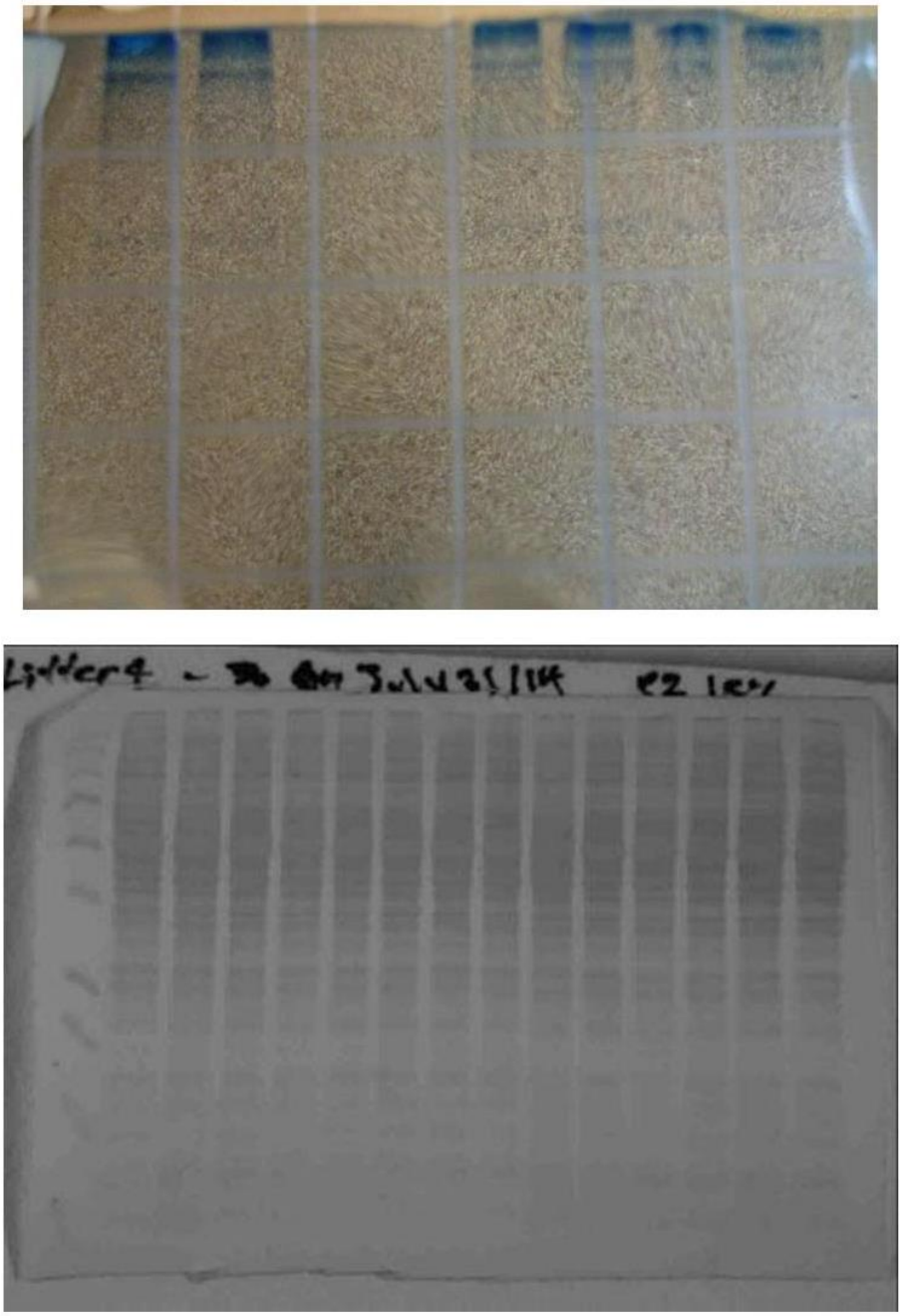

Figure 8: Transfer Efficiency. Transfer conditions were tested via Coomassie Brilliant Blue staining on the polyacrylamide gel (top) and Ponceau S staining on the PVDF membrane (bottom). Results revealed that a transfer of 35 V for 16 hours at 4°C efficiently transfered the proteins of interest from the gel onto the membrane.

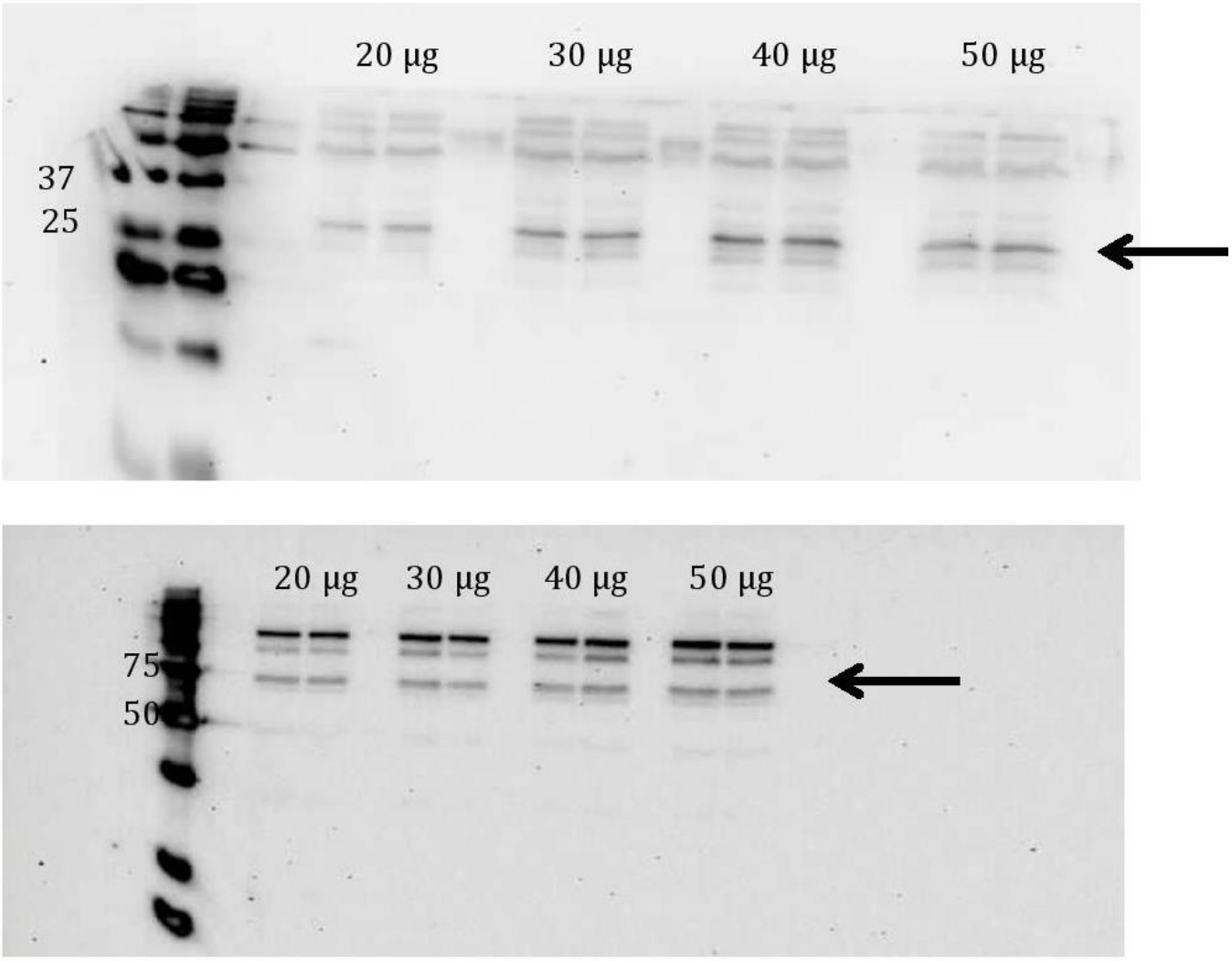

Figure 9: Protein Loading Characterization Blots. A range of 20-50 µg of hippocampal homogenate was loaded for IL-1β (top) and p-AKT/PKB immunoblotting (bottom) to determine the optimal protein concentration to load. A similar process was conducted for all the other antibodies, and it was determined that 40 µg of protein yielded the best signal. Arrows indicate estimated molecular weights of proteins under study.

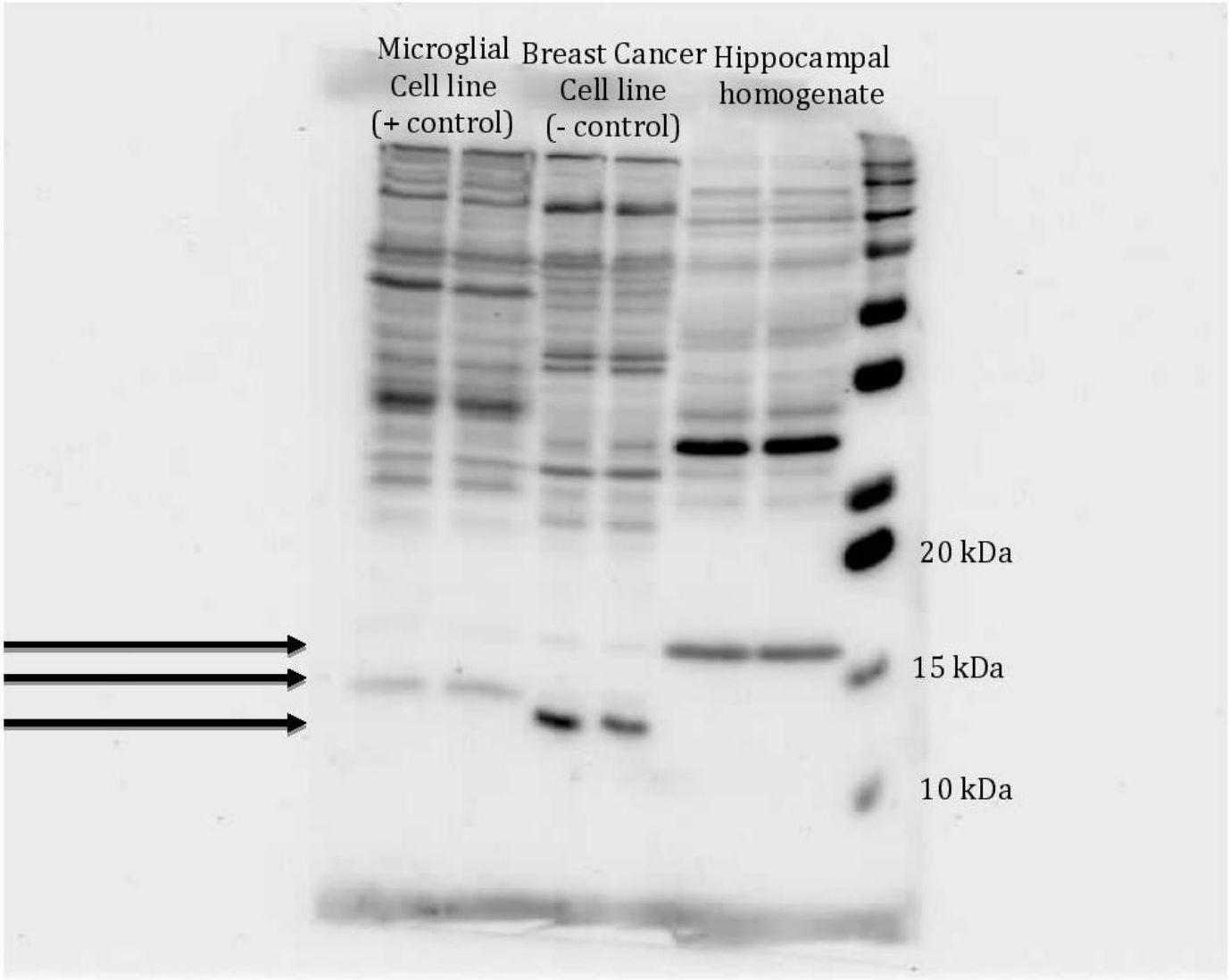

Figure 10: Iba-1 with Loading Controls. Forty µg of hippocampal homogenate was loaded along with MCF-7 breast cancer cell line (negative control) and BV-2 microglial cell line (positive control) homogenates for the rabbit polyclonal antibody Iba-1 from Abcam (Catalogue #ab108539). Although a signal was achieved in the target tissue, the positive control band appeared at a clearly distant molecular weight and the negative control also displayed a strong signal. Arrows indicate bands at approximately the expected molecular weight of Iba-1.

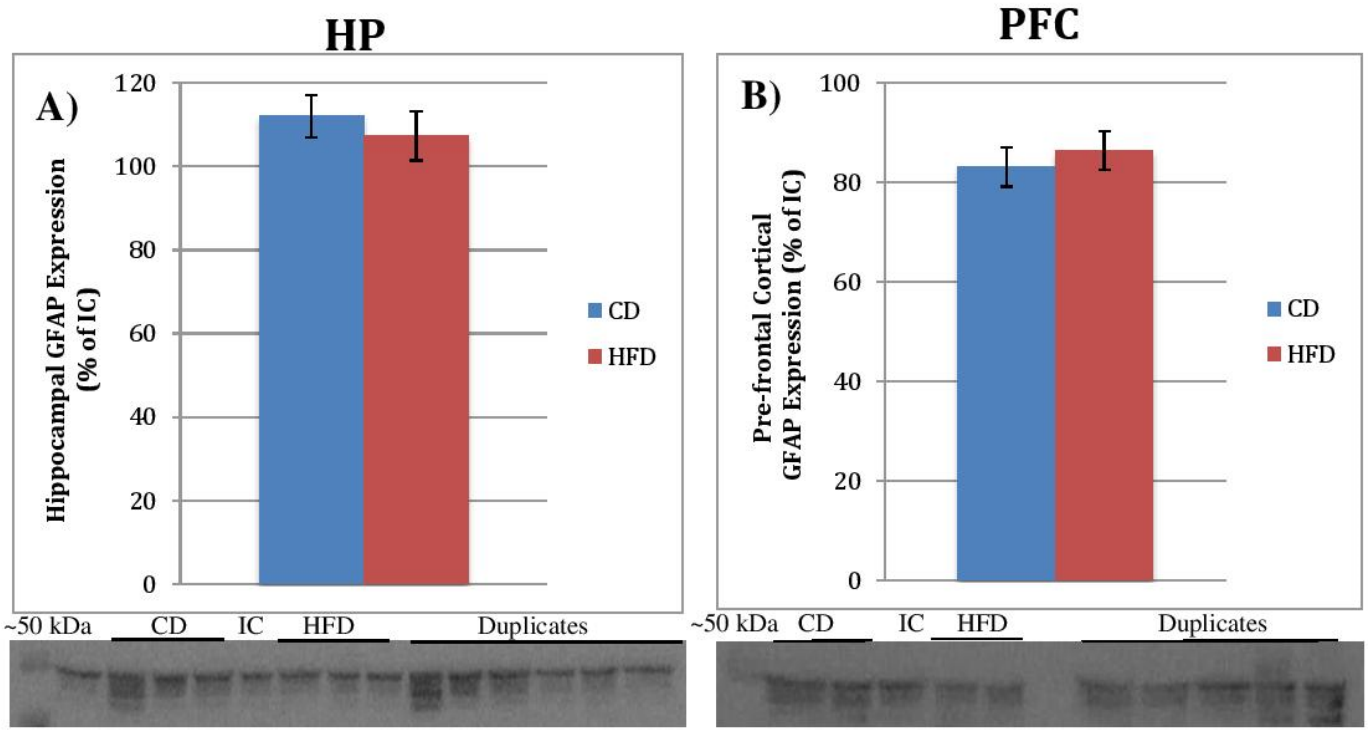

Figure 11: Hippocampal and Pre-frontal Cortical GFAP Expression. The high-fat diet had no effect on expression of GFAP in the hippocampus (A), or the pre-frontal cortex (B; $p > 0.05$ using Student's *t* test). Images are representative immunoblots wherein 40 µg of whole tissue homogenate from each diet was ran in duplicate. Optical densities were normalized to a whole protein stain (Ponceau S) to control for loading variability. Experimental ratios were normalized to a similar ratio constructed for the Internal Control (IC; non-experimental cortical tissue). Data presented are mean ± SEM. HP = hippocampus (N = 14-15); PFC = pre-frontal cortex (N = 10); CD = control diet; HFD = high-fat diet

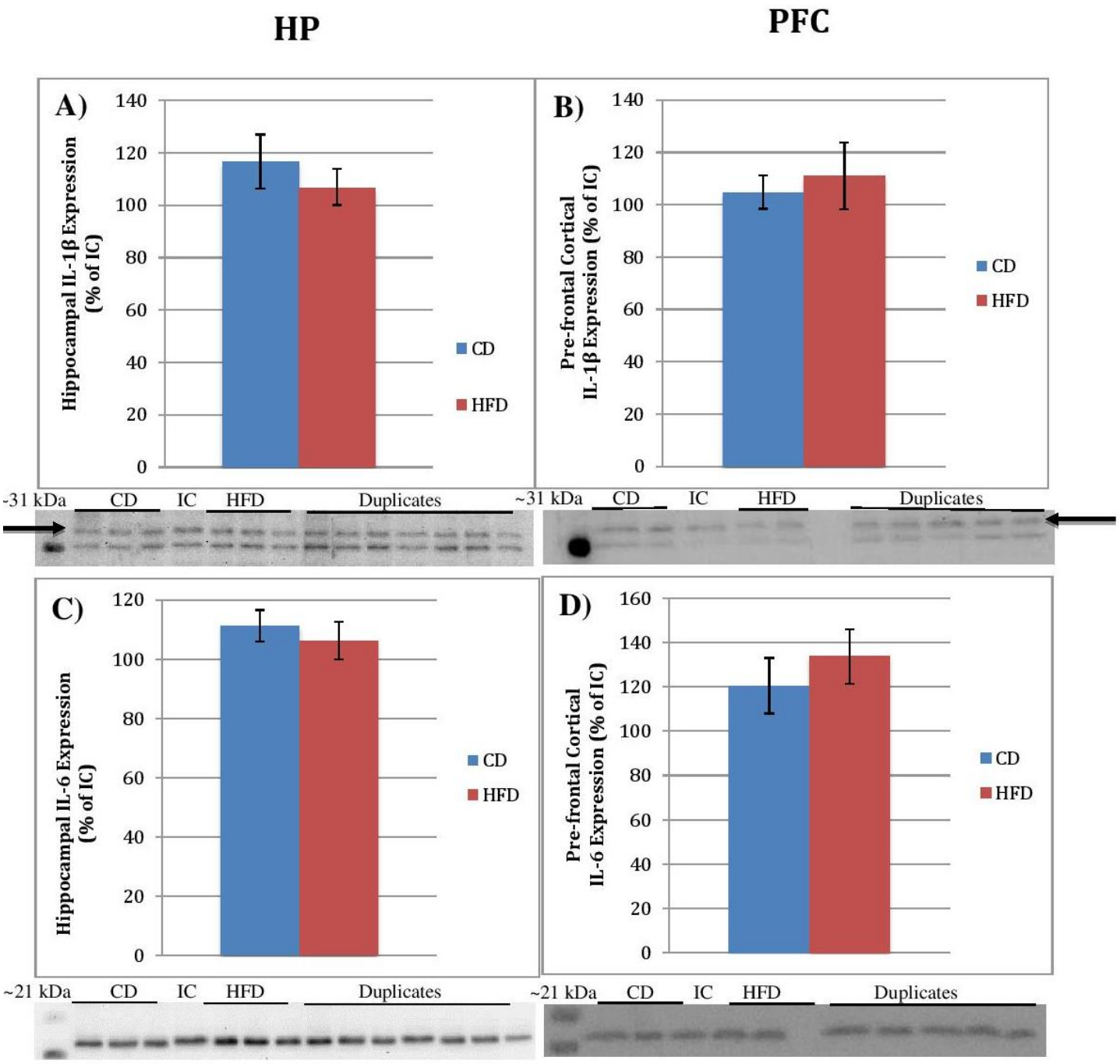

Figure 12: Hippocampal and Pre-frontal cortical IL-1β and IL-6 expression. The high-fat diet had no effect on expression of IL-1β (A, B), or IL-6 (C, D) in either the hippocampus, or the pre-frontal cortex ($p > 0.05$ using Student's *t* test). Images are representative immunoblots wherein 40 µg of whole tissue homogenate from each diet was ran in duplicate. Optical densities were normalized to a whole protein stain (Ponceau S) to control for loading variability. The experimental ratios were then normalized to a similar ratio constructed for the internal control (IC; non-experimental cortical tissue). Data presented in each graph are mean ± SEM. HP = hippocampus (N = 13-15); PFC = pre-frontal cortex (N = 10); CD = control diet; HFD = high-fat diet. Arrows indicate bands at approximately the expected molecular weights of IL-1β.

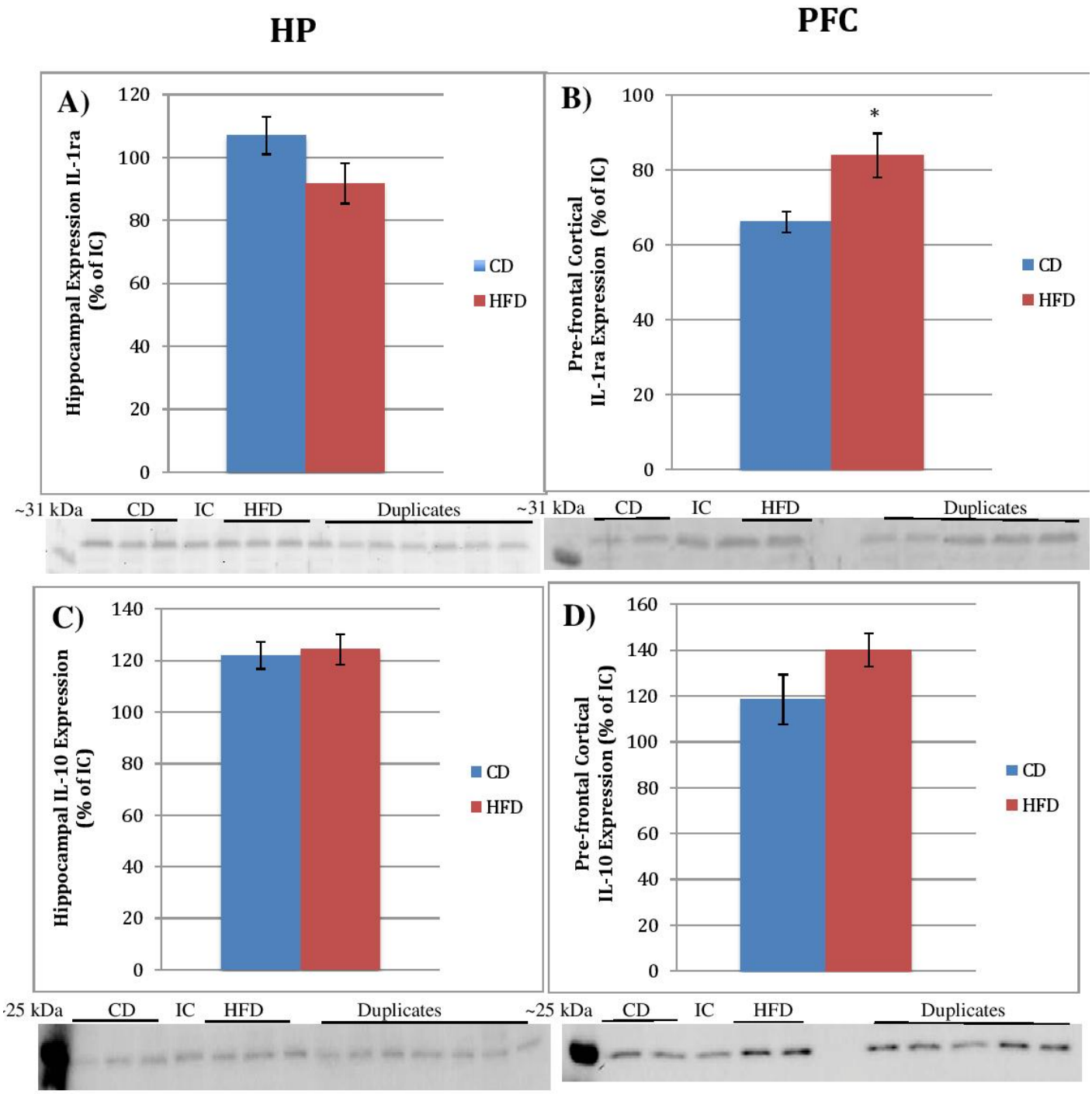

Figure 13: Hippocampal and Pre-frontal Cortical IL-1ra and IL-10 expression. The high-fat diet significantly enhanced the expression IL-1ra (B) in the pre-frontal cortex relative to animals on a control diet ($p < 0.05$). However, no significant differences were found in the hippocampal expression of IL-1ra (A), or expression of IL-10 in either region (C, D) ($p > 0.05$). Images are representative immunoblots wherein 40 µg of whole tissue homogenate from each diet was ran in duplicate. Optical densities were normalized to a whole protein stain (Ponceau S) to control for loading variability. The experimental ratios were then normalized to a similar ratio constructed for the internal control (IC; non-experimental cortical tissue). Data presented are mean ± SEM. HP = hippocampus (N = 14-15); PFC = pre-frontal cortex (N = 9-10); CD = control diet; HFD = high-fat diet, *$p < 0.05$ using Student's *t* test.

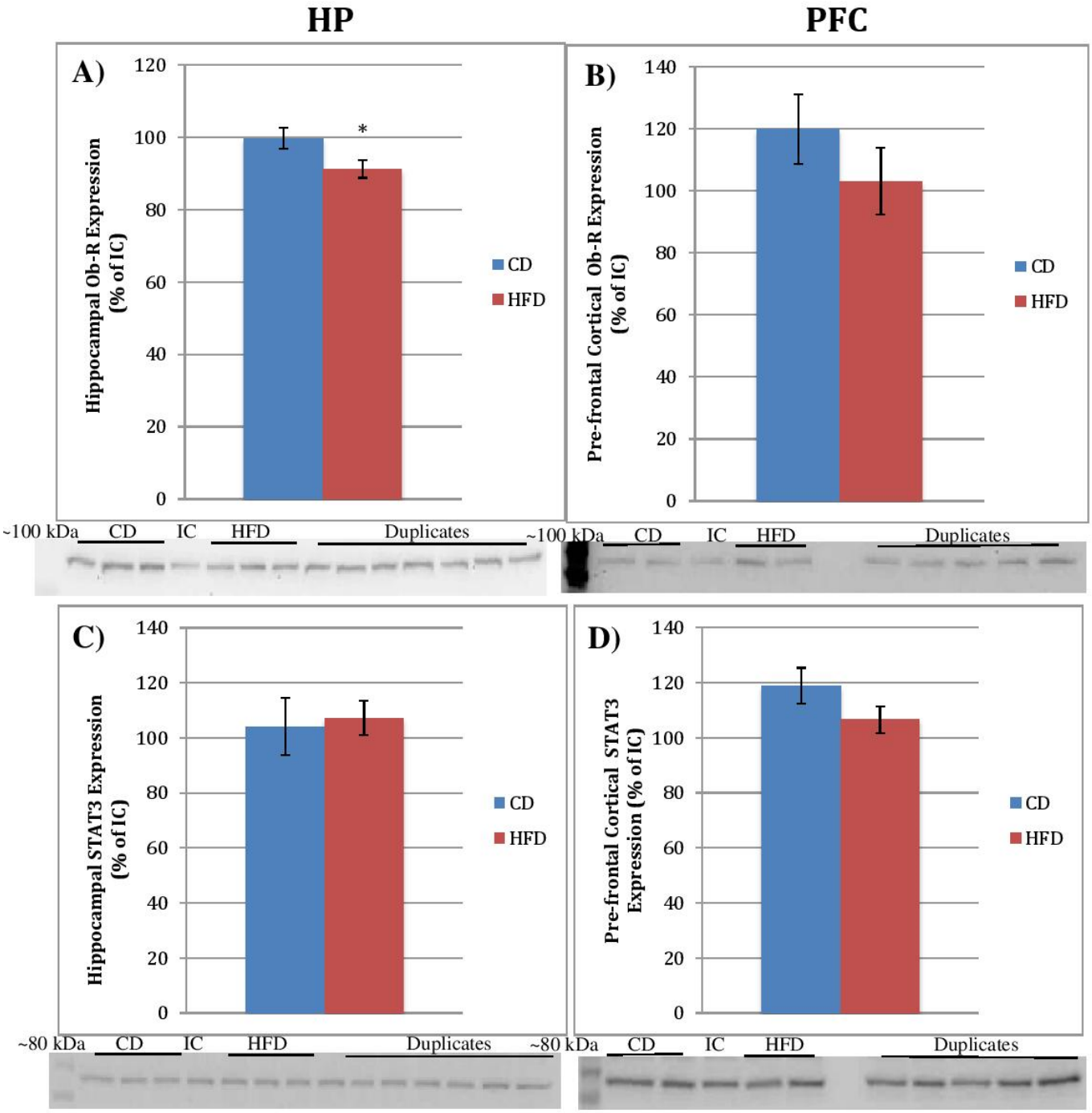

Figure 14: Hippocampal and Pre-frontal Cortical Ob-R and STAT3 expression. The high-fat diet significantly reduced the hippocampal expression of Ob-R (A) relative to animals on the control diet ($p < 0.05$). However, no significant differences were found in the pre-frontal cortical expression of Ob-R (B), or the expression of STAT3 (C, D) ($p > 0.05$). Images are representative immunoblots wherein 40 µg of whole tissue homogenate were ran in duplicate. Optical densities were normalized to a whole protein stain (Ponceau S) to control for loading variability. The experimental ratios were then normalized to a similar ratio constructed for the internal control (IC; non-experimental cortical tissue). Data presented in each graph are mean ± SEM. HP = hippocampus (N = 14-15); PFC = pre-frontal cortex (N = 9-10); CD = control diet; HFD = high-fat diet, *$p<0.05$ using Student's *t* test.

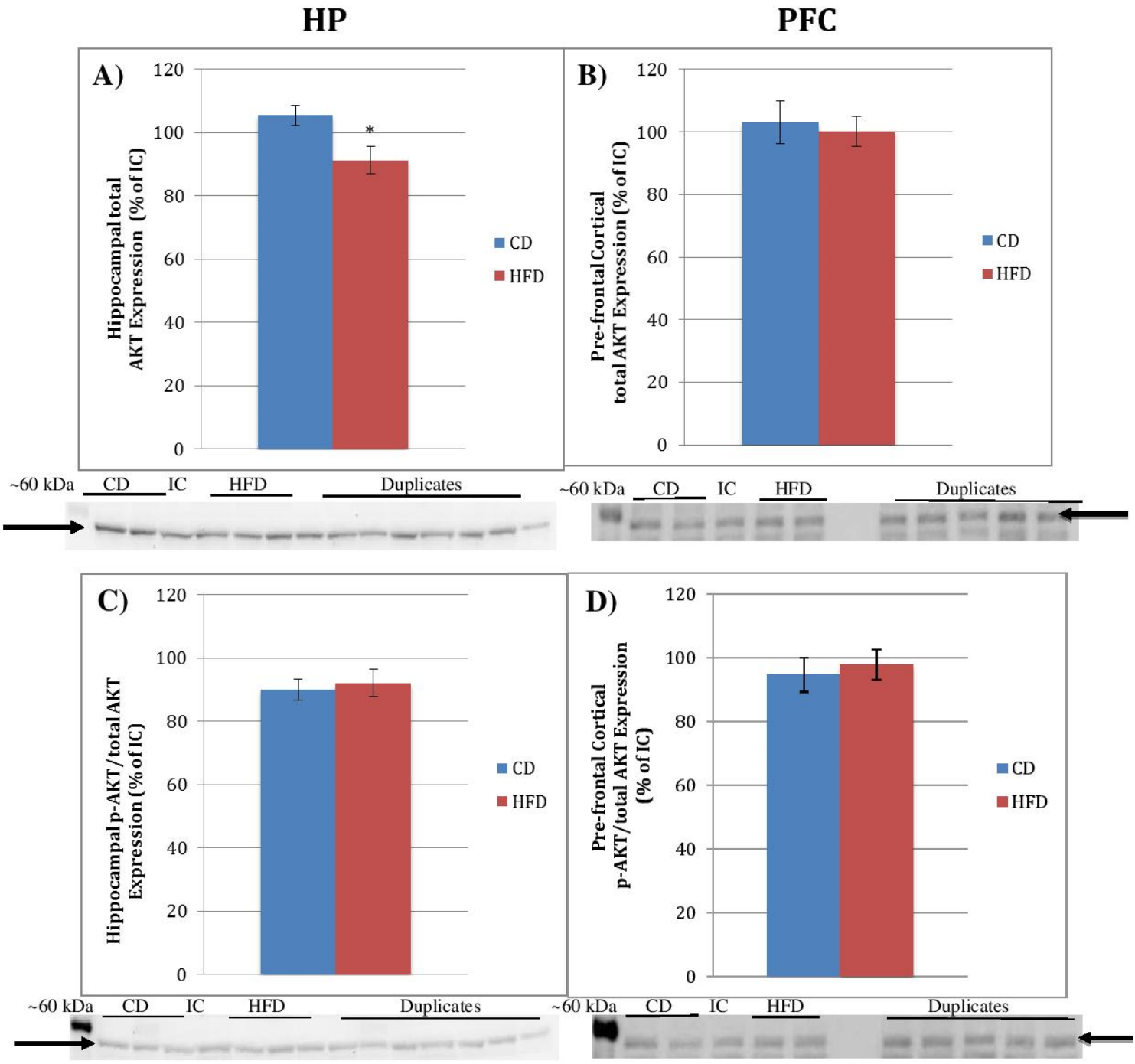

Figure 15: Hippocampal and Pre-frontal cortical AKT/PKB and p-AKT/PKB expression. The high-fat diet reduced the hippocampal expression of AKT/PKB (A) relative to animals on the control diet ($p < 0.05$). However, no significant differences were found in the pre-frontal cortical expression of AKT/PKB (B), or the expression of AKT/PKB phosphorylated on serine 473 of the C-terminal in either region (C, D) ($p > 0.05$). Images are representative immunoblots wherein 40 µg of whole tissue homogenate were ran in duplicate. Optical densities for AKT/PKB were normalized to a whole protein stain (Ponceau S) to control for loading variability while p-AKT/PKB was normalized to total AKT/PKB expression. The experimental ratios were then normalized to a similar ratio constructed for the internal control (IC; non-experimental cortical tissue). Data presented in each graph are mean ± SEM. HP = hippocampus (N = 14-15); PFC = pre-frontal cortex (N = 10); CD = control diet; HFD = high-fat diet, *$p<0.05$ using Student's *t* test. Arrows indicate bands at approximately the expected molecular weights of AKT/PKB and p-AKT/PKB.

6.0 Discussion

In the present study, I examined the effects of a HFD consisting of 45% calories from saturated fat fed to female rats over a period of 10 weeks in order to identify potential neuroinflammatory and leptin signaling effects that could lead to cognitive impairment. The majority of the literature focuses on the neurological effects of HFD on male animals, leaving a noticeable gap that the current study tries to address considering sexual dimorphism in neural development and the development of obesity (Beery & Zucker, 2011). In addition, many researchers often fail to elaborate if their feeding protocol was successful at inducing an obese phenotype, and often use only changes in body weight as their indicator. Here, I studied an array of functional and biometric measures to assess whether the feeding protocol induced an obese phenotype.

Our HFD animals displayed evidence of an impaired oral glucose tolerance based on a significantly higher blood glucose level 30 minutes after an oral glucose bolus following one month on the diet, and 30 and 60 minutes after a glucose bolus following two months on the diet. In addition, our female animals were significantly heavier after the 10 week feeding period. Despite the lack of significance, the HFD animals displayed a trend towards a higher body weight, and significantly heavier retroperitoneal fat pad weights. Based on all the findings, I concluded female animals fed the HFD had developed an obese phenotype.

High-Fat Diet did not Affect Gliosis, but did Increase IL-1ra in the Pre-Frontal Cortex

In spite of developing an obese phenotype, when I assessed the biochemical results from both the hippocampus and pre-frontal cortex, the HFD animals revealed no direct evidence of astrogliosis, or neuroinflammation as seen in the lack of alteration in GFAP and the array of cytokines examined. Even though Iba-1 was not explicitly probed, due to the reasons outlined in

Section 5.4.3, the lack of overt signs of neuroinflammation suggests that Iba-1 would most likely not be altered. Contrary to what was expected, in the pre-frontal cortex I observed an increased expression of the anti-inflammatory cytokine IL-1ra, indicating a possible reduction in neuroinflammation. The results were somewhat unexpected considering that the majority of the literature suggests the primary means of diet-induced cognitive impairment occurs through a neuroinflammatory model, where elevated expression of proinflammatory cytokines is detrimental to normal neuronal health and cognitive processes, such as plasticity (Lyman et al. 2014; Miller & Spencer, 2014). My results leave us with one of three possible explanations: 1) the pre-frontal cortex was in a state of neuroinflammation and the elevation of IL-1ra is indicative of a compensatory mechanism meant to return the brain to a state of basal inflammatory activity, 2) the lack of neuroinflammation suggests that female animals are inherently more resistant to the effects of HFD feeding, or 3) although an obese phenotype was reached in our animals, the duration of the feeding period, or the fat content in our diet was not sufficient to induce neuroinflammatory effects.

To address the first explanation, while I did not observe direct evidence of neuroinflammation via changes in the level of proinflammatory IL-1β, or pleiotropic IL-6, elevation in IL-1ra in the pre-frontal cortex could suggest a compensatory effect in response to an earlier state of diet-induced neuroinflammation. While not significant, HFD animals also displayed a trend towards elevated anti-inflammatory IL-10 production in the pre-frontal cortex. The difference between the two regions of the brain in terms of their inflammatory activity was surprising since the hippocampus and pre-frontal cortex have comparable levels of microglia and astrocytes in healthy adult rodents based on comparisons between different brain region cell densities determined via immunohistochemistry targeting specific glial markers (Lawson et al.,

1990; Savchenko et al., 1997). This would suggest that these two brain regions would also share a similar cytokine profile. Furthermore, when LPS is used to elicit an immune response in neuronal-glial cultures derived from these two brain regions, they show comparable level of TNFα and nitric oxide production, suggesting the microglia from these two regions show comparable activity (Kim et al., 2000).

Instead, my results may suggest that the brain is differentially affected, potentially due to the dietary intervention causing a shift in microglial residency and activity because glial distribution is primarily mediated by environmental cues (Lawson et al., 1990). More specifically, the microglia in the pre-frontal cortex may be shifting towards an alternatively activated (M2) phenotype rather than the classically activated (M1) phenotype (Eggen et al., 2013; Kraakman et al., 2014). M1 macrophages and microglia are typically involved with the enhanced expression of proinflammatory mediators as seen during states of inflammations while the M2 subsets are typically involved with tissue recovery and counteracting inflammation (Eggen et al., 2013; Kraakman et al., 2014). During chronic inflammation and obesity, macrophages recruited in the adipose tissue shift towards the M1 phenotype leading to adipose tissue dysfunction and impaired glucose tolerance (Kraakman et al., 2014). However, populations of macrophages are dynamic, and prolonged exposure to a HFD can cause macrophages to subsequently increase expression of M2 associated transcripts (Kraakman et al., 2014). Similarly, during certain cases of infection, microglia initially adopt the M1 phenotype to clear the pathogen, and then shift to more of an M2-like phenotype (Kraakman et al., 2014). In addition, microglia are also adaptive in their development, where repeated or chronic systemic inflammation can lead to either sensitization (leading to greater proinflammatory response to subsequent immune stimulation), or innate immune tolerance, where microglia develop an attenuated immune response to protect against

neuronal damage (Kraakman et al., 2014). Based on the imbalance in the cytokine profile that was observed, it is likely a combination of both M2 polarization and immune tolerance may be occurring in the microglia in the pre-frontal cortex of our HFD animals as a compensatory effect.

Elevated IL-1ra in this region of the brain most likely reflects the high IL-1ra to IL-1β ratio necessary to counteract the inflammatory effects of IL-1β (Arend 2002). IL-1ra is the natural antagonist to IL-1 signaling, and by binding to its functional receptor, IL-1 receptor type 1 (IL-1R1), blocks its ability to initiate downstream signaling (Arend 2002). Even in the event of infection in healthy adults, IL-1ra concentrations in the circulation must exceed IL-1 by approximately 100 fold in order to negate the effects of IL-1 on target tissues due to the presence of decoy receptors with no signaling properties such as IL-1 receptor type 2 (IL-1R2) (Arend 2002). Furthermore, imbalance between IL-1β and IL-1ra in our animals is consistent with the serum cytokine milieu of individuals with obesity and type II diabetes mellitus, where IL-1ra is elevated in addition to various proinflammatory mediators (Arend et al., 2002; Herder et al., 2009; Donath & Shoelson, 2011). In fact, obese individuals suffering from hyperleptinemia display a level of IL-1ra in their serum that is 7 times greater than that observed in the serum of health weight controls (Juge-Aubry et al. 2003). Elevated IL-1ra may have a role in improving insulin signaling as exposure to IL-1β has been shown to be detrimental and injurious to β-cell function in the pancreas (Arend et al., 2002; Herder et al., 2009; Donath & Shoelson, 2011). As a result, elevated IL-1ra is highly indicative of pre-diabetes, where the body is in a state of subclinical inflammation, and may operate as a means to improve glycemic control (Herder et al., 2009; Donath & Shoelson, 2011). Considering that IL-1ra can also cross the BBB and can be produced centrally by glial cells in the CNS, it is plausible that the pre-frontal cortical cytokine profile I observed in our HFD animals is similar to the inflammatory profile of diabetics (Arend 2002; Herdder et al., 2009;

Donath & Shoelson, 2011). On the other hand, unlike the pre-frontal cortex, I did not observe difference in IL-1ra expression in the hippocampus. However, the pre-frontal cortex and the hippocampus are intimately connected, and, as a result, an altered immune state in the pre-frontal cortex could lead to dysfunction of hippocampal function (Preston & Eichenbarum, 2013). Behavioural data from Goshen et al. (2007) supports such a relationship as mice in their study given an intracerebroventricular administration of a high exogenous dose of IL-1ra (100 μg) showed significantly reduced hippocampal-dependent learning processes such as fear conditioning to a specific context. While I did not measure IL-1ra, or insulin levels in the periphery to support that our animals were in a diabetic state, the impaired OGTT that I observe in our HFD appears consistent with the theory.

An alternative explanation to the compensatory effect of diet-induced neuroinflammation is the possibility that female animals are more resistant to the effects of the HFD as estrogen does exert a regulatory effect on metabolic inflammation by promoting lipolysis, reducing macrophage infiltration of adipose tissue, and supressing the proinflammatory action of IL-6 and TNFα to mitigate potential tissue damage (Lovejoy et al., 2008; Palmer & Clegg, 2015; Moneterio et al., 2014). The regulatory effects on metabolic inflammation of estrogen signaling are primarily mediated by activity on its non-nuclear receptors, as opposed to classical genomic estrogen signaling that regulates reproductive effects (Monterio et al., 2014). Along with the anti-inflammatory effects of estrogen, acute 17 β-estradiol application also has a role in promoting LTP in the CA1 region of the hippocampus in both male and female animals (Foy et al., 2010). Pre-treatment of 17 β-estradiol has also been shown to induce protection against NMDA-mediated excitotoxicity in both cultured neurons and hippocampal slices (Foy et al., 2010). Together, these findings suggest that female animals may be more resistant towards not only the metabolic effects

of a HFD, but also resistant to potential neuroinflammation processes that would be detrimental to synaptic plasticity (Foy et al., 2010; Moneterio et al., 2014). As mentioned earlier, confirming whether an inflammatory cytokine profile is occurring in the periphery of our animals would help to determine whether the animals were indeed protected. Future studies will need to directly examine sexual dimorphism with male animals, or ovariectomized female animals, fed using the same feeding protocol.

To address the last possibility, I compared our feeding protocol to those of other studies. Our feeding protocol was modest compared to the one used by Pistell et al. (2010), where animals fed a diet with 60% of calories from fat over 16 weeks revealed gliosis and elevated proinflammatory cytokine expression. Beilharz et al. (2014) used a HFD similar to ours and fed for a shorter period of time (45% kcal from fat fed over 27-29 days), but supplemented their HFD with a 10% sucrose solution, which could potentially account for the fact they observed elevations in hippocampal TNFα and IL-1β mRNA in their animals. Notably, the one comparable study in terms of both content and duration (45% kcal from fat fed over 4 months), Boitard et al. (2014) found no differences in cytokine mRNA levels under basal conditions, but did observe enhanced inflammatory cytokine mRNA expression upon stimulating the animals with an immune challenge (intraperitoneal injection of LPS). An alternate form of an immune challenge that is also effective at stimulating cytokine expression is the administration of viral mimics like polyriboinosinic-polyribocytidylic acid (PIC) (Konat et al., 2011; Krstic et al., 2012). However, given that PIC activates the immune system through TLR3 activation, rather than TLR4 activation like that of saturated fatty acids, LPS would be a better candidate for an immune challenge due to its homology in immune stimulation (Konat et al., 2011; Krstic et al., 2012). Regardless, had an immune challenge of either LPS, or PIC been applied, I may have been able to see a difference in

proinflammatory cytokine expression, where the HFD animals may have displayed a greater neuroinflammatory response despite showing no differences in basal proinflammatory cytokine expression.

The technique used to measure cytokine expression also needs to be considered. The majority of the literature that analyses cytokine expression typically assesses mRNA expression via quantitative reverse-transcription polymerase chain reaction (qRT-PCR), or protein expression via enzyme-linked immunosorbent assay (ELISA) (Pistell et al., 2010; Baumgarner et al., 2014; Beilharz et al., 2014; Boitard et al., 2014). Only a small proportion of studies examined cytokine profiling via immunoblotting as I have. In spite of this, Lu et al. (2001) were able to show that a HFD feeding protocol was capable of inducing a neuroinflammatory state. HFD mice from their study displayed greater protein expression of proinflammatory TNFα and IL-2, a cytokine which regulates the activity of leukocytes. However, their more extensive feeding protocol (20 weeks of a HFD consisting of 60% saturated fat) may indicate that a more intensive dietary intervention is required to induce similar effects in our female animals (Lu et al., 2011).

When quantifying mRNA expression using qRT-PCR, mRNA is often used as a proxy for the abundance of proteins in a biological sample despite the fact that the relationship between mRNA and protein level is not always linear (Maier et al., 2009). Translation efficiency of mRNA into proteins can be affected by several factors, such as the structure of mRNA, differences in the distribution of mRNA translational regulatory proteins, codon bias, and ribosomal density, while post-translational factors such as protein stability, localization of protein, and post-translational modification, such as ubiquitination, can affect protein abundance and turnover (Maier et al., 2009). Therefore, despite the data presented by earlier reports (Baumgarner et al., 2014; Beilharz

et al., 2014; Boitard et al., 2014), the possibility exists that cytokine protein expression may not actually be affected by a HFD.

Instead of quantifying mRNA, expression of cytokine proteins is most commonly analyzed using the sandwich ELISA technique (Amsen et al., 2009). In this variant of ELISA, homogenates are added to microtiter wells, so that embedded antibodies specific for the cytokines become immobilized (Amsen et al., 2009). A second enzyme-linked antibody is then added to the cytokine complex, which can be chemically converted to produce a colour change (Amsen et al., 2009). The intensity of the colour change is then considered to be proportional to the amount of cytokines present in the homogenate (Amsen et al., 2009). While ELISAs are highly sensitive and high throughput, any technique that utilizes an antibody-antigen interaction does risk a non-specific antibody-antigen reaction, where proteins besides the targeted cytokine bind to the antibodies, resulting in a false positive (Amsen et al., 2009). Immunoblotting offers higher specificity in this regard since homogenates are first separated by weight to aid in the isolation and visualization of the target protein brand (Porsh-Ozcurumez et al., 2004; Chou et al., 2005). For example, to diagnose individuals suspected of being infected with *Francisella tularensis*, the bacterium responsible for tularemia, or human immunodeficiency virus, ELISA is often used as an initial screening tool followed by immunoblotting for confirmation (Porsh-Ozcurumez et al., 2004; Chou et al., 2005).

High-Fat Diet impaired Ob-R and AKT/PKB expression in Female Rat Hippocampus

Our HFD reduced the expression of certain proteins related to leptin signal transduction in the hippocampus, but not the pre-frontal cortex (a trend towards a reduced Ob-R expression was observed, but was non-significant). Although the observed deficits were modest, my results suggest that reduced Ob-R and AKT/PKB expression in response to a HFD may be a compensatory

mechanism to leptin overexposure, and, as a result, may have led to impaired leptin signaling in the hippocampus along the IRS/PI3K pathway. The results are in agreement with the conclusions from previous HFD feeding studies conducted by Valladolid-Acebes et al. (2013) and Lu et al. (2011), who found most of their deficits along the IRS/PI3K pathway, rather than the JAK/STAT pathway, in the hippocampus. However, their deficits were attributed to the phosphorylation of AKT/PKB being reduced rather than AKT/PKB expression (Lu et al., 2011; Valladolid-Acebes et al., 2013). As previously mentioned, Lu et al. (2011) used a more extensive diet regimen compared to ours, and Valladolid-Acebes (2013) stimulated their animals with an acute leptin injection in order to examine differences at the level of phosphorylation. If I were to apply leptin onto brain slices prepared from our animals, further deficits in leptin signaling may be observed, and provide further evidence on how synaptic plasticity could be affected. AKT/PKB is also shared with many other signaling cascades, such as the insulin signaling pathway and tropomyosin receptor kinase (TrkB) signaling, which is activated by BDNF (Camer et al., 2015). As such, a reduced expression of AKT/PKB may imply that a deficit is also occurring at these levels to further reduce hippocampal function and neuronal health, although additional markers from these signaling cascades, such as TrkB or insulin receptor phosphorylation, should be used to confirm this possibility. Similar to the work that is required to clarify cytokines signaling, leptin levels and proteins involved with leptin signal transduction should also be examined in the periphery to confirm if there is indeed elevated serum leptin leading to systemic leptin resistance. Considering that leptin signaling is also involved in stimulating the production of proinflammatory mediators, it would also be of interest to probe for acute phase proteins, such as CRP, which has been previously found to hamper leptin signaling (Li et al., 2006; Martin et al., 2008).

Maternal High-Fat Diet and Effects on Offspring

In addition to filling the gap in the literature in regards to how female brain responds to a HFD, studying female animals provided the means to study another important aspect of obesity. As more individuals continue to develop obesity worldwide, so too does the number of obese women of reproductive age. Considering the developmental origins of health and disease in reference to obesity, a HFD could potentially act as a type of nutritional stress that can permanently alter fetal physiology through the process of developmental programming (Gluckman & Hanson, 2004; Calkins & Devaskar, 2011). Altered hormonal and metabolic effects exerted by maternal HFD may influence early brain development via fetal neuronal modulation (Gluckman & Hanson, 2004; Calkins & Devaskar, 2011). Here, I demonstrated a reduction in Ob-R and AKT/PKB expression in the hippocampus and elevated IL-1ra expression in the pre-frontal cortex in our female animals, which may indicate an altered hormonal and metabolic state that could influence early brain development. Behavioural evidence for the transgenerational effect of diet can be seen in a study conducted by White et al. (2009), where offspring from HFD mothers performed worse on the Morris water maze over the course of 8-9 trials in terms of latency and distance traveled to reach the hidden platform, indicating a deficit in memory retention over the training days. The effect was amplified and revealed a significant difference once the offspring themselves were given a HFD (White et al., 2009).

In terms of neuroinflammation, studies have shown that a maternal diet can have effects on the brain cytokine milieu of the fetus during embryonic development, producing pups with greater levels of activated microglia, leading to detrimental effects on normal neurodevelopment. White et al. (2009) demonstrated that a maternal HFD alone was sufficient to induce significantly elevated levels of IL-6 protein expression in 20 week old pups, while offspring diet was sufficient

to induce an enhanced level of glial activity with elevations in GFAP and Iba-1 protein expression, with no differences found in the level of BDNF and MCP-1. Bilbo & Tsang (2010) have shown that the hippocampus of HFD pups showed significantly higher basal levels of IL-1β protein expression compared to low-fat diet pups that persisted until adulthood. HFD pups also displayed greater hippocampal IL-1β expression in response to an intraperitoneal LPS injection compared to the low fat diet pups (Bilbo & Tsang, 2010). The effect was significantly more pronounced in male pups relative to female pups upon reaching adulthood (Bilbo & Tsang, 2010). The results also coincided with cytokine expression in the periphery, with HFD pups being more sensitive to the LPS immune challenge, and heightened liver IL-1β and serum IL-6 expression compared to immune stimulated, low-fat diet pups (Bilbo & Tsang, 2010).

As for leptin signaling, Kuang et al. (2014) were able to show that two month old pups born from mothers fed a high-sucrose diet showed significant impairments in PI3K and p-AKT/PKB protein expression, and histological evidence demonstrated elevated levels of apoptosis occurring in the CA1, CA2, and CA3 regions of the hippocampus. In this study, the authors concluded that PI3K and p-AKT/PKB, signaling molecules that are downstream to both leptin and insulin-like growth factor-1 (IGF-1), seemed to have neuroprotective effects by limiting the production of caspases and mediating the production of anti-apoptotic factors (Kuang et al., 2014).

Limitations

There are several limitations to consider when interpreting the results I have displayed. As previously mentioned, HFD protocols in terms of fat content and feeding duration are rather variable, making the connection of past literature with my results difficult. In addition, strain differences in the animals used may also play a factor in the difference response to HFD (Jun & Fen, 2006; Kucera & Cervinkova, 2014). Male Sprague-Dawley rats, for example, have been

shown to be more resistant to the effects of a HFD compared to male Wistar rats. When given the same HFD (45% kcal from fat), Wistar rats displayed significantly heavier body weights, impaired oral glucose tolerance, and enhanced lipogenesis compared Sprague-Dawley rats even when the latter strain is fed the HFD for a longer period of time (11 weeks vs. 7 months) (Jun & Fen, 2006). However, other studies have shown Sprague-Dawley rats, especially male animals, are more susceptible to non-alcoholic fatty liver disease induced by HFD consumption compared to Lewis and Wistar rats based on the degree of liver fibrosis and hepatic damage (Kucera & Cervinkova, 2014). Taken together, results from these studies indicate that not only do animal strains respond differently to HFD, but that they could also display different immune profiles as a result. Consequently, our findings in the hippocampus and pre-frontal cortex may be specific only to female Sprague-Dawley rats.

Another limitation that was previously mentioned is the lack of peripheral metabolic measures in relevant biomarkers in the blood serum of our animals. Any potential effects of neuroinflammation and impaired leptin signaling in the brain as a result of HFD would presumably originate in the periphery (Irving & Harvey, 2014; Miller & Spencer, 2014). Therefore, examining cytokine, leptin, and insulin expression in the serum would strengthen the interpretation of the mechanism behind HFD induced impairments.

Lastly, one final limitation to consider is the limited power to detect an effect in many of the protein expression experiments. Post-hoc power analysis of the immunblotting results reveals low power, ranging from 0.03 to 0.41. Since the power calculated is less than the convention of 0.8, the current study design is considered to have insufficient power, and runs the risk that we may falsely accept the null hypothesis (committing a Type II error) (Onwuegbuzie & Leech, 2004).

Therefore, future studies may benefit from the inclusion of more animals to ensure sufficient power.

Conclusion and Future Directions

In this study, I found that female rats fed a HFD over a 10 week period resulted in potential compensatory mechanisms in response to neuroinflammation (characterized by elevated IL-1ra expression in the pre-frontal cortex) and to leptin overexposure (characterized by reduced expression of Ob-R and AKT/PKB). Taken together, these physiological changes suggest that our animals may be predisposed to cognitive deficits as both these factors can contribute to impaired synaptic plasticity. To explore the potential role of estrogen mitigating neuroinflammatory deficits in the CNS, I hope to include male animals in the future to allow for a direct sex comparison. In addition, some of the differences characterized in the literature were not as prominent in our animals, and may require additional treatment (such as LPS injection, or leptin application) to allow differences that may not have been observed at basal levels to be observed. Lastly, to explore the effects of maternal diet on offspring, an additional future experiment could examine the presence of similar protein expression changes in the brains of offspring.

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