SHORT-TERM FEEDING EFFECTS OF HIGH-FAT DIET ON HIPPOCAMPAL NEUROINFLAMMATION: DIFFERENCES AMONG C57BL/6J, C3H/HeN, AND C3H/HeJ MICE

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Abstract

DREW MILLETTE: Short-term Feeding Effects of High-Fat Diet on Hippocampal Neuroinflammation: Differences among C57BL/6J, C3H/HeN, and C3H/HeJ mice
(Under the direction of Dr. Patricia Sheridan)

Neurodegenerative diseases represent a growing public health concern. Increasing evidence has implicated a potential role of dietary intake of saturated fats in Alzheimer's development, possibly through Toll-like Receptor 4 (TLR-4) activation in microglia. We hypothesized that a diet high in saturated fats would increase neuroinflammation, and loss of TLR-4 would protect against this increase. C57BL/6, C3H/HeN, and C3H/HeJ mice were given either a low-fat or a high-fat diet for 8 weeks. HFD increased the expression of TNF-α and decreased expression of IL-10 in the hippocampus of C57BL/6 mice, and increased microglial TLR-2 and TLR-4 receptors. HFD did not increase neuroinflammation in C3H/HeN mice; however, IL-6, MIF and SOCS-3 transcription were decreased. No differences were discovered in C3H/HeJ mice. While microglia isolated from HFD-fed C3H/HeN mice had increased TLR-2 and TLR-4 expression, TNF-α was decreased following LPS-stimulation. In conclusion, C57BL/6 mice are an appropriate model for short-term HFD-induced hippocampal neuroinflammation.

Adrienne, Paul, Mom, Dad, and all my friends,

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List of Abbreviations and Symbols

4-HNE	4-hydroxynonenal
5XRT	5 times reverse transcriptase
Aβ	beta-amyloid
AD	Alzheimer's disease
AGE	advanced-glycation-end
Akt	serine-threonine protein kinase Akt
AMP	adenosine monophosphate
anti-TNF	antibody to tumour necrosis factor
ANOVA	analysis of variance
AP	antigen presentation
AP-1	(also known as JUN, cJun) Jun proto-oncogene
APAF-1	apoptotic peptidase activating factor 1
APO	apolipoprotein
APOE	apolipoprotein E
APOEε4	apolipoprotein E sigma 4
APP	amyloid precursor protein
ATP	adenosine triphosphat

Bax	Bcl2-associated X protein
BBB	blood brain barrier
Bcl-2	B-cell CLL/lymphoma 2
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CA1	Region 1 of hippocampus proper
CD	chow diet
CD11b	(also known as Itgam) integrin, alpha M
CD14	cluster of differentiation 14
CD16/32	(also known as Fcgr1) Fc receptor, IgG, low affinity III
CD45	(also known as PTPRC) protein tyrosine phosphatase, receptor type, C
CD80	CD80 molecule
CD86	CD86 molecule
COX-2	cyclooxygenase-2
cDNA	complementary deoxyribonucleic acid
cJun	(also known as JUN, AP-1) Jun proto-oncogene
CNS	central nervous system

Cre-lox	cyclization recombinase-lysyl oxidase
DCX	doublecortin
DEPC	diethylpyrocarbonate
DHA	docosahexaenoic acid
DIO	diet-induced obesity
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	mix of nucleoside triphosphate
dT	deoxy thymine
EAE	experimentally-induced autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK1/2	mitogen-activated protein kinase
F4/80	(also known as Emr1) EGF-like module containing, mucin-like, hormone receptor-like sequence 1
FA	fatty acid
FABP7	fatty acid binding protein-7
FADD	Fas (TNFRSF6)-associated via death domain
FAT/CD36	fatty acid translocase/CD36 molecule (thrombospondin receptor)

FATP-1	fatty acid translocase protein-1
FATP-4	fatty acid translocase protein-4
FBS	fetal bovine serum
FFA	free fatty acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBP-1	HMG box transcription factor-1
HBSS	Hank's Balanced Salt Solution
HF	high-fat
HFD	high-fat diet
HFL	high-fat lard diet
HMGB1	high-mobility group box-1
HSD	Honestly Significant Difference
HSV-1	herpes simplex virus-1
Iba-1	induction of brown adipocytes 1
ICV	intracerebroventricular
IFN-γ	interferon-gamma
IKBa	(also known as NFKBIA) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKB kinase	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase
IKbKe	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
IKK1	(also known as CHUK) conserved helix-loop-helix ubiquitous kinase
IKKA	(also known as CHUK) conserved helix-loop-helix ubiquitous kinase
IKKB	(also known as IKBKB) inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL-1	interleukin-1
IL-10	interleukin-10
IL-1β	interleukin-1 beta
IL-1R	interleukin 1 receptor
IL-6	interleukin-6
IRAK1	interleukin-1 receptor-associated kinase 1
IRAK4	interleukin-1 receptor-associated kinase 4
IRF-3	interferon regulatory factor-3
JNK	c-Jun N-terminal kinase
Kcal	kilocalorie
LFD	low-fat diet
LTP	long-term potentiation
LPS	lipopolysaccharide

LBP	lipopolysacchardie binding protein
LDLR	low density lipoprotein receptor
LD	low-fat diet
MCI	mild cognitive impairment
MCP-1	macrophage chemotactic protein-1
MD-2	lymphocyte antigen 96
MEK1/2	(also known as Dsor1) downstream of raf1
MetS	Metabolic Syndrome
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP-1α	(also known as CCL-3) chemokine (C-C motif) ligand 3 OR macrophage inflammatory protein -1 alpha
MKK3/6	mitogen-activated protein kinase kinase 3/6
MKK4/7	mitogen-activation protein kinase kinase 4/7
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid

MyD88	myeloid differentiation primary response protein 88
NCI	National Cancer Institute
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
Nrf-2	nuclear factor (erythroid-derived 2)-like 2a
ω-3	omega-3
ω-6	omega-6
p38	(also known as MAPK14) mitogen-activated protein kinase 14
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDGFB	platelet-derived growth factor beta polypeptide
peIF2α	phosphorylated eukaryotic translation Initiation Factor 2alpha
PET	Positron Emission Tomography
PI3K	phosphoinositide-3-kinase
pIKK	phosphorylated IkappaB kinase-like 2
pJNK	phosphorylated c-Jun N-terminal kinase
PPAR-γ	Peroxisome proliferator activated receptor gamma
pPERK	phosphorylated eukaryotic translation initiation factor 2-alpha kinase 3

PRR	pattern recognition receptor
PS-1	presenilin-1
PS-2	presenilin-2
PUFA	polyunsaturated fatty acid
qRT-PCR	quantitative real-time polymerase chain reaction
RANTES	(also known as CCL-5) chemokine (C-C motif) ligand 5 OR regulated upon activation normal T-cell expressed and secreted
RIP	ribosome-inactivating protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SEM	standard error of measurement
SFA	saturated fatty acid
siRNA	small interfering ribonucleic acid
STAT-3	signal transducer and activator of transcription 3
T2D	type 2 diabetes
TAB2	TGF-beta activated kinase 1-binding protein 2
TBK1	TANK binding kinase-1

tGPi	toxoplasma gondii phophoinositide
TIR	toll-interleukin-1 receptor
TIRAP	toll-interleukin-1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-alpha
TRAF6	TNF receptor associated factor-6
TRAM	(Trif)-related adaptor molecule
TRIF	toll-interleukin-1 receptor (TIR) domain containing adaptor inducing interferon-beta
UPR	unfolded protein response
US	United States
WD	Western Diet
WT	wild-type

Chapter I- Background

Introduction

Neurodegenerative disorders, such as Alzheimer's disease (AD) represent a growing issue facing today's population. As compared to other leading causes of mortality, limited knowledge exists regarding risk factors and potential targets for treatment of neurodegenerative disease. With advances in medical treatment of diseases such as cardiovascular disease, diabetes, and cancer, along with changes in the US age-distribution relating to the "baby boom" generation, a growing population over the age of 70 is at risk of AD development. Emphasis on research in this field could lead to better overall health, longer lives, and could lessen the emotional and financial burden on families and the health care system [1].

Neurodegenerative diseases are characterized by loss of structure and function of neurons in various regions of the brain. AD is a neurodegenerative disorder which results in neuronal death within the hippocampal and basal forebrain regions of the brain. These neural regions are generally associated with learning, memory, and emotional states. Cognitive impairment, short-term memory loss, and mood changes are all associated with the progression of the disease [1]. A main hallmark of AD is the formation of beta-amyloid plaques that are associated with neuronal death, and they are a predominant feature in brains of AD patients upon autopsy. Other predictors of AD development include ventricular enlargement, hippocampal atrophy [2], tau protein alterations [3], cerebral amyloid

angiopathy, and silent cerebral infarcts [4]. Though these predictors for AD development have been discovered, both improving screening processes and discovering possible therapeutic targets for treatment remain main research focuses [5].

In order to develop new strategies for disease prevention and treatment of AD, it's important to understand the underlying causative mechanisms driving AD etiology. Considering the complexity of the disease, discovering the underlying mechanisms requires a full understanding of specific risk factors that lead to increased AD development. Our research focuses on the contribution of a high-fat diet, a modifiable risk factor, to potential mechanisms underlying the neurodegenerative disorder.

Risk Factors of Alzheimer's Disease Development

Determining modifiable risk factors, such as nutritional variables, for AD development can be difficult given the long developmental period and complexity of the disease. Many factors likely involved in increased AD risk have varying effects over a long time course. Age is considered the predominant risk factor with AD prevalence drastically increasing as individuals age. AD prevalence is quite common among individuals aged 70 years, continues to increase in the 80 and 90 year age groups, and doubles every 5 years [6].

Like many diseases, genetic components of AD have been proposed. Early-onset AD, which represents only 0.01% of the total number of AD patients, has implicated roles of the amyloid precursor protein (APP) gene as well as presenilin-1 (PS-1) and presenilin-2 (PS-2) genes. These genes represent rare genetic influences that affect AD development in individuals under the age of 65[1,7]. A more common genetic influence in AD development resides in the apolipoprotein (APO) genes, specifically the ε4 gene polymorphisms within the

apolipoprotein E (APOE) gene variant. Individuals with this allele have increased risk of developing the disease; however, this gene polymorphism, unlike those in APP or the presinilin genes, does not guarantee development of AD [7]. For most individuals, many lifestyle risk factors contribute to the development and progression of the disease.

Metabolic syndrome (MetS), a collection of associated risk factors that increase the risk for cardiovascular disease and type 2 diabetes mellitus has been implicated in several different ways in the pathogenesis of AD [8]. Vascular arterial disease has long been hypothesized to be closely related to Alzheimer's development. Dementia is characterized in many different ways including Alzheimer's related dementia as well as non-Alzheimer's related vascular dementia. The vast majority of dementia cases lie between these two types [9]. Growing evidence suggests that pathologies of AD and vascular disease overlap and may be synergistic. Preclinical determination of Alzheimer's development is possible through measuring events of regional-specific cerebral perfusion, or the amount of blood flow in certain regions of the brain at a given time [10,11]. In addition, cerebral amyloid angiopathy, which is the accumulation of beta amyloid (Aβ) plaques in the arteries of the brain, is a characteristic of both stroke and Alzheimer's, bridging the gap between the two diseases [4,10] .

Another aspect of MetS that has been implicated as a possible risk factor of AD development is hyperglycemia and insulin resistance in type 2 diabetes (T2D). Longitudinal studies have reported an association between insulin resistance, T2D, and AD [12,13]. Dysfunctional insulin signaling has been linked to a number of detrimental consequences including oxidative stress, mitochondrial dysfunction, impaired membrane function, as well as increases in cellular glucose, cholesterol, acetylcholine, ATP levels, Aβ accumulation, and

tau hyperphosphorylation. PET studies have demonstrated reduced glucose uptake in AD patients. Hyperglycemia, as a result of impaired insulin signaling or insulin resistance, is associated with disruptive effects in the brain including detrimental effects on cognition and memory. High levels of glucose in the brain are associated with formation of toxic advanced-glycation-end (AGE) products, reactive oxygen species (ROS), and hyperhomocysteinemia. Hyperglycemia perfusing the brain may alter the cerebral vascular system, linking type 2 diabetes, vascular disease, and AD development [10,14].

MetS is a collection of several related pathologies including abdominal obesity linked to an excess of visceral fat, insulin resistance, dyslipidemia, and hypertension [15]. Pathologies associated with MetS may stimulate the development of Alzheimer's disease. A risk factor associated with developing MetS is obesity [16].

Obesity and Alzheimer's Disease

Obesity, a chronic condition characterized by the accumulation of excess adipose tissue, has been associated with increased risk of multiple morbidities, increased mortality, and it is a primary risk factor for both type 2 diabetes and cardiovascular disease. Obesity represents a major, growing epidemic in the United States, with about two-thirds of the adult population being either overweight (body mass index (BMI) $\geq$ 25-30) or obese (BMI $\geq$ 30) [15]. In addition, obesity has been increasing world-wide with the spread of the Western Diet (WD). The WD is characterized by higher levels of both dietary saturated fats and sugars [17]. In addition to its involvement in the Metabolic Syndrome, obesity, i.e. visceral fat expansion, has recently been proposed as a risk factor for the development of AD. In a study by Fitzpatrick et al. (2009), the authors reported that there was an increased risk of dementia in obese (BMI $\geq$ 30) vs. normal weight (BMI 20-25) individuals, after

adjusting for demographics (hazard ratio [HR], 1.39; 95% CI, 1.03 – 1.87). Higher BMI in middle life has been reported to be associated with the development of cognitive impairment and possible neurodegeneration later in life [18]. Another epidemiological study, conducted by Xu et al. (2011), of a population-based twin study in relation to midlife BMI status, found that midlife, overweight BMI (25-30) and obesity (≥30) were associated with increased risk of dementia. They used an adjusted generalized estimating equation with ORs of 1.71 (95% CI 1.30 – 2.25) for overweight and 3.88 (95% CI 2.12 – 7.11) for obesity [19].

Determining associations between AD development and BMI can sometimes be difficult in older age groups, especially if low BMI in older age is an indicator of severe undernourishment. A population-based prospective cohort study conducted by Hughes et al. (2009) as part of The Kame Project reported that subjects with higher baseline BMI and slower declining BMI in late life was associated with reduced risk of dementia. The results of their study suggest that low BMI or a faster decline in BMI in late life may be preclinical indicators of the development of dementing illness, especially for those who were initially overweight or obese [20].

Mouse Models of Obesity and Cognitive Impairment

Mouse models have also given indications that obesity impairs cognition. These particular animal studies have utilized very long-term high-fat feeding models to induce obesity. A study by Farr et al. (2008) tested diet-induced obese mice, as determined by 30% weight gain after 10 months of high-fat diet (HFD) feeding, against non-obese control mice in several spatial memory tests including the hippocampal-dependent Morris Water maze and the T-maze foot-shock avoidance tests. Obese mice performed significantly less well than

non-obese mice in both of these spatial memory tests, indicating a relationship between obesity and hippocampal-dependent, cognitive impairment. Farr et al. (2008) also reported that obese mice performed significantly less well in the non-hippocampal dependent, lever press test [21]. In a report, Hwang et al. (2009) presented evidence that obese male mice, fed a HFD for 9 to 12 months, had significantly lower long-term potentiation (LTP) than normal weight controls. Long-term potentiation is a measure of synaptic plasticity and is important for retention of new memories. Impairments in LTP are a characteristic of Alzheimer's patients [22].

Saturated Fatty Acids, Cognitive Impairment, and AD development

Several studies investigated the effect of diet-induced obesity on AD development. However, in previous studies which had long dietary exposures [21,22], it was difficult to separate the effects of obesity from effects of the dietary components. Diets high in saturated fatty acids have been proposed as a risk factor for AD development in humans and cognitive impairment in mice [23-25].

Morris and co-investigators (2003) observed dietary influences of several types of fatty acids on later AD development in a population of Chicago inhabitants over the age 65 with no pre-clinical AD symptoms observed. After a mean follow-up of 3.9 years, the general trend indicated that diets high in saturated and trans-unsaturated fatty acids, as reported through a food-frequency questionnaire, correlated with increased risk of AD development in the subjects, whereas diets high in monounsaturated and ω-6 polyunsaturated fatty acids had a marginally decreased risk of AD development [23]. In another study, Mattson (2003) stated that low-calorie, low-fat diets have a protective effect against AD development in humans [1].

Several mouse studies examined the effect of high-fat diets on cognition and possible risk factors for AD development [1,24,25]. By attempting to limit the consequences of HFD-induced obesity; however, most studies were unable to fully separate short-term feeding effects of HFD from potential obesigenic effects on cognition.

A study conducted by Gault et al. (2010) presented evidence that high fat diets result in cognitive impairment in object recognition tests. In addition, Gault also presented evidence that high-fat fed mice have compromised LTP induction and maintenance as compared to low-fat fed controls [24]. The Mattson (2003) study reported HFDs promoted cognitive decline as compared to dietary restricted, low-fat controls in rodents [1]. Pistell et al.(2010) presented evidence that C57BL/6 mice fed high-fat diets consisting of 60% fat from lard for 16 weeks produced cognitive impairment and increased proinflammatory cytokine production including Tumor Necrosis Factor – α (TNF-α) and Interleukin-6 (IL-6) in isolated whole-brain tissue compared to low-fat control mice [25].

Proposed Mechanisms Involved in AD Development

Several proposed mechanisms involved in progression of the neurodegenerative state have been postulated. Among these, two predominant mechanisms have been proposed that could generate symptoms of AD. The first mechanism is the formation of Aβ plaques, a process sometimes referred to as abnormal protein aggregation. Aβ is derived from amyloid precursor protein (APP), which was mentioned earlier as one of the genes in which rare mutations can lead to early-onset AD. Production and aggregation of Aβ peptides is believed to be associated with synaptic dysfunction and neuronal death. Aggregation of the Aβ peptides and neurofibrillary tangles begins before the onset of mild cognitive impairment

(MCI) and are associated with generation of ROS, resulting in membrane lipid peroxidation and neuronal death. Aβ accumulation is a hallmark pathological and diagnostic indicator of AD [26-28].

A second proposed mechanism suggests that increased neuroinflammation through activation of resident immune cells results in increased neuronal loss, and subsequent neurodegeneration. Neuroinflammation in different regions of the brain results from the generation of several types of molecules, including pro-inflammatory cytokines, chemokines, and immunoregulators. An exaggerated immune response is considered the predominant source of these inflammatory molecules. In many cases, neuroinflammation is used to fight off infection and in maintaining proper brain function through apoptosis of damaged neurons [29].

The central nervous system (CNS) is tightly regulated in terms of the immune response. For many years, the CNS has been considered an "immune-privileged" organ under strict control by the blood brain barrier (BBB). However, the immune response of the brain may be more dynamic than once believed with new discoveries regarding microglial function, the brain's innate immune cells, and recent reports that certain agents, including WD-feeding, can alter BBB permeability [29,30].

Role of Microglia in Neuroinflammation

Microglia are cells within the brain that can be derived from myeloid cells, particularly monocytes, from the peripheral circulation. They comprise 12% of the cells within the brain, with the highest concentrations of microglia present in the hippocampus and substantia nigra. Microglia provide structural support and can act as immunocompetant cells

within the CNS. Each microglial cell has been hypothesized to function as a hybrid between a white blood cell and a glial cell [29] .

Microglial function is similar to that of macrophage. Microglia express MHC class II molecules and they have the capacity for antigen presentation (AP) to other immune cells for recognition and destruction. Microglia also secrete proinflammatory molecules, i.e. cytokines and chemokines, upon activation. Microglial function helps to clear infectious agents within the CNS as well as facilitate apoptosis of damaged cells. Stimulated microglial cells enable neurons to recover from traumatic stress and injury and have been reported to facilitate guided migration of stem cells to sites of inflammation and injury. Microglia also express TLRs 1-9 on their cell surface and intracellularly, which enables them to respond efficiently to different types of pathogens as part of the innate immune system [31].

Innate Immune Response and Toll-Like Receptor Signaling

The body's response to infection from microbial pathogens relies on both the innate and adaptive immune responses. The innate immune response acts immediately after an infectious challenge; and its actions are conducted largely by modified monocytes, especially macrophage. Macrophages can phagocytose and kill pathogens, as well as produce several inflammatory mediators and cytokines [32] . The ability of these innate immune cells to recognize foreign pathogens and initiate the immune response relies heavily on surface and intracellular receptors that have adapted through time to respond to a wide variety of foreign molecules. Among the pattern-recognition receptors (PRRs), Toll-like receptors (TLRs) have been identified as respondents to a large variety of bacterial, viral, and fungal agents and represent one of the first lines of defenses against invading pathogens [33] .

Mammalian TLRs are highly conserved receptors which share sequence similarity with Toll proteins, essential molecules in embryonic patterning and antifungal protection found in drosophila [34,35]. Mammalian TLRs are type 1 transmembrane protein receptors found on a variety of innate immune cells including macrophages, neutrophils, natural killer cells, and microglia. As part of the innate immune system, TLRs are capable of differentiating between self and foreign pathogens [34] . In addition, TLRs are also related to the IL-1 receptor; however, the extracellular, leucine-rich repeat domains of TLRs are very different from the extracellular domain of IL-1R. TLRs respond to specific molecular motifs called pathogen associated molecular patterns (PAMPs) found on a variety of pathogens in microbial, viral, and fungal organisms [34,36]. As of 2012, 10 human TLRs and 12 mouse TLRs have been discovered and are known to respond to specific ligands, both physiological and synthetic [37]. Microglial TLRs are present either on the plasma membrane (TLR-1:TLR-2, TLR-2, TLR-2:TLR-6, TLR-4, TLR-4:TLR-6, TLR-5, and TLR-11(mouse)) or on the endosomal membrane (TLR-3, TLR-7, TLR-8, and TLR-9). Although some ligands for certain TLRs have yet to be characterized, a list of known TLRs and respective ligands are characterized in **Table 1**.

The first identified and most potent TLR ligand is lipopolysaccharide (LPS), a component of the cell wall of Gram negative bacteria. LPS, also known as endotoxin, which is normally bound to a soluble factor called LPS binding protein (LBP) associates with a phosphatidylinositol-anchored cell surface molecule of microglia called cluster of differentiation 14 (CD14), which in turn activates TLR-4 signaling. In addition, a small secreted molecule called MD-2 associates with TLR-4 and is essential for LPS recognition through TLR-4 homodimerization. TLR-4 activation leads to several different downstream

events that ultimately regulate the innate immune response and inflammation. Of particular interest is the activation of Nuclear Factor Kappa-light-chain-enhancer of activated of B-cells (NF-κB), a proinflammatory transcription factor which enhances cytokine and inflammatory mediator production [33,34]. Downstream events of TLR-4 activation can lead to several different outcomes dependent on localization of several intermediate steps. Initially, it was thought that myeloid differentiation primary response protein 88 (MyD88) was a necessary signaling adaptor for effective TLR-4 signaling; however, MyD88 deficient mice still promote changes that protect them against viral infections through upregulation of IRF-3 upon TLR-4 stimulation and signaling through another adaptor molecule called TIR-containing adaptor inducing interferon-β (TRIF) [33,34] . The TLR-4 signaling pathway is represented in **Figure 1**.

Lipopolysaccharides and Saturated Fatty Acids

Initially, LPS was thought to be the only ligand for TLR-4 stimulation. LPS is highly conserved across species and contains a core of hydrophilic polysaccharides, an O antigen, and a hydrophobic lipid A tail. The lipid A tail is considered the main inducer of the biological responses of LPS [38]. Lipid A of *E. Coli* and *S. 11typhimurium* is a β-1,6 linked disaccharide of glucosamine acylated with R-3-hydroxylaurate or myristate and phosphorylated at positions 1' and 4'. The 3-hydroxyl groups of these saturated fatty acids are further 3-*O*-acylated by lauric acid, myristic acid, or palmitic acid. When the lipid A tail is deacylated, capable through the enzyme, acyloxyacyl hydrolase, it loses its endotoxic properties and acts antagonistic to TLR-4 signaling [39]. This finding, originally reported in a 1986 study, suggests saturated fatty acyl moieties of the lipid A tail may be essential to initiate proinflammatory signaling through the TLR-4 complex [40]. In more recent

Table 1. Microglial Toll-like Receptors, Cellular localization, and Associated Ligands

Toll-like Receptors	Cellular Location	Physiological Ligands	Synthetic ligands
TLR-1:TLR-2	Plasma Membrane	Triacylated lipopeptides	Pam3CSK4
TLR-2	Plasma Membrane	Peptidoglycan, phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan, glucoronoxylomannan, high-mobility group box 1 protein (HMGB1)	Not determined
TLR-2: TLR-6	Plasma membrane	Diacylated lipopeptides, lipoteichoic acid, zymosan	S-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF, macrophage-activating lipopeptide of 2kDa, Pam2CSK4
TLR-3	Endosomal Membrane	Double-stranded RNA	Polyl:C
TLR-4	Plasma Membrane	Gram negative bacterial lipopolysaccharide, vesicular stomatitis virus glycoprotein, mouse mammary tumour virus envelope protein, mannans, glycosylinositol phospholipids, Viral envelope proteins, heat shock proteins, fibrinogen, nickel, HMGB1	Not determined
TLR-4: TLR-6	Plasma membrane	Oxidized low-density lipoprotein, amyloid-β fibrils	Not determined
TLR-5	Plasma Membrane	Flagellin	Not determined
TLR-7	Endosomal Membrane	Single-stranded RNA	Imiquimod, resiquimod, loxoribine
TLR-8	Endosomal Membrane	Single stranded RNA	Resiquimod
TLR-9	Endosomal Membrane	DNA, haemozoin	CpG-A, CpG-B, and CpG-C oligodeoxynucleotides
TLR-11	Plasma membrane	Profilin	Not determined

Adapted from (Lee, Avalos and Ploegh 168-179)

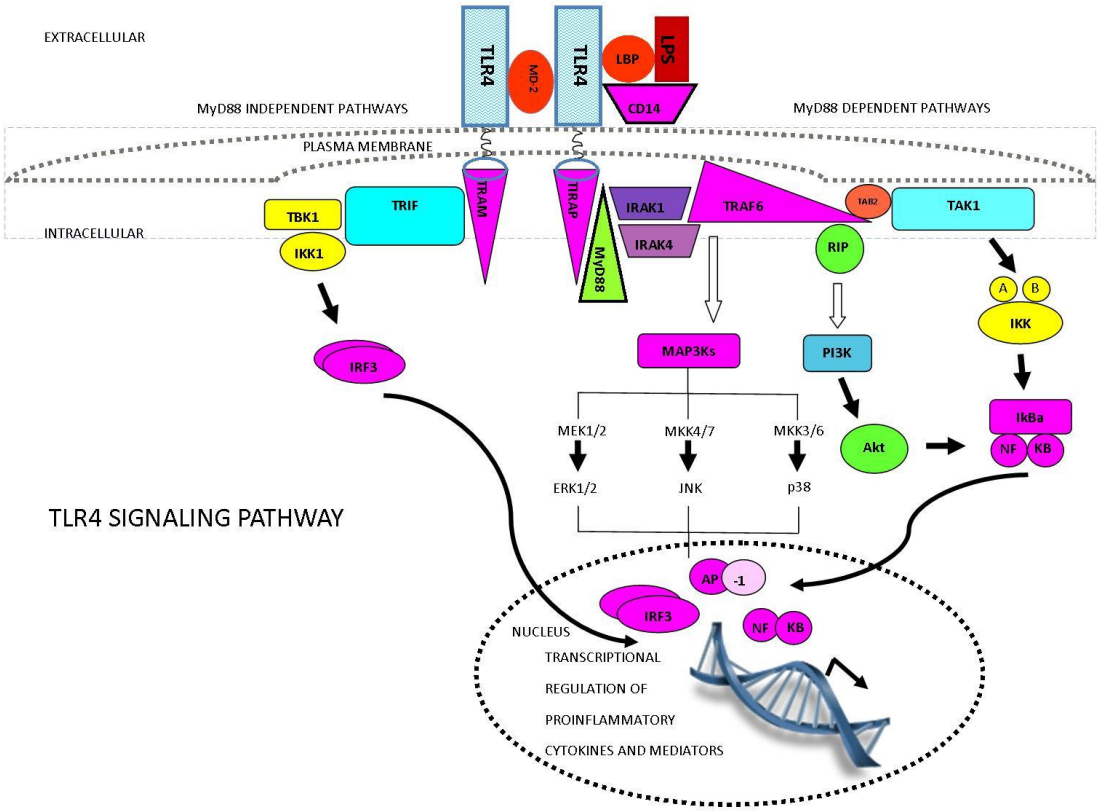

Figure 1. TLR-4 signaling pathway upon LPS stimulation (Adapted from PharmaProjects and ClipArt)

research, greater focus has been placed on the role of dietary saturated fatty acids (SFAs) and TLR-4 signaling as a means for promoting long-term, low-grade inflammation.

Saturated Fatty Acids and Inflammation: a Role for TLR-4

Several studies have investigated high-fat diet induced inflammation in many areas of the body implicating it in the etiology of several diseases related to MetS. High-fat diet induced inflammation has been mainly studied in 3T3-L1 adipocytes and peritoneal

macrophages. Both adipose tissue and macrophages have been reported to produce significant quantities of inflammatory molecules, which are increased in diets high in saturated fats. Ajuwon et al. (2005) investigated the effect of culturing 3T3-L1 adipocytes with palmitate (SFA) on expression of several inflammatory cytokines and mediators. They reported a 3.5-fold increase in NFκB activity as measured through a luciferase enzyme assay, as well as inducing increases in IL-6 mRNA and protein expression. Results from this experiment indicate that palmitate is capable of inducing inflammation in adipocytes; however, a specific mechanism linking palmitate and NFκB activation was not investigated. High-fat diet-induced insulin resistance is thought to be mediated in part through changes in inflammation in adipose tissue and adipocytes, and TLR-4 mutation has been reported to attenuate HFD-induced insulin resistance [43,47,48]. Therefore, to better understand the underlying mechanism, several studies have focused on the role of TLR-4 in HFD-induced changes in the adipocyte inflammatory state [41,42].

Shi and colleagues (2006) linked TLR-4 to inflammatory changes in adipose tissue and adipocytes stemming from either high saturated fat diets or the presence of palmitate. Before investigating the potential role of TLR-4 in adipocytes, the authors wanted to confirm a link between palmitate and TLR-4 signaling. The authors used a 293T cell transfection experiment to confirm that palmitate works through TLR-4 in a MyD88-dependent process to induce NFκB activation. Shi and co-researchers then addressed palmitate induced inflammatory changes through TLR-4 in 3T3-L1 adipocytes. Shi and co-researchers (2006) confirmed that TLR-4 mRNA is significantly expressed in 3T3-L1 adipocytes, isolated mouse adipocytes, and adipose tissue, and confirmed that TLR-4 is functional through LPS stimulation in the 3T3-L1 adipocytes. Shi et al. (2006) reported that 3T3-L1 adipocytes

stimulated with free fatty acids (FFA) had increased cytokine production (TNF-α and IL-6); however 3T3-L1 adipocytes with a TLR-4 knockdown through TLR-4 siRNA did not show any change. In addition, adipocytes were harvested from WT and TLR-4 $^{-/-}$ mice and stimulated with both LPS and FFAs. In WT adipocytes, LPS, and to a lesser extent FFAs, induced both IL-6 and TNF-α production; however this induction was absent in the TLR-4 $^{-/-}$ adipocytes [43].

As part of the *in vivo* portion of the study, Shi et al. (2006) fed WT and TLR-4 $^{-/-}$ mice either a high-fat diet (HFD) or low-fat diet (LFD), and harvested adipose tissue. The authors reported that even though both mouse types gained significantly more weight on the HFD, the TLR-4 $^{-/-}$ mice were more insulin sensitive. In addition, WT mice exhibited increased HFD-induced adipose tissue expression of IL-6, MCP-1, SOCS3, TNF-α, as well as the macrophage marker F4/80. Though adipocytes do express TLR-4 and produce inflammatory cytokines *in vitro*, previous studies have indicated that adipose tissue from obese individuals exhibit higher levels of macrophage infiltration, which could be contributing to increased inflammatory molecules being produced by the tissue [43,44].

Suganami et al. (2006) investigated the interaction between co-cultured adipocytes and macrophage, in order to better understand a mechanism linking saturated fats to reported increases in inflammation produced in adipose tissue. These researchers examined the relative contribution to inflammatory changes of adipocytes and macrophage in co-culture. The investigators used a magnetic cell sorting to completely separate the adipocytes and macrophage, as confirmed through exclusively expressed markers: adiponectin (adipocytes) and F4/80 (macrophage). They assessed relative mRNA levels for several markers in the two cell fractions and found that mRNA of macrophage chemotactic protein-1(MCP-1) was

significantly up-regulated in both macrophage and adipocytes, though at much higher concentrations in adipocytes [44].

In contrast to previous reports, TNF-α mRNA was only up-regulated in the macrophage fraction, indicating that the majority of inflammatory changes that result from circulating FAs (produced by lipolysis in adipocytes) directly stem from macrophages present in adipose tissue, rather than from adipocytes themselves. In addition, Suganami's research team examined the role of TNF-α released by macrophage and the role of palmitate, the most abundant FA released by adipocytes, as a potential paracrine mediator of inflammation. They found that TNF-α was capable of inducing NFκB activation in adipocytes and that palmitate was capable of inducing TNF-α production through a NFκB-dependent process in RAW264.7 macrophages, a standard macrophage culture cell model [44].

Suganami et al. (2007) harvested macrophages from C3H/HeJ and C3H/HeN mice to assess the role of TLR-4 in this process. C3H/HeJ mice possess a natural loss of function mutation in TLR-4, whereas C3H/HeN mice are their respective TLR-4 intact controls. Suganami and co-workers (2007) also reported that both LPS (positive control) and palmitate induced TNF-α in macrophages isolated from the C3H/HeN mice. In contrast, this increase in TNF-α expression was greatly attenuated in the macrophages isolated from TLR-4 mutant C3H/HeJ mice. Suganami and co-researchers (2007) also investigated levels of lipolysis by adipocytes in the presence of macrophage from either C3H/HeJ or C3H/HeN mice. FA release was significantly decreased in the adipocyte co-cultured with C3H/HeJ macrophage as compared to co-cultures of adipocytes with C3H/HeN macrophage, indicating a role of TLR-4 in regulating both inflammation and lipolysis in adipose tissue [45].

Many reports have demonstrated the role of TLR-4 in regulating inflammation in individuals with HFD-induced obesity, as well as playing a role in several disorders associated with MetS. A study by Tsukomo et al. (2007) investigated the role of TLR-4 as a candidate between metabolic signals, inflammation, and insulin resistance. The investigators gave C3H/HeJ and C3H/HeN mice either a chow control diet or a HFD (55% from fat) experimental diet. Tsukomo et al. (2007) reported that the C3H/HeJ mice were protected against diet-induced obesity, as well as exhibiting decreased adiposity, increased oxygen consumption, decreased respiratory exchange ratio, greater insulin sensitivity, and increased insulin signaling capacity in adipose tissue, muscle, and liver. Furthermore, TLR-4 mutation in the C3H/HeJ mice prevented IκB kinase and c-Jun NH_2-terminal kinase activity, which are both involved in NFκB activation and production of inflammatory molecules. Taken together, results indicated a critical role of TLR-4 in the development of HFD-induced insulin resistance, potentially mediated through increased inflammation [42,45]. Considering the relationship between MetS-associated disorders, such as insulin resistance, with AD development, TLR-4 could be playing a role in regulating HFD-induced neuroinflammation, which may lead to increased AD risk.

TLR-4, HFD, and Neuroinflammation

Few studies have addressed the role of TLR-4 in HFD-induced changes in neuroinflammation. Considering the similarity between macrophage and microglial function and that both cell types express TLR-4, as well as many other TLRs, it's likely that many of the same processes exist. In addition, dietary fats are transported into the brain through a process still under investigation, but reported to involve both active transport through FATP1, FAT/CD36 and passive diffusion [46].

A Milanski et al. (2009) study presented evidence that TLR-4 signaling is upregulated and activated by long chain saturated fatty acids and this contributes to increased ER stress in the brain (hypothalamus) of TLR-4 intact C3H/HeN mice, but not C3H/HeJ mice [47]. This study followed up a report by Tsukomo et al. (2007) hypothesizing that dietary fats act through TLRs 2/4 and endoplasmic reticulum stress to induce cytokine expression in the hypothalamus, leading to functional resistance to the anorexigenic hormones, insulin and leptin [42]. Milanski et al. (2009) used both Wistar rats and the C3H/HeJ and C3H/HeN mice fed either a chow or one of two high fat (36.0 g% fat from lard or 36.0g% oleic acid rich) diets. The results of their study indicated that long chain saturated fatty acids act through TLR-4 to promote increased inflammatory protein expression in the hypothalamus, increased cytokine production, increased ER stress, and TLR- 2/MyD88 and TLR-4/MyD88 upregulation in rats. In addition, loss or inhibition of TLR-4 protected against diet-induced body mass gain, impaired leptin resistance, and arachidic acid-induced hypothalamic cytokine expression. The Milanski (2009) study clearly indicated that TLR-4 plays a major role in regulating inflammation in the hypothalamus, as well as contributing to disorders related to obesity and MetS [42,47].

Milanski et al. (2009) reported that both TLR-2 and TLR-4 were upregulated and constitutively activated in the hypothalamus, and that TLR-2 and TLR-4 were exclusively expressed on microglia within the hypothalamus following a high fat diet. In addition to increased or constitutive activation of TLRs caused by high saturated fats, the microglia will also respond more vigorously to agonists such as LPS or peptidoglycans, and produce increased levels of proinflammatory molecules [47]. This phenomenon is known as "microglial priming." Many events can occur which result in more reactive immune cells

such as "primed microglia." In addition, another study with similar dietary conditions to the Milanski study (2009) indicated that 8 weeks of high-fat feeding was sufficient to increase apoptotic markers in the hypothalamus, microglial activation through increased F4/80 expression, and TLR-4 upregulation. In addition, this study reported that loss of TLR-4 activity in C3H/HeJ mice, compared to C3H/HeN control mice, prevented body mass gain with equivalent dietary food intake and altered expression of apoptotic markers [48].

Preliminary data from the Sheridan lab (unpublished data) reported that early infection with herpes simplex virus 1 (HSV-1), primed microglia isolated from C57BL/6 whole brain display increased pro-inflammatory gene expression including TLR-4. Microglia harvested from HFD, HSV-infected mice had higher expression levels of several markers of priming and inflammatory cytokines including TLR-4, MHCII, CD80, CD86, CD11b, IL-1β, and TNF-α compared to LFD, HSV-infected mice, implicating a role of HFD in contributing to increased TLR-4 surface receptor expression.

Hypotheses

Previous research has focused on either the role that TLR-4 plays in other areas of the body than the brain, or how HFD can contribute to increased neuroinflammation in areas of the brain *less related* to Alzheimer's development. We examined the effect of HFD on neuroinflammation within the hippocampus, an important site in AD development. Furthermore, investigation of the role that TLR-4 plays in mediating HFD-induced neuroinflammation was warranted, since previous reports have indicated a role of TLR-4 in mediating HFD-induced inflammation in other tissues. Our primary hypothesis was that mice fed a high-fat diet (HFD) for 8 weeks will exhibit increased levels of neuroinflammation within the hippocampal region compared to low-fat fed controls. In

addition, we postulated that 8 weeks of HFD-feeding will result in increased surface expression of TLR-2 and TLR-4. Our secondary hypothesis was that loss of TLR-4 activity would prevent HFD-induced increases in neuroinflammation.

In order to investigate the effect of HFD-feeding on neuroinflammation in the hippocampus, C57BL/6 mice were fed either a high fat diet (HFD) consisting of 45% calories from lard/soybean oil, or a low-fat control diet (LFD) consisting of 10% calories from the same fat source. This LFD control diet was more reflective of a true low-fat control diet, as previous studies, including the Milanski (2009) and Moraes (2009) studies, utilized normal chow diets with highly variable fat sources. After assessing status of neuroinflammatory mediators and changes in microglial surface receptor expression in the C57BL/6 mice, the role of TLR-4 in mediating HFD-induced neuroinflammation was examined by using C3H/HeJ mice, which as mentioned previously, have a natural loss-of-function mutation in TLR-4. C3H/HeJ mice and C3H/HeN mice, and their respective TLR-4 intact controls, were fed the same HFD and LFD for 8 weeks. We measured cytokine transcriptional changes and microglial reactivity.

The results of these two experiments are presented in Chapter 2.

Chapter II – Journal Manuscript[1]

INTRODUCTION

Neurodegenerative disease such as Alzheimer's disease represents a growing public health concern with prevalence increasing both domestically and world-wide. Neurodegenerative disease is characterized by loss of structure and function of neurons within various regions of the brain, leading to symptoms such as cognitive impairment/dementia. Age has long been considered the most predominant risk factor for Alzheimer's development. Considering shifts in the age distribution of the US population towards older aged individuals, the population at risk for this disease continues to increase. This has placed greater economic burden on the health community, emphasizing the importance of research in this field towards developing new screening techniques, better treatment options, as well as discovering preventative measures. Unlike other diseases such as cancer, diabetes, or cardiovascular disease, treatment for neurodegenerative disease remains scarce as many of the risk factors are either unknown or insufficiently characterized [1] . Determining risk factors for diseases with long developmental periods such as Alzheimer's disease can be a difficult task. In recent years, several potential risk factors for neurodegenerative disease development have been proposed and investigated.

Alzheimer's is considered a multi-factorial disease with both genetic and environmental influences. Early onset AD is characterized by rare mutations in the *APP* gene, as well as mutations in *PS-1* and *PS-2*; however, these represent only a small percentage of the population who develop AD. A more common gene polymorphism,

[1] (to be submitted to the Journal of Nutrition): High Fat Diet for 8 weeks Increases Inflammation in the

APOEε4, has been associated with increased AD development, representing a main genetic influence for late-onset AD. This polymorphism is not sufficient to cause AD development on its own [7]. In general, environmental factors likely play a larger role in AD etiology over the course of a patient's lifetime.

Disorders associated with Metabolic Syndrome (MetS) have been given greater focus within this research field as potential risk factors. Insulin resistance, Type II Diabetes, Vascular Disease, Atherosclerosis, and Obesity have all been associated with increased risk of AD development. Obesity contributes as a primary risk factor for all disorders related to MetS [8,12,13]. Recent longitudinal studies have associated obesity/BMI, as well as, increased dietary intake of saturated and trans-unsaturated fatty acids with increased AD risk [23,49,50,51]. Though it may be difficult to dissociate each MetS disorder and dietary intake in order to identify the true underlying mechanism, it is possible to address potential pathways that contribute to increased AD development.

One proposed pathway that has been implicated in several MetS disorders is high fat diet-induced activation of innate immunity through Toll-like Receptor 4 (TLR-4). Polymorphisms in TLR-4 in the context of diet have been associated with several MetS disorders and age-related diseases, indicating that this may be a relevant target in AD disease pathology [47,52,53] TLR-4 is part of a family of Toll-like receptors that respond and activate the innate immune response upon binding to various pathogen associated molecular patterns (PAMPs) found on bacteria, viruses, and fungi. Activation of the innate immune response increases levels of proinflammatory cytokines, chemokines, and immunoregulators. Classically, lipopolysaccharide (LPS), a main component in cell walls of gram-negative bacteria, is considered the ligand for TLR-4. Binding of LPS to CD14, a TLR-4 co-receptor,

activates a downstream cascade of TLR-4, ultimately leading to increases in Nuclear Factor Kappa-light-chain-enhancer of activated B-cells (NF-κB), a transcription factor that promotes production of pro-inflammatory cytokines and chemokines [34]. LPS contains three domains; an O antigen, a core polysaccharide, and a lipid A tail. Evidence has shown that the lipid A tail, which contains multiple saturated fatty acyl moieties, is the endotoxic component of LPS [38]. When LPS is deacylated, it loses its endotoxic properties, indicating that saturated fats may be responsible for activating the inflammatory cascade associated with TLR-4 activation [40,54].

Microglia, the central nervous system's primary innate immune cells, express TLRs 1-9 and are capable of mediating inflammation in response to several different kinds of stimuli [29]. A primary proposed mechanism for the development of neuronal degradation, and subsequent neurodegenerative disease, is the over-activation of microglia in producing persistent, low-level inflammation within the brain. Over-activation of microglia can be achieved through several different mechanisms. Microglial priming is the hypothesis that certain stimuli either early on or consistently presented, causes the microglia to be more responsive and inflammatory when presented with future stimuli. Another consideration is constitutive activation of TLR-4 through constant ligand recognition, as would be the case with components of an individual's diet. A 2009 article published by Milanski et al., demonstrated that high fat diet resulted in constitutively activated microglia in the hypothalamus, as well as increased ER stress [47].

Several studies have addressed the role of saturated fatty acids (SFAs) in activating TLR-4 in the context of several diseases [33,36,38,39,41-45,47,48,54-57]; however, the role of TLR-4 in high-fat diet-induced neuroinflammation in the hippocampus has not been

thoroughly addressed. We hypothesized that HFD would increase inflammation in the hippocampus after only 8 weeks of feeding, Further, loss of TLR-4 will protect against HFD-induced neuroinflammation.

METHODS AND MATERIALS

Animals and animal care. Weanling, male C3H/HeJ mice containing a naturally-occurring, loss-of-function TLR-4 mutation, their respective C3H/HeN control mice (NCI, Bethesda, MD); and C57BL/6J (Jackson Laboratories, Bar Harbor, ME) were housed at the University of North Carolina Animal Facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Animals were housed 4/cage under pathogen-free conditions and maintained under protocols approved by the Institutional Animal Care and Use Committee. The mice were randomized to either a high-fat diet (HFD) consisting of a 45% kcal by fat diet with soybean oil and lard as the fat source (D12451, Research Diets) or a 10% fat diet from the same fat source (D12450B). Water and food were available *ad libitum*. Body weights were measured at 6 and 12 weeks of age. After 8 weeks, mice were killed by rapid cervical dislocation and the brain was removed, quickly dissected and flash frozen.

Quantitative RT-PCR. Total RNA was isolated using a commercially available acid-phenol reagent (TRIzol; Invitrogen, Carlsbad, CA). Potential DNase contamination was removed with Amplification Grade Dnase 1 (Sigma Aldrich, St. Louis, MO). RNA was re-extracted for purification using phenol: chloroform (5:1) in isoamyl alcohol, chloroform: isoamyl alcohol (24:1), and re-precipitated using Molecular Grade ethanol (200 proof) and 9M glycogen. First-strand cDNA reverse transcription was synthesized using a master mix of M-MLV Reverse Transcriptase 200 u/μL (Promega, Madison, WI) with 5X RT buffer

(Promega), Oligo $(dT)_{15}$ primers, 10mM dNTP mix, and DEPC-treated water. Thermal conditions were 42°C for 60 min followed by 95°C for 5 min, and held at 9°C until storage at -20° C. Quantitative Real-Time PCR (qRT-PCR) was carried out with previously described TaqMan Assays on Demand Primer Probe pairs (Applied Biosystems, Branchburg, NJ) to determine the mRNA expressions of GAPDH, IL-1β, IL-6, IL-10, TNF-α, MIF, SOCS-3 in each tissue fraction for all three mouse strains. In addition, IFN-γ, MCP-1/CCL2, MIP-1α/CCL3, and RANTES/CCL5 mRNA expression was determined for C3H/HeN and C3H/HeJ mice. Real-time reactions were carried out on a CFX96 Real-Time System (BioRad, Hercules, CA) with BioRad CFX Manager using TaqMan Universal PCR Master Mix (Applied Biosystems). All genes of interest were normalized to GAPDH mRNA expression.

Microglial Isolation. Microglia were isolated following a previously published method (17). Briefly, whole brains were removed and homogenized in HBSS. Single cells suspensions were filtered through 70 μM nylon mesh and centrifuged at 500Xg for 5 min. The cells were re-suspended in 70% Isotonic Percoll (Sigma) and then layered with 50%, 35% and 0% isotonic Percoll. The Percoll gradient was centrifuged at 2000Xg for 20min at room temperature. The microglia layers were located at the 70/50% interphase and were carefully collected, washed 2 times with HBSS, and finally re-suspended in complete RPMI.

Microglial TNF-α and IL-6 ELISA Immunoassay. Isolated microglia were plated with 1 X 10^5 cells per well on a 96-well plate. Microglia were incubated with either media, Pam2CSK4 (a synthetic TLR2 ligand), or purified LPS for 16 hours. Supernatants were collected for quantifying TNF-α and IL-6 protein expression through ELISA assays (R & D systems).

Splenocyte TNF-α and IL-6 ELISA Immunoassay. Splenocytes were isolated in unsupplemented HBSS. Samples were processed into single-cell suspensions by mechanical agitation of a Stomacher (Seward) and strained througha 40-µM nylon filter. Cells were subjected to RBC lysis using ACK lysis buffer for 6 min at room temperature, washed, and counted. Isolated splenocytes were plated 1 X 10^6 cells per well on a 96-well plate. Splenocytes were incubated with either media, Pam2SCK4, or purified LPS for 48 hours. Supernatants were collected for qualifying TNF-α and IL-6 protein expression through ELISA assays (R & D systems).

Flow Cytometry. Previously plated microglia were washed and re-suspended in DMEM, 5% FBS, and incubated with anti-CD16/32 to block non-specific binding to F_c receptors. Microglia were then surface-stained with anti-CD11b, CD45, TLR-2, and TLR-4 antibodies for flow cytometric analysis to determine the Mean Fluorescence Intensity (MFI). Cells are analyzed using an Accuri Flow Cytometer (Accuri, Ann Arbor, MI) and data analysis is performed using Flow Jo software (Tree Star, Ashland, OR).

Serum Leptin and Resistin ELISA Immunoassay. To determine markers of obesity status and leptin resistance, serum leptin and serum resistin were measured by ELISA (R & D systems) in serum collected from fed mice.

Statistical Analysis. Weight data for C57BL/6 mice were analyzed using a Student's T test. Percent weight gain data and qRT-PCR data were analyzed using the Kruskall-Wallis test. Serum ELISA data were analyzed using 2 way ANOVA with post hoc Students' T test. Microglial TNF-α and IL-6 ELISA data were analyzed using 3 way ANOVA with post hoc Tukey's HSD. Flow Cytometry data was analyzed using Flow Jo Analysis software.

Microglial TLR-2 and TLR-4 MFI data were analyzed using a Student's T test, comparing treatment to media control. All statistical analyses were performed using JMP Statistical Software (SAS Institute). Values are the mean ± SEM. Data were considered statistically significant if $P < 0.05$.

RESULTS

C57BL/6 mice gain significantly more weight on the high fat diet after 8 weeks. After 8 weeks of high fat feeding, C57BL/6 mice gained significantly more weight ($p = 0.0002$) than low-fat fed C57BL/6 mice (Figure 2).

C57BL/6 mice have increased neuroinflammation in the hippocampus following a high-fat diet. In order to assess potential changes in neuroinflammation as an effect of HFD, qRT-PCR was performed on RNA extracted from hippocampi of C57BL/6 mice fed either a LFD or a HFD. TNF-α mRNA was significantly increased in the hippocampus of HFD-fed mice ($p = 0.03$) as compared to LFD-fed mice (Figure 3A). In addition, IL-10 was significantly decreased ($p = 0.006$) in the hippocampus of the HFD-fed mice (Figure 3B). There were no significant differences in hippocampal mRNA levels of IL-1β, IL-6, MIF, or SOCS-3 comparing LFD-fed and HFD-fed mice (data not shown).

HFD increased TLR-2 and TLR-4 on microglia harvested from C57BL/6 whole brain tissue. HFD significantly increased the expression of TLR-2 and TLR-4 on microglia isolated from whole brains compared to LFD-fed mice (Figure 4).

C3H/HeJ mice gained significantly more weight than C3H/HeN mice following a high-fat diet, corresponding to differences in serum leptin. In order to investigate the potential role of TLR-4 in mediating the observed increases in neuroinflammation in the hippocampus, we

utilized C3H/HeJ mice, which have a natural loss-of-function mutation in TLR-4, compared to C3H/HeN mice, their respective TLR-4 wild-type controls. After 8 weeks on the high fat diet, both C3H/HeN mice and C3H/HeJ mice gained significantly more weight ($p \leq 0.0001$) (Figure 5A). In addition, HFD-fed C3H/HeJ mice gained significantly more weight (20.8 ± 0.7%) compared to HFD-fed C3H/HeN mice (12.4 ± 0.8%) in respect to LFD-fed controls ($p \leq 0.0001$) (Figure 5B). Leptin was significantly increased in the serum of HFD-fed C3H/HeJ mice, but not in the HFD-fed C3H/HeN serum samples (Figure 5C). The leptin data corresponds to the weight gain data presented in Fig. 5B. Leptin was also increased in the C3H/HeN mice; however this increase was not significant.

HFD decreased IL-6 mRNA levels in hippocampi of C3H/HeN mice, but not C3H/HeJ mice. IL-6 mRNA was decreased ($p = 0.05$) in hippocampi isolated from HFD-fed C3H/HeN compared to LFD-fed controls (Figure 6A); however, this dietary effect was not seen in C3H/HeJ mice. Furthermore, there were no changes as an effect of diet in any of the pro-inflammatory cytokines tested, including TNF-α (Fig. 6B), IL-1β (Fig. 6C), IL-10, MCP-1, MIP-1α, or RANTES (data not shown).

HFD decreases MIF mRNA in the hippocampus of C3H/HeN mice, but not C3H/HeJ mice. MIF transcription was significantly decreased ($p \leq 0.01$) in the hippocampus (Figure 7) of HFD-fed CH3/HeN mice compared to LFD-fed controls; however, there was no difference between HFD-fed C3H/HeJ and LFD-fed C3H/HeJ mice.

HFD decreases SOCS-3 mRNA expression in the hippocampus of C3H/HeN mice, but not C3H/HeJ mice. SOCS-3 transcription is decreased ($p = 0.0562$) in the hippocampus of

HFD-fed C3H/HeN mice, but not in HFD-fed C3H/HeJ mice (Figure 8), indicating a potential role of TLR-4.

TLR-2 and TLR-4 receptor expression is upregulated following stimulation with LPS in microglia isolated from HFD-fed C3H/HeN mice in a TLR-4 dependent manner. TLR-2 and TLR-4 surface receptor expression was significantly upregulated ($\alpha = 0.05$) following stimulation with purified LPS in microglia isolated from HFD-fed C3H/HeN mice compared to media controls (Fig. 9A and Fig. 9B). There were no significant differences between LPS and media stimulation in microglia isolated from LFD-fed C3H/HeN mice, LFD-fed C3H/HeJ mice, or HFD-fed C3H/HeJ mice. Pam2CSK4, a synthetic TLR-2 ligand, did not increase TLR-2 or TLR-4 receptor expression in any samples compared to media controls (Fig. 8C and Fig. 8D).

TNF-alpha production was significantly decreased in microglia isolated from HFD-fed C3H/HeN mice following LPS and Pam2CSK4 stimulation compared to LFD-fed C3H/HeN microglia. LPS stimulation increased TNF-α expression in microglia isolated from both LFD-fed and HFD-fed C3H/HeN mice compared to media stimulated microglial controls. As expected, LPS did not increase TNF-α in microglia isolated from C3H/HeJ mice. In C3H/HeN microglia, TNF-α expression was decreased in HFD-fed microglia compared LFD-fed microglia following LPS stimulation (Fig. 10A). Pam2CSK4 increased TNF-α expression in microglia isolated from both LFD-fed and HFD fed C3H/HeN mice; however, there were no significant differences between Pam2CSK4 and media stimulation in microglia isolated from C3H/HeJ mice. TNF-α expression decreased in microglia isolated from HFD-fed C3H/HeN mice compared to LFD-fed C3H/HeN mice following Pam2CSK4 stimulation (Fig. 10B).

IL-6 production in microglia isolated from C3H/HeN and C3H/HeJ mice fed either a low-fat diet or a high-fat diet. LPS significantly increased IL-6 in microglia isolated from LFD-fed C3H/HeN mice compared to media controls. As expected, LPS did not induce IL-6 production from microglia isolated from C3H/HeJ mice. There were no differences in IL-6 expression between LFD and HFD in either C3H/HeN or C3H/HeJ mice following LPS stimulation (Fig. 10C). Pam2CSK4 significantly increased IL-6 protein expression in both LFD and HFD-fed C3H/HeN microglia but only LFD-fed C3H/HeJ microglia. There was no significant difference in IL-6 in microglia isolated from HFD-fed C3H/HeJ. There were no differences in IL-6 expression between LFD and HFD in either C3H/HeN or C3H/HeJ mice following Pam2CSK4 stimulation (Fig. 10D).

Effect of HFD-feeding on inflammatory response in isolated splenocytes. TNF-α production was significantly increased following LPS stimulation in splenocytes from both LFD-fed and HFD-fed C3H/HeJ mice as compared to media controls. As expected, LPS did not induce TNF-α production in splenocytes isolated from C3H/HeJ mice. (Fig. 11A). Pam2CSK4 stimulation significantly increased TNF-α production in all four treatment groups as compared to media controls; however, HFD did not augment this response (Fig. 11B). LPS stimulation resulted in significantly higher production of IL-6 in HFD-fed C3H/HeN mice only, indicating a role of TLR-4 in HFD-induced increases in IL-6 production (Fig. 11C). Stimulation with Pam2CSK4 increased IL-6 production only in C3H/HeN mice only, and there was no effect of HFD-feeding (Fig. 11D).

DISCUSSION

While many studies of the effect of HFD on the brain have focused on the hypothalamus [47,48,58], fewer have focused on the effects on the hippocampus. However,

there is a growing body of literature, in both rodents and humans, that points to a role for obesity and/or the consumption of a diet high in saturated fat on increased neuroinflammation, memory impairment, and dementia including Alzheimer's disease [1,8,9,15,18,19,21-23,25,30,49-51,59-72]. Excessive neuroinflammation caused by prolonged microglial activation has been hypothesized to play a major role in many neurological disorders and diseases including AD. The goal of this study was to determine if relatively short-term feeding of HFD could increase inflammation in the hippocampus. We found that after 8 weeks of HFD, C57BL/6 mice have increased inflammation in the hippocampus, as well as increased TLR-2 and TLR-4 receptor expression on isolated microglia. Several studies have reported that saturated fatty acids (SFA) drive expression of inflammatory molecules through TLR-4 activation, specifically activation of the pro-inflammatory transcription factor, Nuclear Factor Kappa-light-chain-enhancer of activated B-cells (NF-κB) [9,42,44,47,54,73]. Both TNF-α and IL-10 transcription are controlled, in part, through TLR-4 signaling in microglia [47]. In our study, 8 weeks of HFD-feeding upregulated both TLR-2 and TLR-4/MD-2 surface expression on microglia isolated from whole brain from C57BL/6 mice. Though our findings of elevated TLR-2 and TLR-4 in isolated microglia is not specific to the hippocampal region of the brain, it does indicate that microglia in the whole brain respond to HFD by upregulating inflammatory surface receptors. The increase in TLR-2 and TLR-4 receptors on HFD microglia could account for the increase in TNF-α mRNA expression in the hippocampus.

These findings are important as a very recent study has found that there may be a critical period for HFD exposure to result in learning and memory impairments [74]. In this study, only mice exposed to a HFD early in life showed deficits in learning and memory

tasks and altered neurogenesis in adulthood. Hence, we hypothesize that the inflammation in the hippocampus early in life, as seen in our study, could contribute to the deficits in learning and memory.

Previous studies have shown that in macrophage and hypothalamic microglia, SFAs are capable of signaling through TLR-4 to increase inflammation [39,44,47,54]. Therefore, we utilized the well-characterized C3H/HeJ model and the control C3H/HeN mice. In contrast to C57BL/6 mice, HFD for 8 weeks did not increase pro-inflammatory gene expression, nor decrease anti-inflammatory gene expression in C3H/HeN mice. In the C3H/HeN mice, which have intact TLR-4 signaling, we would have expected TNF-α transcription to increase in the hippocampus similar to C57BL/6 findings; however, we observed no significant changes at this time point. In addition, transcription of IL-6, another pro-inflammatory cytokine, was significantly decreased in the TLR-4 intact mice, indicating a possible role of TLR-4 in decreasing IL-6.

While there was no increase in pro-inflammatory cytokines in the HFD-fed C3H/HeN mice, we observed a decrease in Macrophage Migration Inhibitory Factory (MIF) and Suppressor of Cytokine Signaling-3 (SOCS-3). These decreases were abrogated in the C3H/HeJ mice, suggesting a role for TLR-4. MIF plays a role in many pathways including the immune system as well as lipid and glucose metabolism [74-81]. MIF expression is typically increased in obesity, and can be induced by the presence of TNF-α, glucocorticoids, PPAR-γ, HBP-1 and resistin [76,77,83]. In this present study, a diet high in fat caused a significant decrease in MIF transcription in a TLR-4 dependent manner. In addition, MIF transcription was significantly downregulated in a TLR-4 dependent manner after 8 weeks on the HFD in the liver of C3H/HeN mice (data not shown). Several studies have shown a

strong association between TNF-α production and MIF transcription. Though there were no differences in TNF-α mRNA expression, microglial activation assays reported a significant decrease in TNF-α production following stimulation with either a TLR-2 or TLR-4 agonist in the HFD-fed C3H/HeN microglia. It is possible that the less inflammatory microglia in the HFD-fed, TLR-4 intact mice could be contributing to the observed differences in MIF mRNA levels through decreased TNF-α production.

SOCS-3 is upregulated in the presence of pro-inflammatory cytokines, as a means of negative feedback. In addition, SOCS-3 has been implicated as one of the underlying mechanisms that leads to leptin and insulin resistance. Brain-specific SOCS-3 deficiency in mice has been shown to increase leptin sensitivity through increased STAT3 tyrosine phosphorylation and confer protection from diet-induced obesity, insulin resistance, and hyperleptinemia [84]. Our study demonstrated that 8 weeks of HFD-feeding decreased SOCS-3 expression in the hippocampus in a TLR-4 dependent manner. While leptin was increased in HFD-fed C3H/HeN mice, lower levels of SOCS-3 expression in HFD-fed, TLR-4 intact hippocampus is the opposite of what we expected. However, the high fat diet increased SOCS-3 expression in the C3H/HeJ mice in the liver (data not shown), which is expected considering a significant increase in serum leptin levels in these mice. The data pertaining to hippocampal SOCS-3 expression could be specific to this region of the brain. Additional studies are needed to further elucidate these results.

In our study, the HFD exposure increased microglia surface expression of both TLR-2 and TLR-4 following either LPS or Pam2CSK4 stimulation in a TLR-4 dependent manner. In C3H/HeN mice, despite upregulation in these inflammatory receptors, TNF-α production following either LPS or Pam2CSK4 stimulation was decreased in the microglia isolated from

HFD-fed C3H/HeN mice as compared to LFD-fed controls. In contrast to the microglia, there was a significant increase in IL-6 production and no difference in TNF-α production in splenocytes isolated from HFD-fed C3H/HeN mice as compared to respective LFD-fed controls. This may imply that 8 weeks of HFD-feeding was sufficient to increase the robustness of the inflammatory response in the periphery, but was not enough time to observe similar increases in the hippocampus.

While we were not able to determine if HFD-induced inflammation is induced by TLR-4 signaling, two recent studies have implicated that AMP-kinase and oxidative stress may be alternative mechanisms by which HFD increases inflammation. In one study, C57BL/6 mice fed resveratrol along with a HFD for 20 weeks, had decreased hippocampal TNF-α and improved insulin and energy metabolism within the hippocampus. Resveratrol-treated mice also had improved memory function compared to HFD mice. Importantly, resveratrol, while normalizing insulin and glucose levels, did not decrease weight, suggesting that obesity alone does not increase neuroinflammation [64]. Another study demonstrated that treatment of obese mice with ursolic acid, which has anti-inflammatory and antioxidant properties, improved the hippocampal-dependent learning and memory as measured by the Morris water maze task [85].

High-fat diets have been recognized as a potential risk factor for AD development, and increased neuroinflammation is hypothesized to contribute to AD [8,15,21-23,30,49-51,59,60,62,64,69,70,72,74,85-91]. We observed increased neuroinflammation in the hippocampus of C57BL/6 mice after 8 weeks of HFD-feeding. The C3H mouse model may not be a good model for understanding the effect of high-fat diet on neuroinflammation in the hippocampus and subsequent AD development/cognitive impairment. Future studies

utilizing TLR-4 knockout mice and treating with anti-oxidants or drugs that improve insulin sensitivity will further elucidate the underlying mechanism involved in HFD-induced hippocampal inflammation.

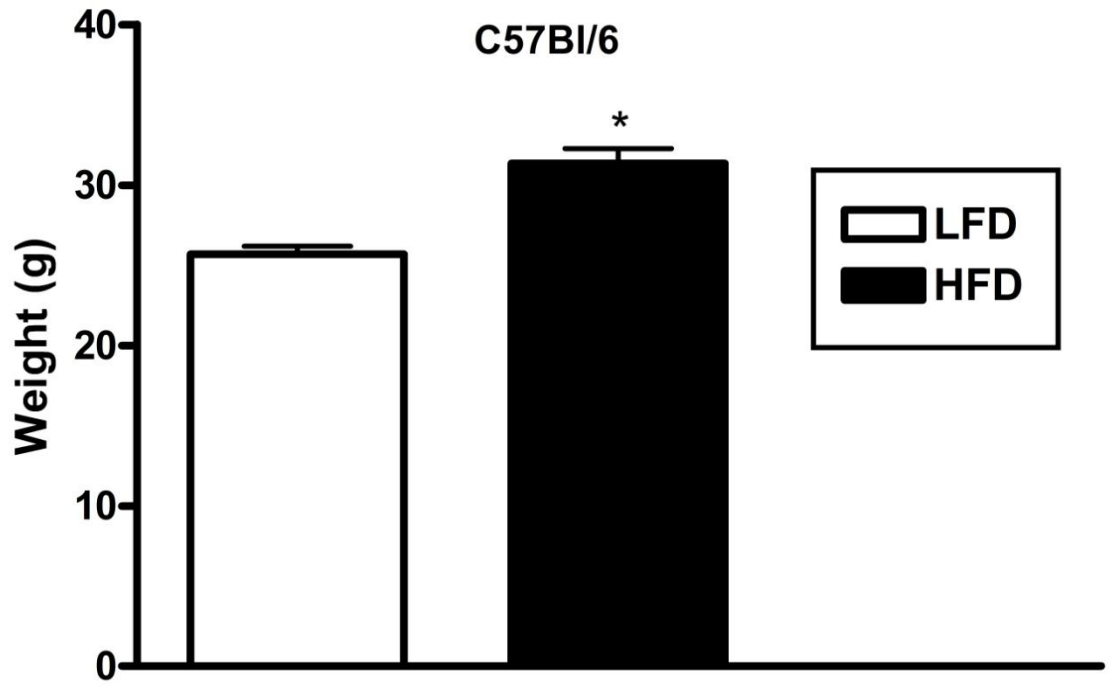

Figure 2. Mean weights of C57BL/6 mice fed for 8 weeks on either a low-fat or high-fat diet. Data are mean ± SEM, n = 8, 7. *different from LFD, $P < 0.05$.

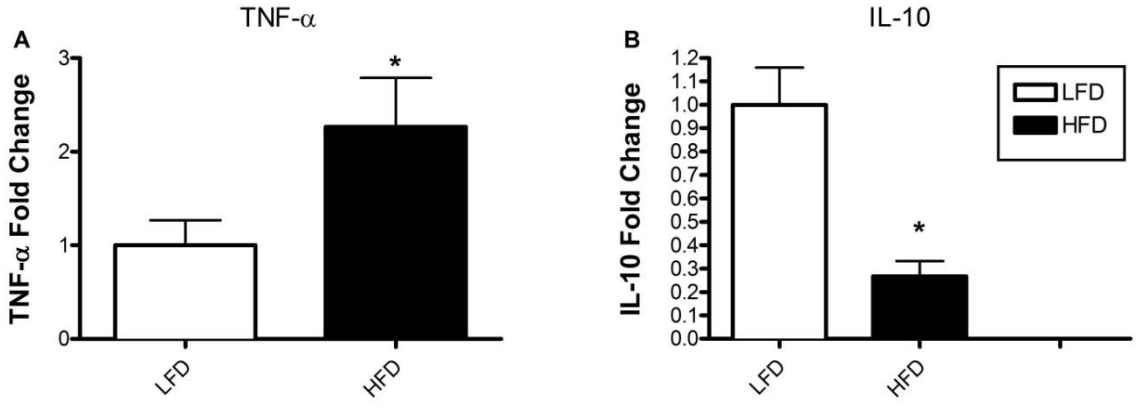

Figure 3. TNF-α expression (*A*) and IL-10 expression (*B*) in hippocampus from C57BL/6 mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM, n = 8, 7. *different from LFD, $P < 0.05$.

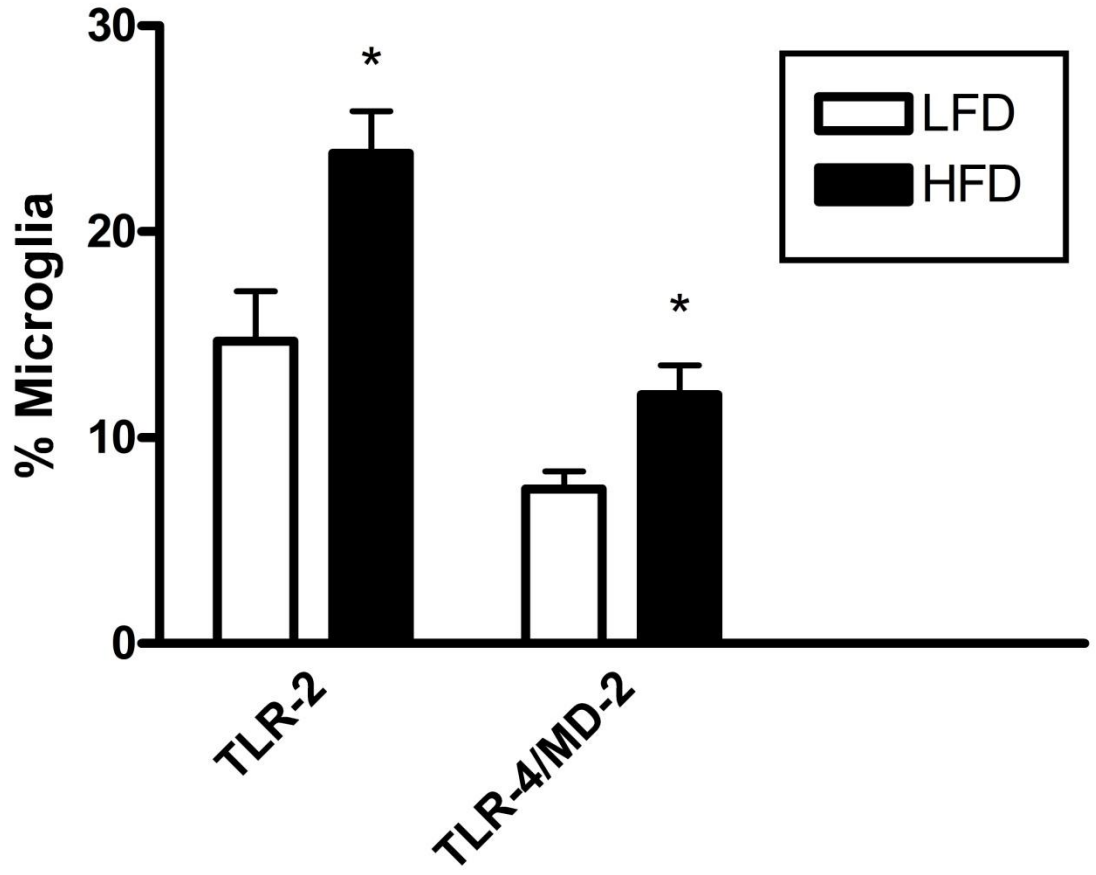

Figure 4. Percent of microglial population expressing TLR-2 and TLR-4/MD-2 surface receptors isolated from whole brain of C57BL/6 mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM, n = 8. *different from LFD, $P < 0.05$.

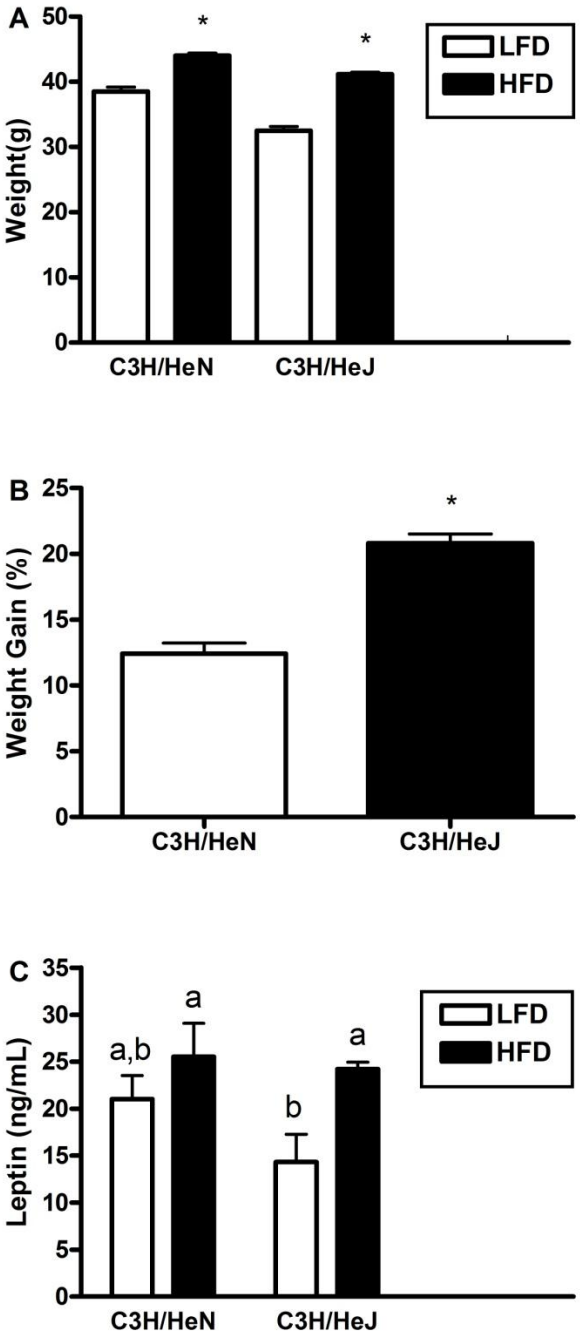

Figure 5. Mean Weights of C3H mice fed 8 weeks on either LFD or HFD (*A*), Percent Weight Gain after 8 weeks of HFD-feeding compared to LFD control (*B*), and Serum Leptin of C3H mice fed 8 weeks of either LFD or HFD (*C*). Data are mean ± SEM, n = 8.

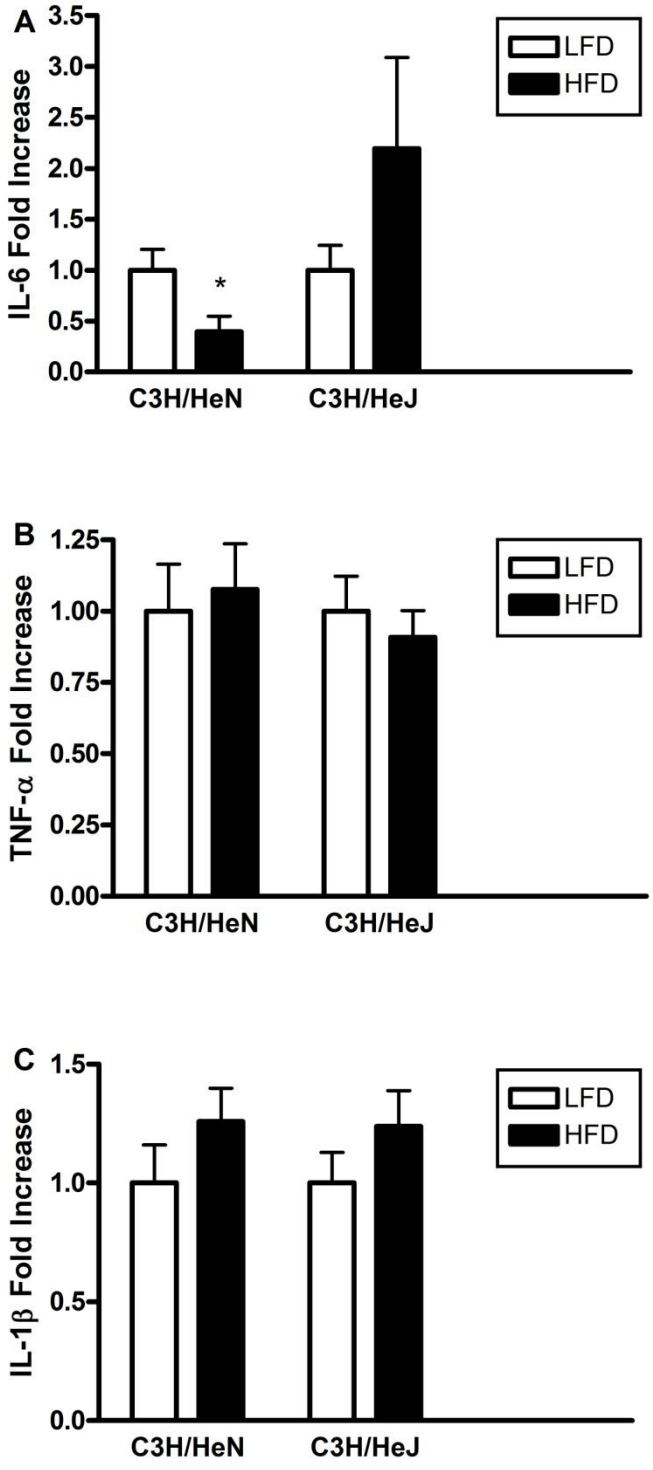

Figure 6. IL-6 expression (*A*), TNF-α expression (*B*), and IL-1β expression (*C*) in hippocampus of C3H mice fed 8 weeks of either LFD or HFD. Data are means ± SEM, n = 8. *different from respective LFD control, $P < 0.05$.

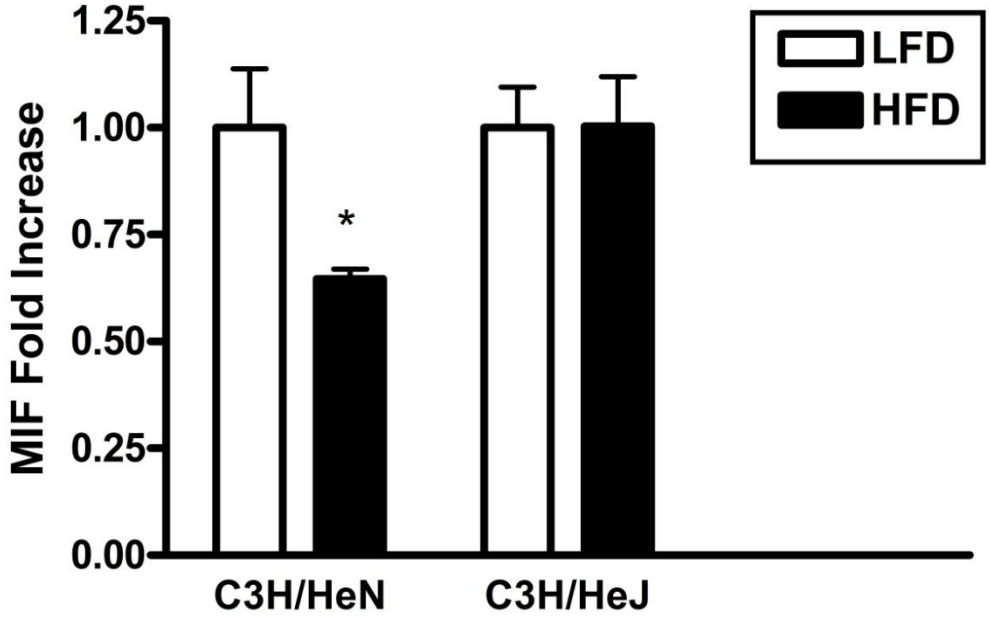

Figure 7. MIF expression in hippocampus from C3H/HeN and C3H/HeJ fed 8 weeks of either LFD or HFD. Data are means ± SE, n = 8. *different from respective LFD control, $P < 0.05$.

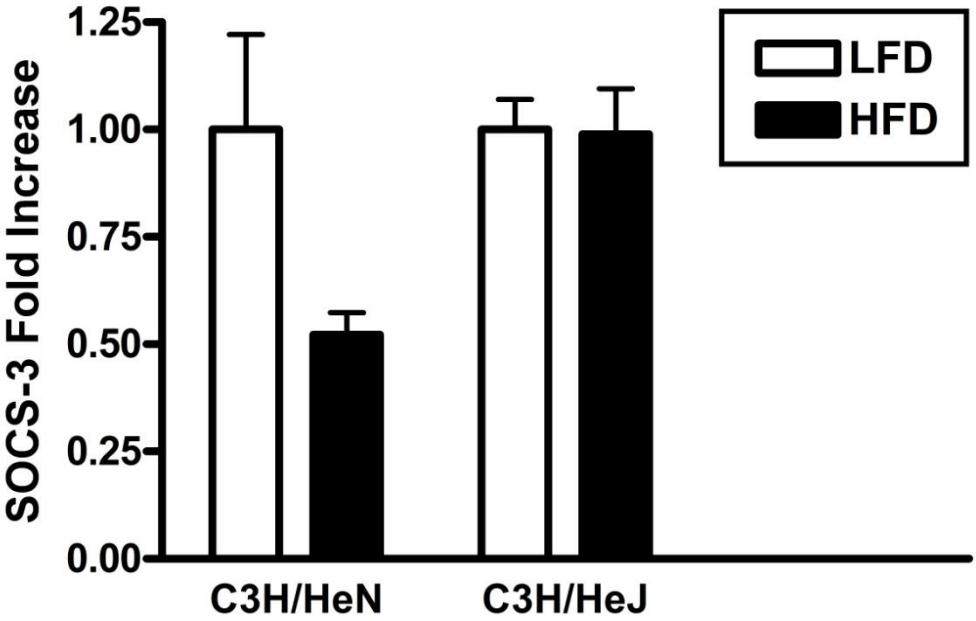

Figure 8. SOCS-3 expression in hippocampus of C3H/HeN and C3H/HeJ fed 8 weeks of either LFD or HFD. Data are means ± SEM, n = 8. $P = 0.0562$.

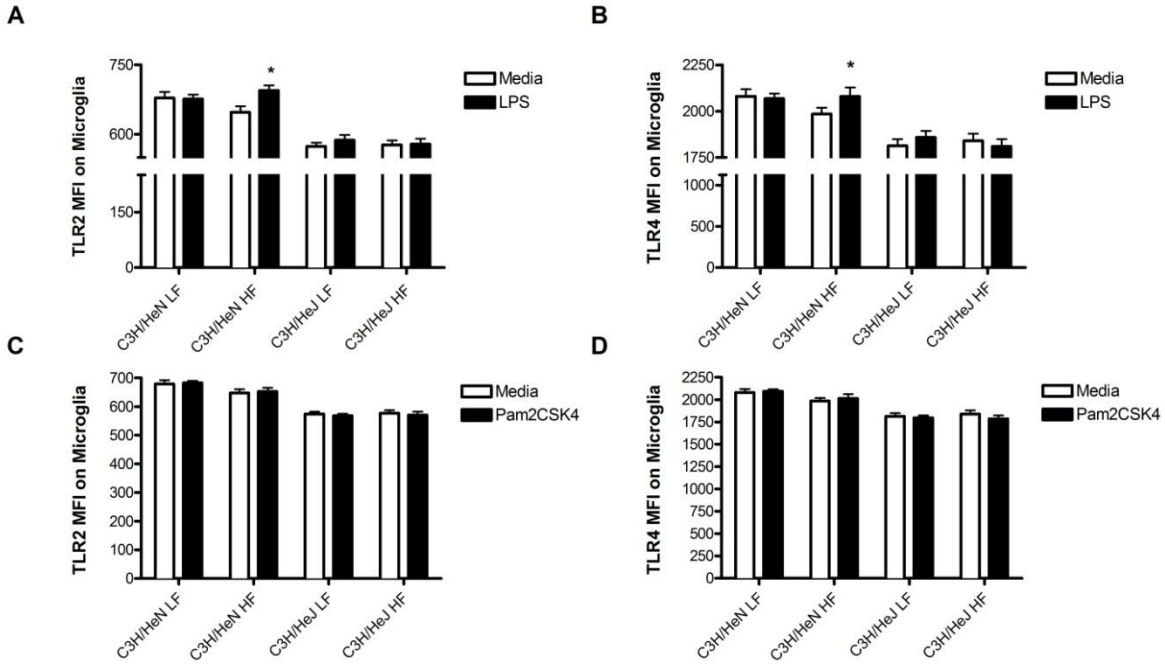

Figure 9. TLR-2 (*A, C*) and TLR-4 (*B, D*) surface expression (Mean Fluorescence Intensity) on microglia isolated from whole brain of C3H mice fed 8 weeks of either LFD or HFD. Data are means ± SEM, n = 6 (Media), 8 (LPS, Pam2CSK4). *different from Media stimulation, $P < 0.05$.

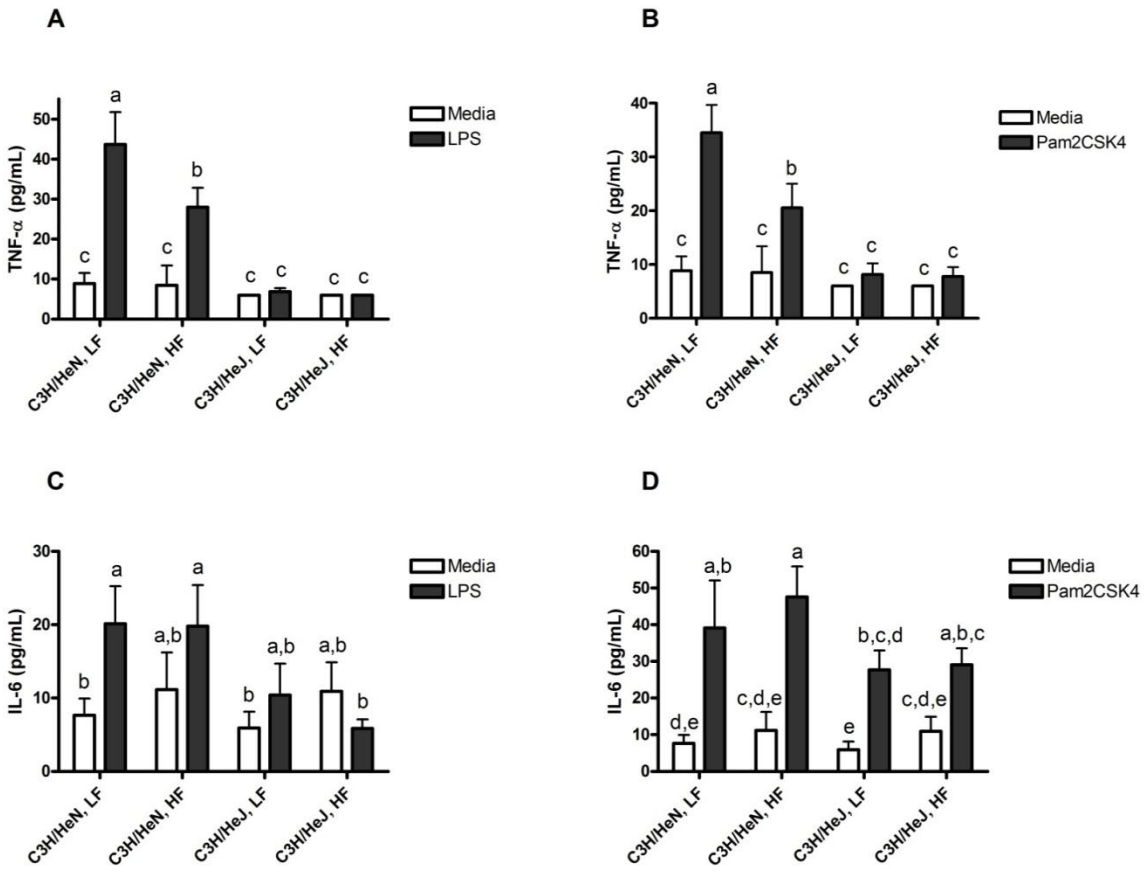

Figure 10. TNF-α and IL-6 production (pg/mL) in isolated microglia following LPS stimulation (*A, C*) or Pam2CSK4 stimulation (*B, D*). Data are means ± SEM, n = 6 (Media), 8 (LPS, Pam2CSK4). Labeled means without a common letter differ, $P < 0.05$.

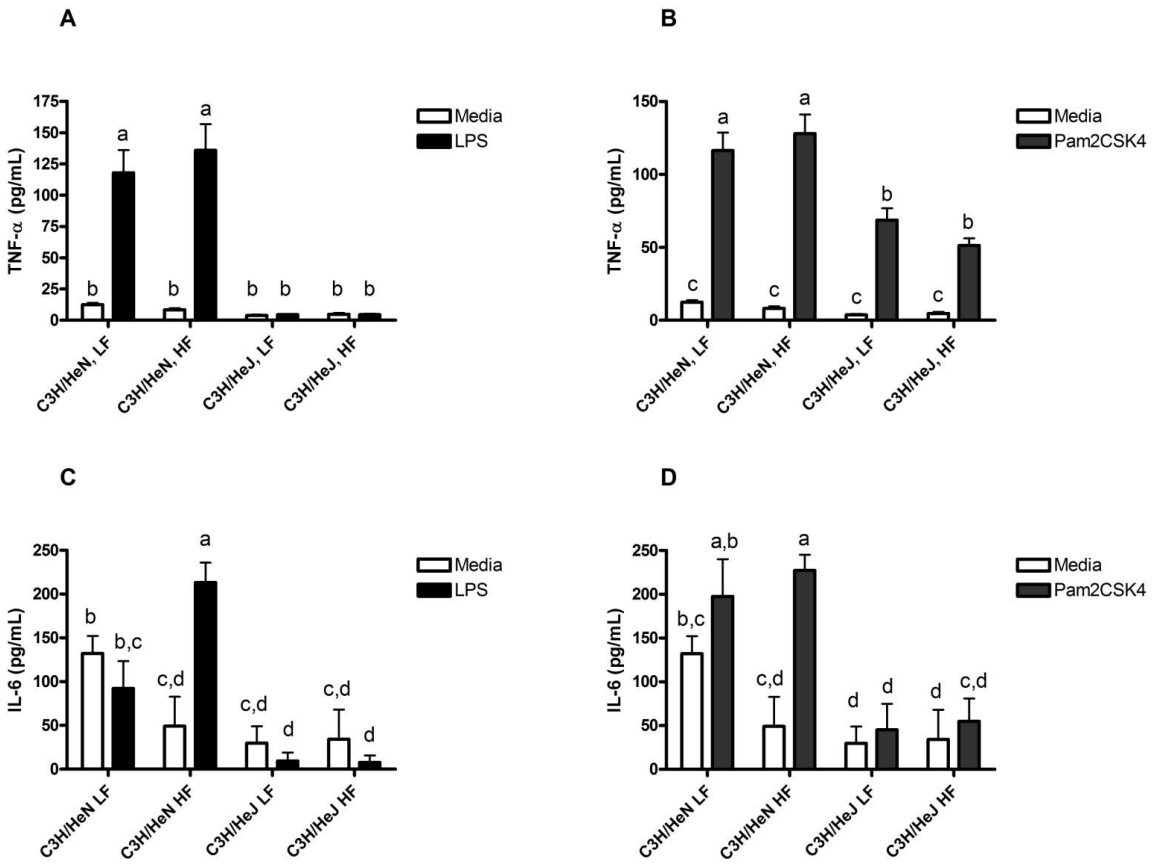

Figure 11. TNF-α and IL-6 production (pg/mL) in isolated splenocytes following LPS stimulation (*A*, *C*) and Pam2CSK4 stimulation (*B*, *D*). Data are means ± SEM, n = 6 (Media), 8 (LPS, Pam2CSK4). Labeled means without a common letter differ, $P < 0.05$.

Chapter III – Discussion

Determining the causative mechanisms underlying the development of neurodegenerative disorders is a primary research focus in the AD community. Previous investigations have identified several potential risk factors for AD including obesity, high-fat dietary patterns, cardiovascular disease, insulin resistance, type 2 diabetes, MetS, and genetic factors. Most of these potential risk factors, like AD itself, are manifested over long time periods and may be modifiable through lifestyle changes. The focus of this study is to investigate the effect of short-term HFD-feeding on neuroinflammation within the hippocampus. This preventable dietary risk factor may be responsible for other reported risk factors for AD development.

The results reported in Chapter 2 are presented in the context of current and previous investigations, and this chapter also includes discussion of the pertinent issues and research questions in this field of study. Some of the difficulties in studying the effects of HFD-feeding on parameters of neuroinflammation are in separating the effects of obesity *per se* from the acute effects of the dietary fat components, as well as determining the optimal duration of the diet needed to assess the adverse effects of short term HFD-feeding on the hippocampus.

Considerations for Designing Studies Investigating Short-term HFD-feeding effects on the Hippocampus

At the start of our study, the shortest high-fat diet duration to report neuroinflammatory changes within the brain was 8 weeks [47,48] which was used in determining the experimental protocol. A more recent study reported changes in pro-inflammatory cytokine expression after 1 and 3 days of HFD-feeding [58].

However, the Milanski (2009), Moraes (2009), and Thaler (2011) studies reported changes within the hypothalamus, an area of the brain relatively unprotected by the blood brain barrier (BBB). Diet-induced neuroinflammation and microglial activation in the hypothalamus could be important factors in the development of nutritional diseases such as obesity, insulin resistance, or leptin resistance. Though these risk factors are associated with AD development, the hypothalamus is not a brain area directly related to cognitive impairment or AD pathology. In order to study implications of short-term HFD-feeding on neuroinflammation in relation to potential causes of AD, we assessed inflammatory markers within the hippocampus, a region of the brain directly related to cognition.

The BBB is a physical, protective barrier between cerebral blood vessels and areas of the CNS. In contrast to hypothalamic areas of the brain, in which the BBB is "leaky" [106], the BBB regulates the entrance of specific molecules, including dietary fatty acids, into the hippocampus, indicating that short-term feeding effects like those seen in the Thaler study (2011), may be more difficult to evaluate [58]. Many dietary fatty acids, including SFAs, need prolonged exposures to cross the barrier. The BBB poses a potential obstacle in designing studies investigating acute HFD effects on hippocampal neuroinflammation, as well as other measures within the hippocampus. If the BBB regulates the entrance of certain

types of dietary fats, then an optimal diet duration exists for observing the effects of specific dietary fats on neuroinflammation, while minimizing the effects associated with HFD-induced obesity. This research question has important implications in interpreting the data.

Research investigating fatty acid transport through the BBB is sparse. The mechanism that regulates the transport of fatty acids across the tight junctions of the BBB is still unknown. Two prevailing theories are (1) passive diffusion using a "flip-flop" mechanism independent of protein-mediated transport and (2) protein-mediated fatty acid transport [46]. Passive diffusion would entail fatty acids separating from albumin at the BBB, since most proteins do not readily pass through the BBB's tight junctions. Upon separation from albumin, evidence supports the notion that the free fatty acid moves through the BBB in a transmembrane, "flip-flop" manner. In contrast, the active transport theory could include specific protein transporters including fatty acid transport proteins 1-6 (FATP 1-6), fatty acid translocase/CD 36 (FAT/CD36), and/or a variety of fatty acid binding proteins (FABPs). Though short and medium chain fatty acids, less than 12 carbons in length, move readily across the BBB, transport of long chain fatty acids, such as palmitic, stearic, or linoleic acid, is likely more regulated. FATPs and FAT/CD36 have high affinities for long-chain fatty acids such as palmitic or stearic acid, and are expressed in neural tissue. FABPs, specifically FABP7, which is expressed in the brain, have high affinities for polyunsaturated fatty acids such as docasahexaenoic acid (DHA) or ω-6 PUFAs such as linoleic acid [46].

Studies have explored the capacity for BBB penetration of certain types of dietary fats. A study conducted by Edmond et al. (1998) reported that perdeuterated

polyunsaturated fatty acids, particularly linoleic acid but also other ω-6 PUFAs, were readily transported into the developing brains of Sprague-Dawley rats. In addition, the brain is enriched in polyunsaturated fats, which are utilized for essential components i.e. structural membranes of brain cells. Edmond et al. (1998) also reported that in young, developing rats, perdeuterated monounsaturated and saturated fats did not cross the BBB [92]. In another experiment utilizing primary brain microvessel endothelial cells, Mitchell et al. (2011) reported that oleic acid (MUFA) was actively transported. In addition, RNA interference of FATP-1 or FAT/CD36 significantly prevented the transport of oleic acid. Taken together, research suggests that a combination of passive diffusion and active transport is likely involved in the process of fatty acid uptake into the brain [46].

Several reports have indicated that prolonged feeding exposure to a Western Diet (WD) increases BBB permeability [30]. The WD used in the Kanoski and Davidson study (2011) was high in both saturated fatty acids and simple sugars. Other reports have indicated that prolonged exposure to LPS can also increase BBB permeability, and several similarities exist between molecular structures of LPS and SFAs [30,94]. If the BBB becomes more permeable, hypothetically, increased SFA penetrance through the BBB would allow effects of HFD-feeding on potential cytokine production. Though transport of long-chain SFAs across the BBB is highly debated, HFDs, specifically high in saturated fats, have reported detrimental effects in cognition, neuroinflammation, and AD development. Changes in the BBB may allow for HFD effects to take place. To date, the proper diet duration to affect hippocampal neuroinflammation from acute HFD-feeding remains unknown

Effects of Short-term HFD-feeding on Neuroinflammation in the Hippocampus

Results from this study, specifically in C57BL/6 mice, indicate that 8 weeks of HFD-feeding is sufficient to increase TNF-α expression, a pro-inflammatory cytokine, in the hippocampus. In addition to increases in TNF-α expression, significant decreases were found in IL-10 expression, an anti-inflammatory cytokine. The combination of increased TNF-α expression and decreased IL-10 expression results in an increased neuroinflammatory state within this region of the brain. Cytokine production at the protein level within the hippocampus was not directly measured; however, transcriptional changes indicate an effect of HFD-feeding on activation of an inflammatory signaling pathway. Though 8 weeks of HFD-feeding limited obesogenic effects of the diet, C57BL/6 mice fed the HFD gained significantly more weight than their LFD-fed controls. Some of the effects reported may be due to changes related to diet-induced weight gain/obesity, rather than acute effects of the HFD.

Other studies have also utilized 8 weeks to assess changes in the hippocampus; however, either in different models or with very different diets. A 2008 study conducted by Thirumangalakudi et al. (2008) investigated the effect of a high-fat/high-cholesterol diet (1.25% cholesterol) compared to normal chow (0.05% cholesterol) in C57BL/6 mice LDLR WT, non-transgenic mice, as well as LDLR -/- and PDGFB-App^{wt} mice. Thirumangalakudi and co-researchers (2008) reported that C57BL/6 mice fed the high-fat/high-cholesterol diet for 8 weeks had increased TNF-α, IL-1β, IL-6, Nitric oxide synthase 2, and COX-2 expression in the hippocampus. The increase in neuroinflammation was accompanied by increased microglial and astrocyte activation, and these high-fat/high-cholesterol fed C57BL/6 mice were deficient in handling an increasing working memory load [72].

Another study conducted by Granholm et al. (2008) used a similar dietary strategy as the Thirumangalakudi study (2008), but in 16 month old Male Fischer rats. They used a high saturated fat diet that consisted of 2% cholesterol and 10% hydrogenated coconut oil compared to 12% soybean oil. Like in the Thirumangalakudi study (2008), Granholm et al. (2008) also reported increased microglial activation in the high saturated fat fed group, in addition to impairment in the cognition tests. Both studies utilized diets that were very different from our study, but indicated that 8 weeks is sufficient to detect neuroinflammatory effects of relatively, high-fat feeding [62].

Though reports of short-term HFD-feeding effects on neuroinflammatory changes in the hippocampus are limited, the Thirumangalakudi (2008) and Granholm (2009) studies indicated that diet durations of 8 weeks, an equivalent diet duration to our study, generated comparable results.

Studzinski et al. (2009) also investigated the short-term effects of HFD-feeding, specifically a 40% kcal from fat Western Diet for only 4 weeks, on oxidative stress and Aβ levels in the hippocampus. Studzinski and co-researchers (2009) used AD mouse models including a Cre-lox knock in of mutated, "humanized" APP and PS1 genes on a C57BL/6J background compared to wild-type (WT), non-transgenic littermates. Studzinski et al. (2009) reported that after only 4 weeks of HFD-feeding, both mouse strains, transgenic and WT mice, had significantly increased oxidative stress as measured through lipid hydroperoxides in the hippocampus. Oxidative stress is often accompanied by heightened levels of inflammation, indicating that the results of our study could be consistent with this report [71]. In addition, after 4 weeks of HFD-feeding both mouse strains had significantly increased weight gain, fasting glucose, and resistin levels. Plasma insulin, leptin, and adiponectin were

not significantly changed; however, Studzinski noted an upward trend in plasma leptin in HFD-fed mice. Like in the present study, Studzinski et al. (2009) were unable to fully separate the effects of short term WD-feeding from possible obesogenic effects. No changes in Aβ levels in either mouse type after only 4 weeks were reported [71], which suggests that HFD-effects on oxidative stress may precede alterations to Aβ levels in the hippocampus, even in genetically-predisposed mice.

Effects of Short-term HFD-feeding on Microglia

Another main finding of the C57BL/6 experiment presented herein was that 8 weeks of HFD-feeding increased the number of microglia expressing TLR-2 and TLR-4/MD-2 in whole-brain tissue. These toll-like receptors respond to various pathogen-associated molecular patterns (PAMPs) as part of the innate immune system [34]. It has been hypothesized, that in addition to recognition of PAMPs that are associated with bacterial, viral, and fungal pathogens, these receptors also can be activated through binding of SFAs [39,41,44,47]. Our data clearly indicated that the HFD upregulated the expression of these receptors and a greater number of microglia would potentially respond to TLR-2 and TLR-4 ligands.

In the C3H experiment, TLR-2 and TLR-4 was also upregulated by 8 weeks of HFD-feeding; however, this upregulation was only present in the C3H/HeN mice. This result suggests that HFD-induced TLR-2 and TLR-4 upregulation is mediated through TLR-4 signaling. Another study also reported that TLR-2 expressed on endothelial cells was upregulated in a TLR-4 dependent manner through neutrophil NADPH oxidase [93]. An unexpected finding in the C3H experiment was that even though TLR-2 and TLR-4 receptors were upregulated on the microglia isolated from whole brain tissue of HFD-fed C3H/HeN

mice, the microglial inflammatory response upon LPS stimulation was significantly decreased. In support of this finding, there was a lack of increased neuroinflammation in the HFD-fed C3H/HeN mice in the PCR data. It may be possible that downstream signaling molecules of the TLR-2 and TLR-4 pathways were downregulated as a result of 8 weeks of HFD-feeding in the C3H/HeN mice. Further study would be needed to elucidate this finding.

Both the Milanski (2009) and Moraes (2009) articles also assessed TLR-2 and TLR-4 levels, as well as activation of microglia; however, in contrast to our study which assessed these markers in C57BL/6 and C3H mice, both Milanski (2009) and Moraes (2009) assessed these changes in Wistar rats [47,48]. Moraes and co-investigators (2009) demonstrated that 8 weeks HFD-feeding with 55% kcal from lard was sufficient to increase levels of TLR-4 expression in hypothalami from Wistar rats, as well as F4/80, a glial cell activation marker [48]. Milanski et al. (2009) demonstrated that acute intracerebroventricular administration of arachidic acid resulted in increased TLR-2/MyD88 and TLR-4/MyD88 associations/activations in the hypothalami of Male Wistar rats [47]. The Granholm (2009) and Thirumangalakudi (2008) studies also reported increased microglial activation through immunohistochemical staining, indicating that HFDs results in increased microglial activation across several different rodent models [62,72].

Effects of Long-term HFD-induced Obesity on Neuroinflammation

As mentioned, it is often difficult to separate the effect of obesity from the acute effects of HFD on neuroinflammatory changes within the hippocampus. Both the Studzinski paper (2009) and our study limited these potential obesogenic effects on hippocampal neuroinflammatory markers [71]. However, in both models, significant weight gain occurred as a result of HFD-feeding. Obesity is associated with many physiological changes that have

a wide array of adverse effects on organs of the body and increases the risks of chronic diseases such as type 2 diabetes mellitus and heart disease.

One of the main findings in the C57BL/6 mice was increased TNF-α expression in the hippocampus. TNF-α is a potent pro-inflammatory cytokine and adipokine that is normally increased with higher adiposity. TNF-α plays an important role in initiating and regulating the cytokine cascade during an inflammatory response, mediated through NF-κB activation [107]. In addition, increased TNF-α expression is associated with increased AD risk and is found in the brains of AD patients. TNF-α also has been reported to increase BBB permeability [94], which in turn will increase the amount of TNF-α that can enter the brain after a given exposure time. It may be possible that some or all of the observed increases in TNF-α expression were the result of changes in peripheral inflammation. A future study could assess serum levels of TNF-α between different treatment groups. In order to separate effects of peripheral TNF-α expression on hippocampal TNF-α expression from hippocampal TNF-α expression due to HFD, a neutralizing anti-TNF antibody could potentially be used by injecting into peripheral circulation [95].

Though 8 weeks of HFD-feeding is a relatively short period for inducing obesity, studies investigating the effects of long-term HFD-induced obesity on various neuroinflammatory markers and cognition have shown clear detrimental effects over the long run. Long-term diet feeding introduces more potential factors into the development of both neuroinflammation and AD pathologies; however, they may also be representative of the etiology of AD, which occurs over a full life-time and is generally manifested after 65 years of age [22,25,67,69,70,74,86,].

Boitard et al. (2012) fed both weanling 3 week-old C57BL/6J and 12 week-old C57BL/6J mice either a standard chow diet (CD) consisting of 3 g% (approximately 6.5% kcal) from fat or a high-fat diet (HFD) (D#12451, Research Diets) consisting of 45% kcal from fat diet for 11 weeks. The high-fat diet used in this study was the same high-fat diet used in our work. Boitard and co-investigators (2012) tested mice in a two-stage relational memory flexibility arm maze test after 11 weeks of HFD-feeding. In addition, they also measured doublecortin (DCX) levels, an endogenous marker of newborn neurons, in dentate gyrus of the hippocampus. After 11 weeks of HFD-feeding, all HFD-fed treatment groups gained significantly more weight. Boitard et al. (2012) found that more weanling C57BL/6J mice were unable to habituate to the first stage of the relational memory flexibility test than the other 3 treatment groups. These mice were subsequently excluded from the second stage of testing. In the second stage of the behavioral test, they found of the mice that remained, weanling C57BL/6J mice were less able to associate visual cues in similar, but slightly different, arms of the maze. This indicated impairment in relational memory flexibility in HFD-fed, weanling C57BL/6J mice compared to CD-fed controls. These two behavioral findings were further supported by the finding that there was a significant decrease in DCX, indicating less neurogenesis, in the HFD-fed, weanling C57BL/6J mice compared to CD-fed controls. Neurogenesis is important in both development and cognitive ability. There were no differences between CD and HFD-fed adult C57BL/6J mice in any of the tests. This study conducted by Boitard et al. (2012) indicates that the age of the mice upon HFD-feeding is important in determining both neuronal and behavioral consequences [74].

Hwang et al. (2009) fed both weanling C57BL/6N and C3H/HeN mice either a low-fat diet (4.2% fat by weight) or a high-fat diet (34.9% fat by weight) for 12 weeks. Hwang

and co-researchers (2009) reported that HFD-fed C57BL/6N mice were more susceptible to body weight gain and lipid peroxidation in the CA1 region of the hippocampus than the LFD-fed C57BL/6N, LFD-fed C3H/HeN, or HFD-fed C3H/HeN mice. Lipid peroxidation is often accompanied by increased neuroinflammation. Given the similar design with differences mainly in diet duration, it is not surprising that the results in Hwang et al. (2009) would be in agreement with our data, which showed increased hippocampal neuroinflammation in the HFD-fed C57BL/6 mice; however, we were unable to replicate this increase in the C3H/HeN mice fed the same diet [86].

Another study conducted by McNay et al. (2010) fed Sprague-Dawley rats on either standard chow or a high-fat diet (31.8% kcal by butter and corn oil) for 14 weeks and tested cognitive performance. McNay et al. (2010) reported that HFD-feeding, in the presence of hyperinsulinemia or increased body mass, impaired cognition in several behavior tests. McNay (2010) also reported that HFD-feeding, in the absence of hyperinsulinemia or increased body mass, did not cause cognitive impairment and that microinjections of insulin into the hippocampal region improved cognitive performance in obese, cognitively impaired rats. These findings indicated an important role of insulin in the memory process [67].

A study conducted by Puig et al. (2011) fed 6 week old C57BL/6J mice either a high-fat diet (21.2% fat by weight) or a low-fat control diet (5.5% fat by weight) for 22 weeks. Puig and co-researchers reported diet-induced obesity contributed to increased TNF-α and activation of microglia and macrophage in both adipose tissue and hippocampal brain tissue, respectively. In addition, these pro-inflammatory changes were accompanied by increased brain levels of APP. The results in the Puig study (2011) agree with our hippocampal C57BL/6 data [70].

A study reported by Morrison et al. (2010) investigated the effects of a Western Diet (WD), a high-fat lard (HFL) diet, or corresponding low-fat control diets on hippocampal oxidative stress and cognition in "aged" mice. They fed 20 month old C57BL/6 mice the diets for 16 weeks. Morrison and co-researchers (2010) reported that even though both the WD and HFL treatment groups had increased body weight, adiposity, and fasting glucose, only the HFL group had increased age-related oxidative damage and impaired retention in behavioral testing [69]. Another study conducted in the same lab led by Pistell et al. (2010) tested middle-aged, 12 month old C57BL/6 mice with the same WD and HFL diets used in the Morrsion study (2010) for 21 weeks. The Pistell study (2010) reported that both WD and HFL treatment groups had increased body weight and astrocyte reactivity, but only the HFL group had increased cytokine production, impaired cognition, and decreased brain-derived neurotrophic factor (BDNF) [25]. Our diet was most similar to the WD used in this study, though differences did exist in the fat composition between each diet. Their findings when compared to our findings confirm that the age of the mice upon dietary treatment and diet durations is important in determining the neuroinflammatory effects, because we observed neuroinflammatory effects in our weanling C57BL/6 mice after only 8 weeks, whereas a similar diet was ineffective in increased neuroinflammation in older mice over a longer, dietary duration.

Additional studies have investigated the effects of very long-term HFD-feeding. Hwang et al. (2009) placed 3 week-old weanling male and female C57BL/6J mice on either a normal chow or a HFD for a 9 to 12 month dietary period. Hwang et al. (2009) reported that male mice were more vulnerable to the effects of the HFD. Obese male mice developed hypercholesterolemia, hyperglycemia, hyperinsulinemia, and hyperleptinemia, but not

hypertriglyceridemia. The obese female mice had less hypercholesterolemia and hyperinsulinemia than the obese males. In addition, obese male mice on a HFD had decreased learning performance compared to LFD-fed, normal controls. The decreased learning performance in HFD-fed obese males corresponded to impaired synaptic plasticity [22].

Another study conducted by Farr et al. (2008) fed 8 week old CD-1 male mice for 10 months on either a 5% fat regular chow or a 10% fat diet. The investigators determined obesity status based on more than a 30% weight gain over the feeding period. The obese CD-1 male mice had hypertriglyceridemia. Farr and co-researchers reported that obese CD-1 male mice displayed impaired acquisition in 3 different cognitive paradigms, and when triglycerides were lowered through administration of the drug, gemfibrozil, cognition was improved, indicating a potential role of hypertriglyceridemia in cognitive performance. Both studies investigated the effects of very long-term HFD-feeding, which may be representative of the long-term exposure of HFDs in contributing to increased AD risks, as reported in human epidemiological studies [21].

In addition to normal mouse and rodent models, i.e. C57BL/6 male mice, several studies previously mentioned investigated the effects of HFD-feeding and diet-induced obesity in AD mouse models. The Studzinski article (2009) used a humanized double-mutant of both the APP and PS-1 gene, both genes with implications in AD development. Studzinski and authors found that even though both models exhibited detrimental effects of 4 weeks of HFD-feeding, that the double-mutant exhibited the highest levels of lipid peroxidation. They also found that the changes in oxidative damage preceded the formation of Aβ plaques [71]. Another study conducted by Fitz et al. (2010) fed 9 month old APP23

mice, a transgenic AD mouse model on a C57BL/6 background, with a high fat/high cholesterol diet for 4 months. Fitz et al. (2010) demonstrated that high-fat feeding exacerbated cognitive performance, increased amyloid load, and increased insoluble Aβ levels. In both studies, HFD exacerbated AD pathologies in the AD mouse models indicating a potential role of HFD in AD pathogenesis [61].

The review of these mouse and rodent reports, partly summarized in **Table 2**, clearly indicates that high-fat feeding for periods of several weeks can lead to increased neuroinflammation in the hippocampus, impaired cognition, and can lead to pathology similar to neuronal changes observed in Alzheimer's disease. In addition, the effects of HFD-feeding are altered by numerous factors including, but not limited to, genetics, dietary composition of the HFD, dietary exposure time, and the age at exposure. Each of these factors can contribute to different results.

Differences between C57BL/6 mice and C3H mice in response to HFD-feeding

Several studies have reported a role of TLR-4 in mediating HFD-induced inflammation. The Milanski study (2009) reported that loss of TLR-4 activity in C3H/HeJ mice largely prevented increases in neuroinflammation within the hypothalamus [47]. In order to investigate how TLR-4 could mediate the observed neuroinflammation in the hippocampus, we utilized the same C3H/HeJ mice and their respective TLR-4 intact controls, C3H/HeN mice.

After 8 weeks of HFD-feeding, we were unable to replicate the increased hippocampal neuroinflammation reported in the C57BL/6 mice in the C3H/HeN mice. We hypothesized that C3H/HeN mice, which have intact TLR-4 signaling, would have had

comparable results to the C57BL/6 mice. In addition, we observed decreased neuroinflammation in the hippocampus of the C3H/HeN mice, specifically decreased IL-6 expression, and the results indicated that TLR-4 may have mediated this HFD-effect. Differences in the results between the C57BL/6 mice and the C3H mice imply that these two mouse models may have reacted differently to the experimental diet, because of unexplained genetic and/or environmental factors.

That two different mouse models would have experienced different effects of the same HFD was not unexpected. Inbred mouse strains can react differently given similar experimental conditions. C57BL/6 mice have been characteristically used in studies investigating the effects of diet-induced obesity (DIO), as well as HFD-induced diabetes/insulin resistance studies, because of their propensity to gain weight and become insulin resistant. In addition to a propensity for developing associated risk factors of AD from HFD-feeding, C57BL/6 mice have also successfully been used to demonstrate HFD-induced neuroinflammation in the hippocampus in many different experimental set-ups [64,69,70,72,74,86]. In future studies investigating the role of TLR-4 in mediating HFD-induced neuroinflammation in the hippocampus, a TLR-4 knock-out on a C57BL/6 background might be employed. Increased expression of neuroinflammation has been reported in AD patients [90]. The C57 mouse model may be a more suitable model than the C3H mouse model for representing relative short-term HFD-feeding effects on hippocampal neuroinflammation.

It appears that the brain of C3H mice are more protected against the acute effects of HFD-feeding, despite increases in peripheral inflammation as we reported in liver (data not shown) and isolated splenocytes. The Hwang study (2009) also reported that C3H/HeN mice

were more resistant to weight gain, oxidative damage, and microglial reactivity than C57BL/6N mice following 12 weeks of HFD-feeding. Given our results as well as the comparative analysis conducted by Hwang et al. (2009), definitive differences between the two mouse models in studying the effect of HFD on hippocampal neuroinflammation were evident [86].

This major difference in model responses to HFD is further supported by comparative studies investigating the effects of different infection models on both C3H/HeN and C57BL/6 mice. For example, a study conducted by Packiam et al. (2010) reported that both Balb/C and C57BL/6 mice were susceptible to *Neisseria gonorrhoeae* infection that resulted in increased cytokine production; however, these authors reported that C3H/HeN mice were resistant to infection with *N. gonorrhoeae* [96]. Many studies have compared C3H and C57 mice in different infection models of *Chylmadia*, with several reporting increased susceptibility in C3H mice [97,98]. In one study, however, the C3H mice did not produce as much TNF-α as the C57 mice, which the authors believed may have resulted in increased susceptibility to infection [97]. Genetic strain clearly plays a large role in determining the host response to certain pathogenic stimuli, including the induction of cytokine expression.

Another difference between these two mouse strains was their origin. C57BL/6J mice were purchased from Jackson Laboratories, whereas C3H mice were obtained from NCI. Both mouse strains were placed on the experimental diets at weanling, which was 3 weeks for C3H mouse strains and 4 weeks for C57BL/6J mice. It is possible, but unlikely, that the difference in age at weanling contributed to differences in neuroinflammatory response utilizing the same diets. It is more likely that differences occurred in how each

laboratory housed the dams for the two mouse types. Maternal diet can affect brain development during both pre-natal and post-natal, weanling periods.

One study conducted by Ranade et al. (2008) investigated the effects of three types of malnourishment diets (caloric restriction, low in protein, and low in iron content) on hippocampal development in Swiss albino mice. Ranade et al. (2008) reported that hippocampal sub-regions of the F1 generation were differentially vulnerable to changes in hippocampal volume, depending upon the particular malnutrition diet. Ranade et al. (2008) also reported that the volume differences in the hippocampal sub-regions of the F1 progeny corresponded with differences in working memory errors in behavioral testing. This study demonstrated that differences in diets can have different effects on hippocampal development [99]. It is not likely that either facility providing mice fed maternal diets to C3H or C57 dams that may have malnourished the pups; however, if the dietary composition was different in each group, developmental differences may have resulted.

Another study conducted by Tian et al. (2011) specifically addressed the effects of maternal diet differences in the ω-6/ω-3 PUFA ratio on brain development. Different ω-6/ω-3 PUFA ratio diets were given 2 months before conception and through the lactation period. Tian et al. (2011) reported that each diet produced different expression of several brain developmental genes, and that diets highest in ω-3 PUFAs may have been beneficial for early brain development [100]. Though previous studies are lacking, investigation of the particular effects of standard maternal mouse diets on brain development or brain lipid profiles across different mouse strains are needed. A future study might investigate how small differences inherent in breeding colonies maintained by different facilities could affect different brain

studies including developmental changes, brain lipid profiles, as well as later responses to experimental stimuli.

Differences between our results and the reported results of the Moraes study (2009) and the Milanski study (2009) require further examination. Moraes et al. reported increased inflammation and apoptotic gene expression in the hypothalamus of C3H/HeN mice after 8 weeks of HFD-feeding, and the Milanski article (2009) reported protection from DIO in the C3H/HeJ mice [47]. We were unable to replicate either result. Though Moraes et al. (2009) and Milanski et al. (2009) utilized similar HFDs to the one employed in this study, they both compared to standard rodent chows [47,48]. Our comparisons were made against a more relevant, low-fat defined control diet. The fat source used in our HFD and LFD were the same, whereas the fat, protein, and carbohydrate sources in their LFD controls could have been different from their experimental HFDs. Controlling for the type of fat source in the LFD would likely limit differences seen between the two treatment groups, in comparison to the diets used in the Milanski (2009) and Moraes (2009) studies. In addition, the age of the C3H mice used in the Milanski (2009) and Moraes (2009) studies were older, both 8 weeks old as compared to the 3-week old, weanling mice that were used in this study [47,48]. Developmental differences in the mice may have existed in the studies, as was demonstrated in the Boitard et al. study (2012) that could have affected comparisons of the research data [74].

Even though BBB permeability was not measured in either the C57 or C3H mice, one may postulate that the increased SFAs present in the HFD were able to enter the hippocampus of the C57 mice, but not the C3H mice, which would in part explain the

differences in the effects of the diets. As mentioned previously, a limited number of studies investigating fatty acid uptake in the brain exist, but the limited evidence that has been presented implies that PUFAs are readily taken up, whereas SFAs may only pass through BBB when it becomes leakier. In addition to increases in SFA content, the HFD used in our experiments with the C57 and C3H mice were particularly high in PUFAs, which have been reported to have some anti-inflammatory, neuroprotective effects [23,101,102]. The results reported for the C3H mice may reflect only the neuroprotective effects of PUFAs, and that SFAs were not readily taken up into the hippocampus; whereas for the C57BL/6 mice, 8 weeks was sufficient to allow SFAs to enter the hippocampus and increase neuroinflammation. One of the research questions that might be tested by another experiment would be if genetic strain or weanling age of the mice affects fatty acid uptake into the brain on experimental HFDs. A labeling experiment utilizing perdeuterated fatty acids could be employed in a time-course dependent manner to test the capacity for specific types of fatty acids to transport across the BBB into the hippocampi of different inbred strains over time.

The C3H model was unsuccessful in replicating and investigating the role of TLR-4 in HFD-induced neuroinflammation; however, it did provide some interesting results that add further support investigating the potentially neuroprotective effects of ω-6 PUFAs. Though these particular ω-6 PUFAs, i.e. linoleic acid, have been hypothesized to be pro-inflammatory through increased production of prostaglandins, leukotrienes, and prothromboxanes, associations have been previously reported in epidemiological studies between increased ω-6 FA intake with decreased AD risk. The experimental diet utilized in our study had particularly increased linoleic acid content as reported in **Table 3** in appendix.

A human study conducted by Morris et al. (2003) examined the associations between intake of different types of dietary fats with incidence of AD in Caucasian and African-American populations. Morris (2003) reported increased AD risk with increased dietary intake of saturated and *trans*-unsaturated fats, whereas there was a decreased AD risk with increased intake of monounsaturated and ω-6 polyunsaturated fats. Furthermore, Morris et al. (2003) reported an inverse linear association between AD risk and intake of vegetable fat ($p = 0.002$), and after adjusting for specific types of fats, a marginally significant inverse linear association was found between ω-6 PUFAs and AD risk [23]. In addition, animal studies have indicated that feeding with higher amounts of omega-6 PUFAs is protective against clinical incidence and histological manifestations of experimentally-induced autoimmune encephalomyelitis (EAE) [101] . Omega-6 fatty acids, specifically supplemental linoleic acid, were reported to be protective in human cases of multiple sclerosis [102]. Therefore, though studies are limited, some beneficial effects of ω-6 fatty acids have been noted in the brain.

Future Studies and Implications of Our Findings

Further study and experimentation is needed to elucidate several of the findings reported in this study. Testing tissues of C57 and C3H mice concordantly would be useful, which could allow to control for as many environmental factors as possible. Body weights and age of the mice should be held within a limited range of each other at the start of the study; however, inherent differences always exist between strains. In addition to replicating the data, a third treatment group of TLR-4 knockout mice on a C57BL/6J background could be used to test the functional role of TLR-4 in mediating increased neuroinflammation in the C57 strain. Data from this experiment would likely demonstrate differences between the

strains after 8 weeks of HFD-feeding and a role of TLR-4 in HFD-induced TNF-α expression in the C57 mice.

Serum leptin and resistin levels were measured in the C3H mice; however, additional serum measurements would be useful. Serum leptin and resistin levels should be measured in the C57 mice to make comparisons between strains. In addition, insulin plays important roles in the etiology of AD, memory processing, and obesity [13]. Insulin has also been reported to modulate the innate immune response and cytokine expression levels in several different models [103,104]. Serum insulin should be measured in all treatment groups, which may provide important information as to why differences in inflammatory response were seen between the two mouse models. A likelihood of differences in serum insulin will exist due to the propensity for C57 mice to develop insulin resistance after HFD-feeding. Downstream insulin signaling in all the treatment groups may also provide interesting data. In addition, as mentioned previously, a measurement of serum TNF-α and IL-6, and possibly other markers, may be important in all treatment groups.

Another key aspect of the mice which was not studied in the present experiment was the lipid profile of the mouse, both in the periphery and the brain. Lipid profiles would be an important variable because of differential effects on tissues. High-fat diets are known to affect peripheral lipid profiles. Docosahexaenoic acid, arachidonic acid, linoleic acid, and α-linolenic acid can all have major effects on brain lipid profiles during development [105]; however, there are far less studies investigating and reporting changes in brain lipid profiles as a result of increased dietary intake of saturated fatty acids. If brain lipid profiles differ between LFD-fed and HFD-fed mice in the hippocampus, this could contribute to potential differences in effects. There could also be differences in brain lipid profiles at baseline

between each genetic strain. Tissue lipid profiles may differ over time, as the BBB changes with prolonged exposure to the HFD.

Even with several uncertainties, the data from this study can be extrapolated to the human population. First of all, in the C57BL/6 mice, we demonstrated that even short-term HFD-feeding can be detrimental and have consequences. Neuroinflammation can lead to increased neuronal death, and after only 8 weeks of HFD-feeding, increased levels of neuroinflammation were found in the hippocampus. The fact that short-term feeding can have effects within the hippocampus implies that dietary interventions against detrimental diets and transitions to potentially beneficial diets could positively affect AD-prone individuals.

A major finding of this paper seems is that genetic strain of the mice can play a large role in determining the outcome of a particular diet on neuroinflammation, which is a known risk factor for the development of neurodegenerative disease. In addition, it is well established that certain genetic factors predispose individuals to be more at risk for AD later in life.

For at-risk individuals, it may be possible, with more supportive data, to design diet plans to limit risk for AD. For instance, for individuals carrying the APOE ε4 polymorphism, dieticians may promote diets that are low in fat, particularly in SFAs. Also, considering epidemiological data as well as some of the data presented in both the C57 and C3H mice, dieticians may want to promote a transition between the types of fats an at-risk individual may consume towards a diet higher in PUFAs than SFAs. Still, much remains to be learned, but early indications tend to support the notion that individuals with dietary

intake lower in SFAs tend to have better health outcomes including decreased AD risk. As more data begin to accrue, especially mechanistic data from animal models, new dietary strategies can be designed that will ultimately decrease the incidence of AD.

Table 2. Summary of Cited Studies Investigating Effects of High-fat Feeding on Neuroinflammation and Cognition

Investigators	Mouse/Rodent Model	Diet	Relevant Findings
Thaler et al. (2011)	Weight-matched Male Long-Evans Rats, weight-matched male C57BL/6 (ages unspecified)	Standard lab chow (PMI Nutrition International), high-fat diet containing 60% kcal fat (D#12492, Research Diets) Duration: Various with 1d, 3d, 7d, 14d, 28d, 4 weeks, 20 weeks	In Long-Evans rats • 4 and 20 weeks of HFD-feeding caused increased proinflammatory cytokine expression in hypothalamus, but 4 weeks was not sufficient to increase liver or adipose tissue inflammation • HFD-feeding acute time course experiment indicates an "on-off-on" pattern of hypothalamic cytokine expression with increased TNF-α, IL-6, and Ikbkb at 1d, increased IL-6, SOCS-3, Ikbkb, and Ikbke at 3d, decreased Il-1β at 7d, increased Ikbkb at 14d, and increased TNF-α, IL-6, SOCS-3, Ikbkb, and Ikbke expression at 28d • Microglial activation markers were increased in accordance with cytokine induction with increases at 1d, 3d, 14d, and 28d • Immunohistochemistry with Iba-1 indicated that activation of microglia after 3d In C57BL/6 mice • Proinflammatory cytokine expression in hypothalamus followed a similar expression pattern during the first 7 days of HFD-feeding
Studzinski et al. (2009)	1 month old APP X PS1 transgenic mice, 1 month old C57BL/6 WT littermates	Standard chow containing 10% fat (Diet #98052602), typical WD mix containing 40% fat (Diet #D12079B) Duration: 4 weeks	• WD resulted in increased protein carbonyls, protein nitrosylation, and lipid hydroperoxides in both WT and APP X PS1 mice hippocampi, with the highest levels of each oxidative stress marker in hippocampi of APP X PS1 mice • WD did not increase soluble or insoluble Aβ levels in either mouse strain
Thirumangalakudi et al. (2008)	4 month old C57BL/6J mice, 4 month old LDLR $^{-/-}$ mice	Normal chow (5% fat and 0.05% cholesterol), High fat/high-cholesterol (21% fat and 1.25% cholesterol) Duration: 8 weeks	C57BL/6J mice fed the high-fat/high-cholesterol diet • Were deficient in handling increased working memory loads compared with controls • had increased microglial and astrocyte activation • had increased TNF-α, IL-1β, IL-6, Nitric oxide synthase-2, COX-2, and β-site APP cleaving enzyme 1
Moraes et al. (2009)	8 week old Male Wistar Rats, 8 week old C3H/HeN mice, 8 week old C3H/HeJ mice, 8 week old Swiss	Standard chow (9% kcal saturated fat), High-Fat chow (HF) (55% kcal saturated fat) Duration: 8 weeks	In Male Wistar Rats • HF diet led to increased hypothalamic expression of TNF-α, IL-1β, IL-6, protein levels of SOCS-3, pJNK, pIKK, and glial cell activation marker F4/80. In C3H/HeJ mice • gained significantly less weight than C3H/HeN mice • Increased expression of Bax, association of APAF1 with caspase-9,

	mice		association of FADD with caspase-8, and decreased Bcl-2. • Increases in pro-apoptotic markers in C3H/HeJ mice were accompanied by ER stress markers pPERK and pJNK.
Milanski et al. (2009)	4 week old Male Wistar Rats, 8 week old C3H/HeN mice, 8 week old C3H/HeJ mice	Standard rodent chow (CD) (4.0 g% or approx. 9% kcal), a high-fat chow (HF) containing 36.0 g% fat (or approx. 56% kcal) from unspecified animal source, a unsaturated fat-rich chow (OL) containing 36.0 g% from olive oil Duration: 8 weeks for C3H/HeN and C3H/HeJ, 16 weeks for Male Wistar Rats	In Male Wistar Rats • 16 weeks of HF-feeding increased expression of TNF-α, IL-1β, IL-6, but not IL-10 in the hypothalamus • Increased cytokine expression was accompanied by increased F4/80 expression in cells mostly present in the medial eminence and arcuate nucleus of the hypothalamus • Intracerebroventricular (ICV) injection of arachidic acid for 1 to 3 days resulted in increased TLR-2/MyD88, TLR-4/MyD88 associations, and levels of ER stress markers including UPR, pJNK, pPERK, and peIF2α • Most TLR-4 positive cells in the hypothalamus were found to be microglia cells expressing F4/80 • TLR-4 inhibiting antibody completely restrained the capacity for ICV injection of arachidic acid to induce TNF-α, IL-1β, IL-6, and IL-10 expression
Milanski et al. (2009)			In C3H/HeN and C3H/HeJ mice • HF-fed, C3H/HeN mice presented 50% body mass gain compared to 30% body mass gain in HF-fed, C3H/HeJ mice • C3H/HeJ mice failed to induce a remarkable increase in IL-6 or IL-10 expression after a 3 day ICV treatment of arachidic acid
Granholm et al. (2008)	16 month-old Male Fischer Rats	12% soybean oil diet, "Sat-fat" diet consisting of 10% hydrogenated coconut oil and 2% cholesterol Duration: 8 weeks	• "Sat-fat" fed rats committed more working memory errors in the radial arm maze, especially at higher memory loads • Increased microglial activation in the hippocampi of "Sat-fat" fed rats was observed through immunohistochemical staining
Boitard et al. (2012)	3 week-old C57BL/6J, 12 week-old C57BL/6J	Standard chow diet consisting of 3 g% from fat (approx. 6.5% kcal), high-fat diet (HFD) consisting of 45% kcal from fat (D#12451) Duration: 11 weeks	• After 11 weeks of HFD-feeding, both weanling and adult C57BL/6J mice had similar morphometric and metabolic changes • Only HFD-fed weanling mice were impaired in both stages of the two-stage relational memory flexibility test • Only HFD-fed weanling mice had decreased neurogenesis as measured through doublecortin levels in the dentate gyrus of the hippocampus • Results indicate a critical period during development for HFD-effects on hippocampus

Hwang et al. (2009)	Weanling C3H/HeN mice, weanling C57BL/6N mice	Low-fat diet (LD) consisting of 10% kcal from fat, high-fat diet (HD) consisting of 60% kcal from fat Duration: 12 weeks	• HD-fed C57BL/6N mice body weights and weight gains were significantly higher than LD-fed C57BL/6N, LD-fed C3H/HeN, or HD-fed C3H/HeN • C57BL/6N were more susceptible to HFD-induced lipid peroxidation in CA1 region of the hippocampus than C3H/HeN
McNay et al. (2010)	1 month-old Sprague-Dawley Rats	Standard lab chow, high-fat diet (HFD) consisting 31.8% kcal from butter and corn oil Duration: 14 weeks	• HFD, in the absence of hyperinsulinemia or increased body mass, did not affect memory performance • Diet-induced obese rats showed impaired cognition compared to controls • Insulin injection into the hippocampus improved cognition in cognitively impaired rats
Morrison et al. (2010)	20 month-old C57BL/6 from National Institute of Aging	Low-fat control diets (98052602 or D12450B), Western Diet (WD) (D12079B) consisting of 40% butterfat and 29% sucrose, high fat lard diet (HFL) (D12492) consisting of 60% animal fat Duration: 16 weeks	• Both WD and HFL fed mice had increased body weight, adiposity, fasting blood glucose • Only HFL fed mice had increased age-related oxidative damage (protein carbonyls) and impaired retention in a behavioral test • Results implicated a potential role of an antioxidant enzyme, Nrf-2
Puig et al. (2011)	6 week-old APP^{tmlDbo}/J homozygous ($APP^{-/-}$) mice, 6 week-old wild-type C57BL/6J mice	Low-fat diet (LFD) consisting of 5.5% fat by weight (approx. 11.6% fat by kcal), High-fat diet (HFD) consisting of 21.2% by weight (approx. 37.7% fat by kcal) Duration: 16 weeks	• Adipose and brain tissue from HFD-fed mice had increased TNF-α, microglial (brain) or macrophage (adipose) activation • HFD-fed mice had increased APP proteins in brain and adipose
Jeon et al. (2012)	4 week-old C57BL/6J mice	Low-fat chow (LFD) consisting of 10% kcal from fat, High-fat diet (HFD) consisting of 60% kcal from fat (Diets from Research Diets) Duration: 20 weeks	• HFD-fed mice had decreased serum adiponectin and glucose tolerance, and increased serum leptin, serum glucose, and serum insulin • HFD increased TNF-α and Iba-1 protein levels • HFD increased microglial activation in hippocampus • HFD increased 4-HNE levels compared to LFD-fed controls • HFD increased neurodegeneration in hippocampus
Hwang et al. (2010)	3 week-old, weanling	Normal chow consisting of 13.5% kcal from fat, High-fat diet (HFD)	• Obese male mice developed hyperglycemia, hyperinsulinemia, hypercholesterolemia, and hyperleptinemia

	C57BL/6J, both male and female	consisting of 45% kcal from fat Duration: 9 to 12 months	• Obese female mice had less hyperinsulinemia and hypercholesterolemia than obese males • Obese male mice had decreased learning performance than LFD-fed normal controls • Obese male mice had impaired synaptic plasticity compared to controls • Males were more vulnerable to HFD effects
Farr et al. (2008)	8 week-old CD-1 mice	Regular chow (13.5% kcal from fat), High-fat breeder chow (27% kcal from fat) Duration: 10 months	• Obesity status was determined by 30% weight gain compared to regular chow controls • Obese mice showed impaired acquisition in 3 different cognitive paradigms: active avoidance T-maze, Morris Water maze, and a food reward lever press • Intracerebrovascular (ICV) injection of triolein (triglyceride) caused significant cognitive impairment • ICV injection of palmitate did not significantly differ from saline control ICV injection • No differences were found in protein carbonyl levels or 3-nitrotyrosine levels between mice on the 5 and 10% fat diets • ICV injection of gemfibrozil, a triglyceride lowering drug, improved cognition in impaired, obese mice

Appendix – Supplementary Data

The following figures and table include additional PCR data collected throughout the course of this research project, a full breakdown of the dietary composition of the research diets, and additional serum ELISA data. In addition to collecting the hippocampus, we conducted PCR on tissue from the brain stem and hypothalamus of the C3H mice.

In **Table 3** and **Table 4**, a full breakdown of the dietary composition is listed for the study's research diets (D12450B and D12451) as reported by ResearchDiets Inc.

Table 3. Fatty Acid Composition of LFD (D12450B) and HFD (D12451) from Research Diets

Ingredient	D12450B (low fat control)	D12451
Lard	20	177.5
Soybean Oil	25	25
Total Fat Added	45	202.5
C2 – Acetic	0	0
C4 – Butyric	0	0
C6 – Caproic	0	0
C8 – Caprylic	0	0
C10 – Capric	0	0.1
C12 – Lauric	0	0.2
C14 – Myristic	0.2	2.0
C14:1 – Myristoleic	0	0.0
C15	0	0.1
C16 – Palmitic	6.5	36.9
C16:1	0.3	2.4
C16:2	0	0
C16:3	0	0
C16:4	0	0
C17	0.1	0.7
C18 – Stearic	3.1	19.8
C18:1 – Oleic	12.6	64.4
C18:2 – Linoleic	18.3	56.7
C18:3 – Linolenic	2.2	4.3
C18:4 – Stearidonic	0	0
C20 – Arachidic	0	0.3
C20:1	0.1	1.1
C20:2	0.2	1.4
C20:3	0	0.2
C20:4 – Arachidonic	0.1	0.5
C20:5 – Eicosapentaenoic	0.2	0
C21:5	0.0	0
C22 – Behenic	0	0

C22:1 – Erucic	0	0
C22:4 – Docosapentaenoic	0	0
C22:6 – Docosahexaenoic	0	0
C24 – Lignoceric	0	0
C24:1	0	0
Total	43.7	191.1
Saturated (g)	9.9	60.0
Monounsaturated (g)	13.0	68.0
Polyunsaturated (g)	20.7	63.2
Saturated (%)	22.7	31.4
Monounsaturated (%)	29.9	35.6
Polyunsaturated (%)	47.4	33.0

Table 4. Macronutrient and Micronutrient Composition of LFD (D12450B) and HFD (D12451) from Research Diets

	D12451		**D1240B**	
Macronutrient	gm%	kcal%	gm%	kcal%
Protein	24	20	19.2	20
Carbohydrate	41	35	67.3	70
Fat	24	45	4.3	10
Total		100		
kcal/gm	4.73		3.85	
Ingredients	gm	kcal	gm	kcal
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	315	1260
Maltodextrin 10	100	400	35	140
Sucrose	172.8	691	350	1400
Cellulose, BW200	50	0	50	0
Soybean oil	25	225	25	225
Lard	177.5	1598	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H20	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Red Dye #40	0.05	0	0.05	0
Total	858.15	4057	1055.05	4057

Figures 12 – 19 include additional non-significant PCR data collected in the hippocampus of C57BL/6 and C3H mice, as well as PCR data collected in the brain stem and hypothalamus. Brain-stem and hypothalamus gene expression was assayed using the protocol previously described in Chapter 2. Expression of pro-inflammatory cytokines including IL-1β, IL-6,

TNF-α, and IFN-γ, expression of the pro-inflammatory mediator, MIF, expression of an anti-inflammatory cytokine, IL-10, and expression of anti-inflammatory mediatory, SOCS-3, were all assayed. Of particular note, MIF expression was decreased in the hypothalamus of HFD-fed C3H/HeN mice compared to LFD controls. There were no other significant differences in expression of any of the genes assayed in C3H or C57BL/6 mice.

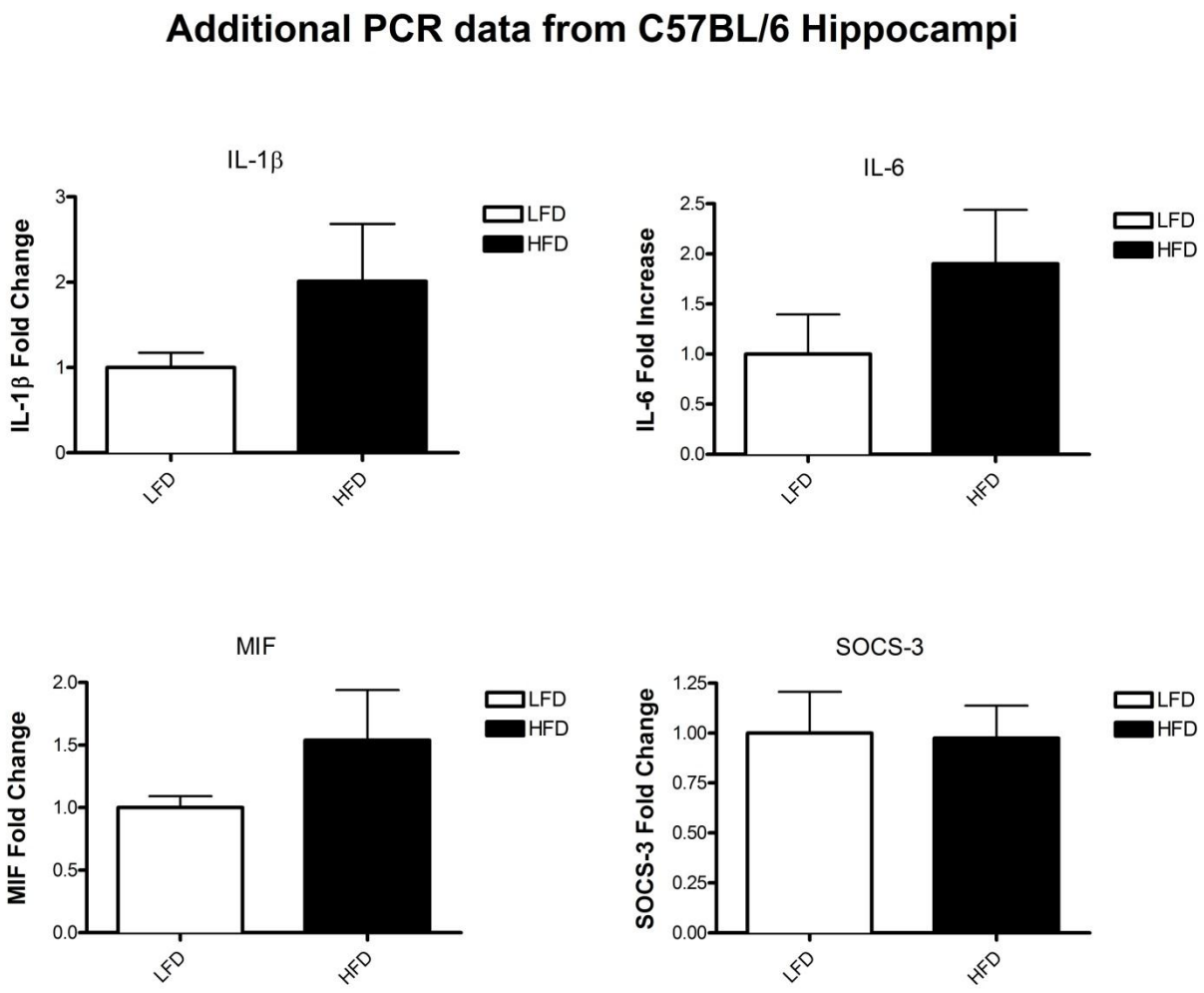

Figure 12. IL-1β expression, IL-6 expression, MIF expression, and SOCS-3 expression in the hippocampus of C57BL/6J mice fed 8 weeks of either LFD or HFD. Data are means ± SEM. *difference from LFD control, $P < 0.05$.

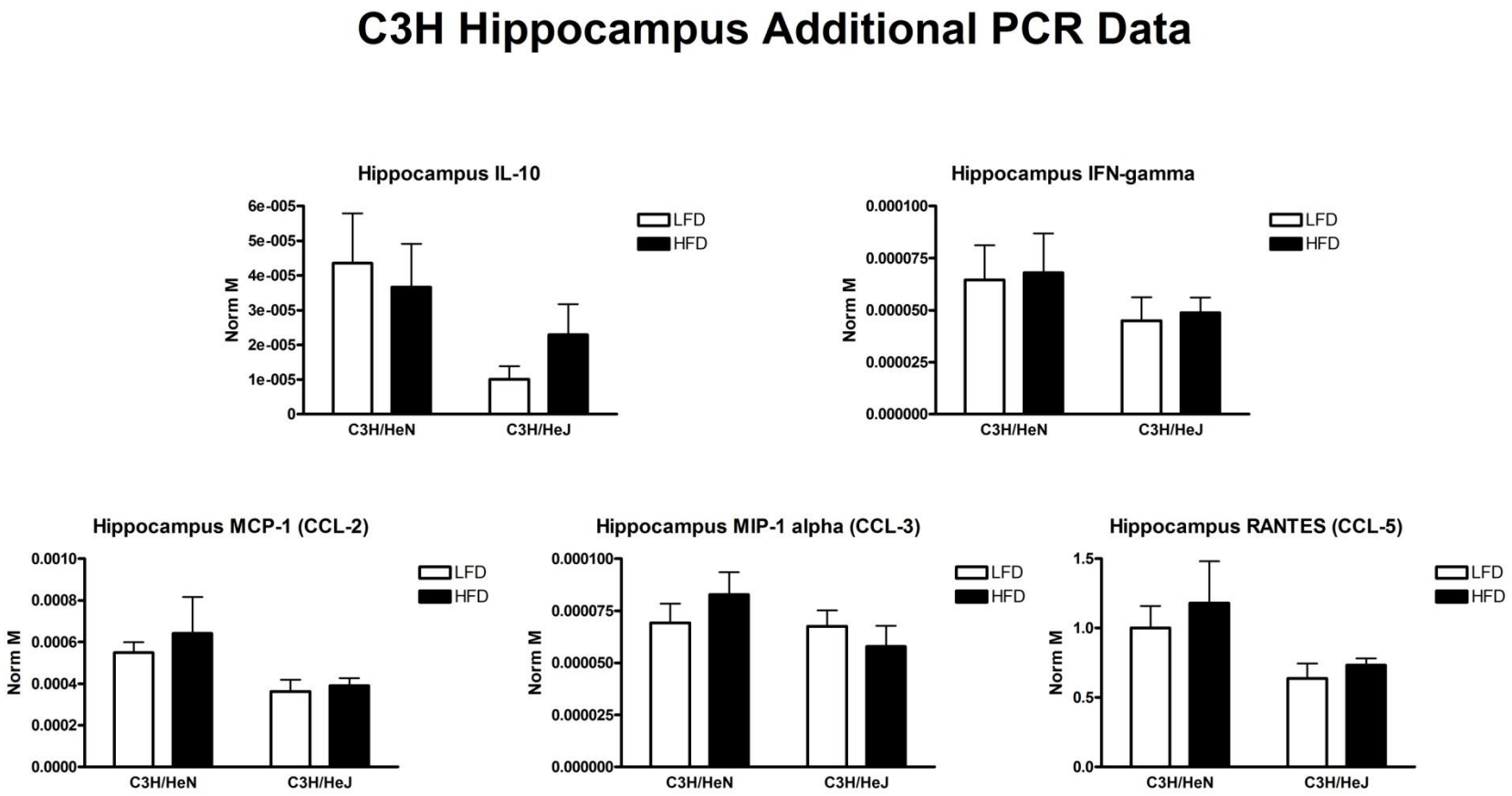

Figure 13. IL-10 expression, IFN-γ expression, MCP-1 (CCL-2) expression, MIP-1α (CCL-3) expression, and RANTES (CCL-5) expression in the hippocampus of C3H mice fed 8 weeks of either LFD or HFD. Data are means ± SEM. *difference from LFD control, $P < 0.05$.

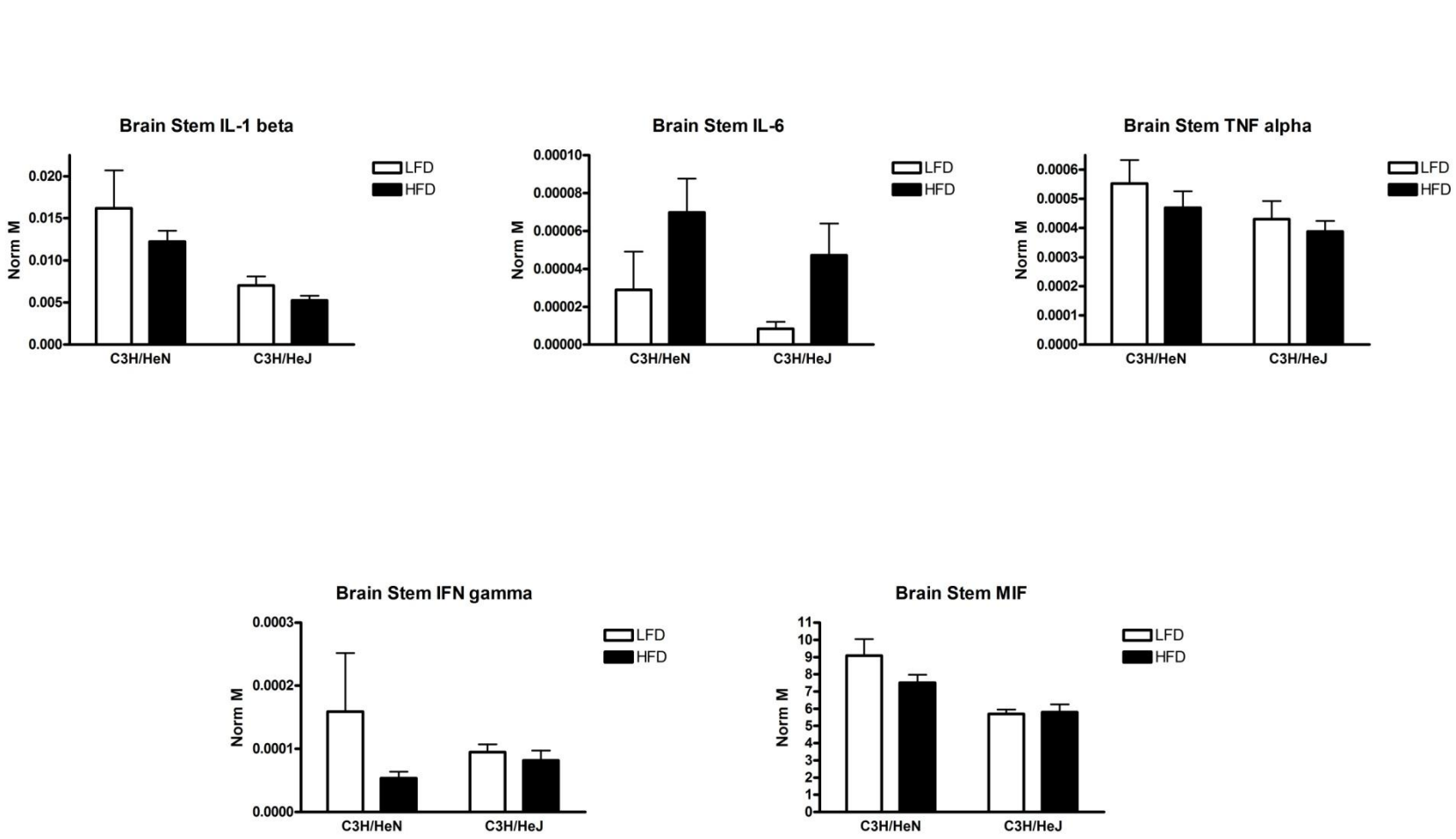

Figure 14. IL-1β expression, IL-6 expression, TNF-α expression, IFN-γ expression, and MIF expression in the brain stem of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

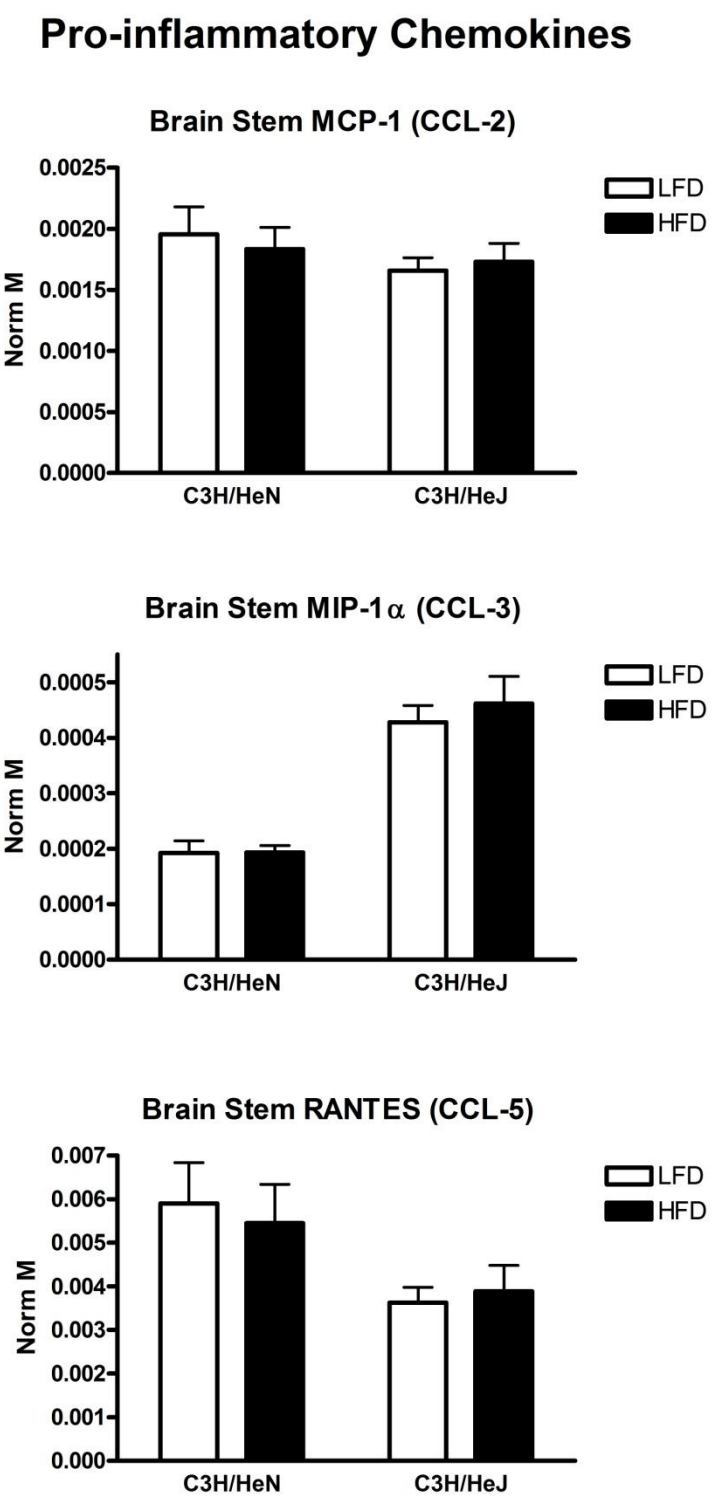

Figure 15. MCP-1 (CCL-2) expression, MIP-1α (CCL-3) expression, and RANTES (CCL-5) expression in the brain stem of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

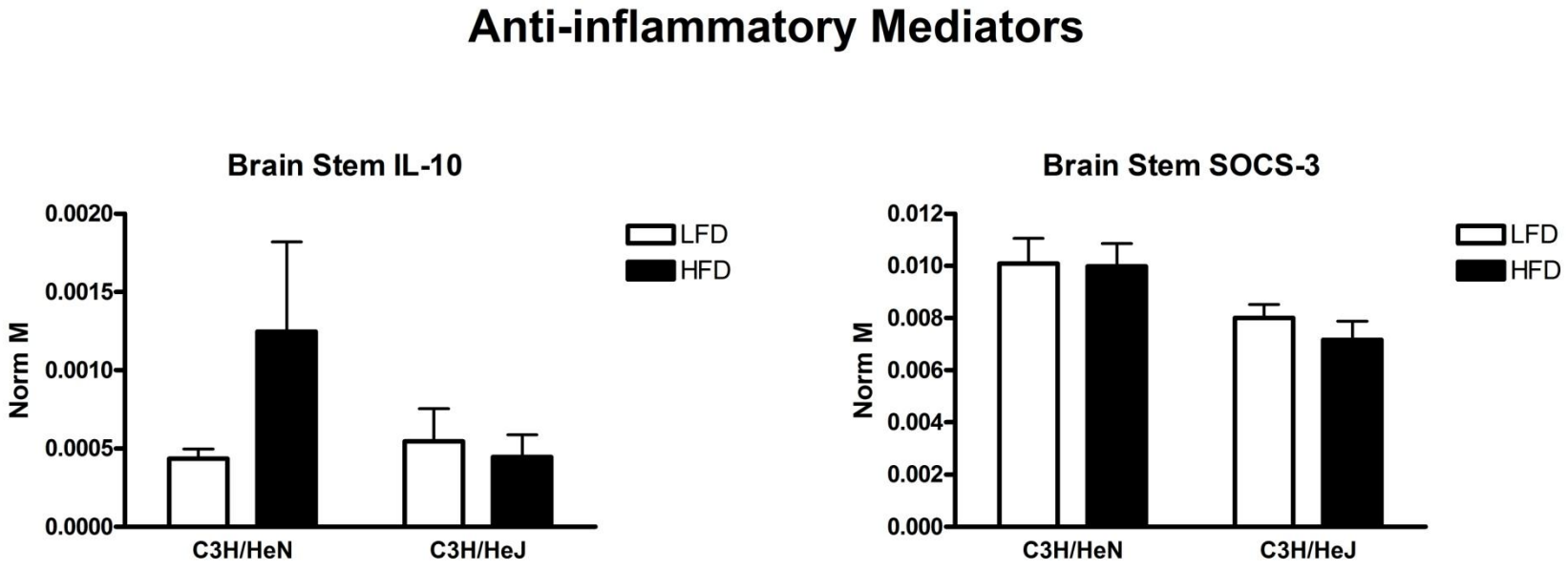

Figure 16. IL-10 expression and SOCS-3 expression in brain stem of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

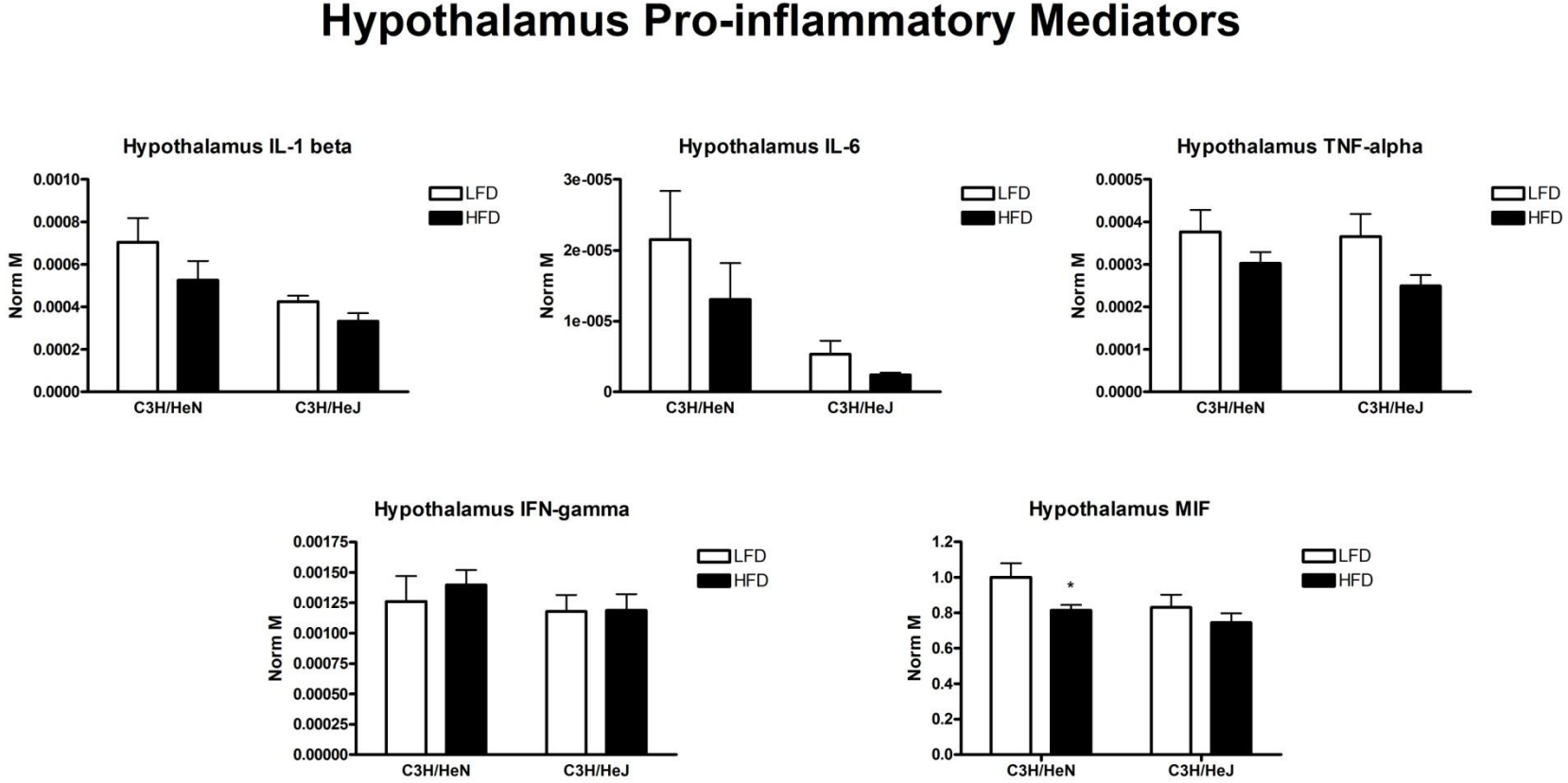

Figure 17. IL-1β expression, IL-6 expression, TNF-α expression, IFN-γ expression, and MIF expression in hypothalamus of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

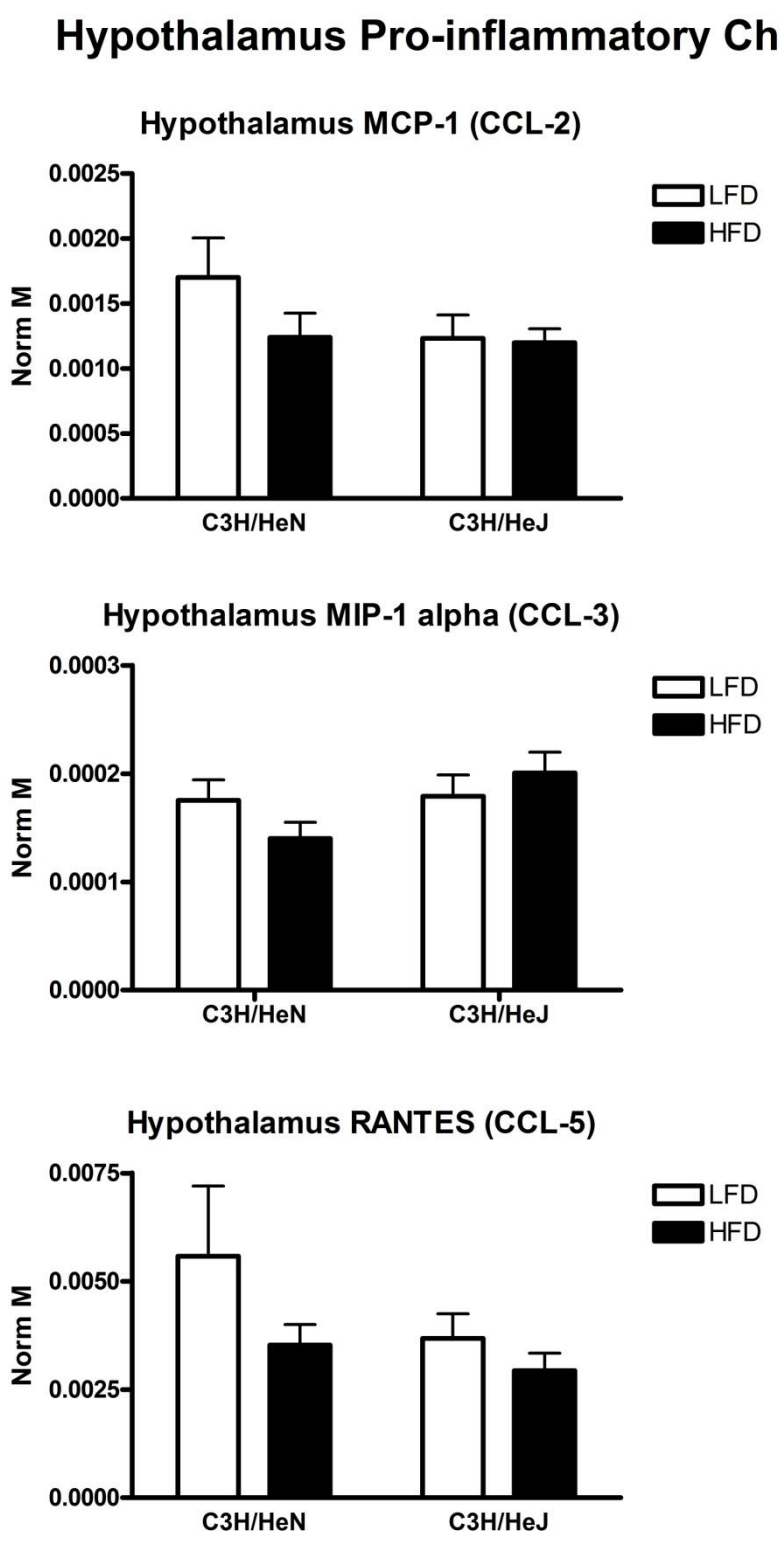

Figure 18. MCP-1 (CCL-2) expression, MIP-1α (CCL-3) expression, and RANTES (CCL-5) expression in the hypothalamus of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

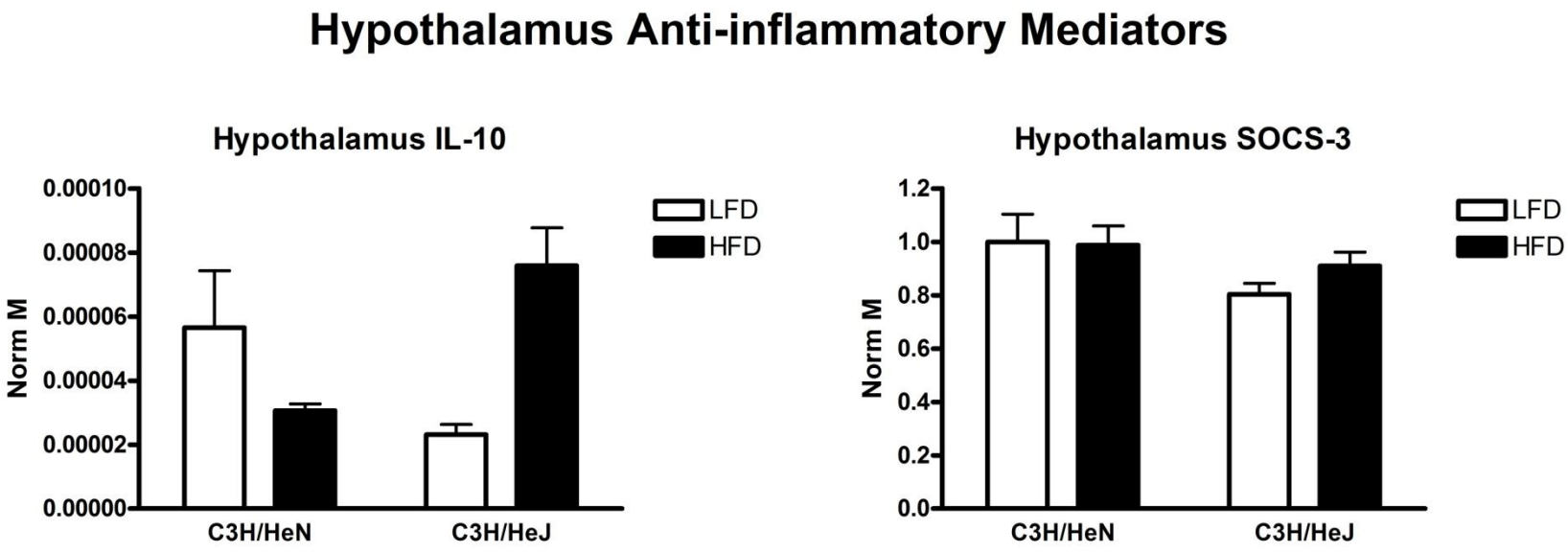

Figure 19. IL-10 expression and SOCS-3 expression in hypothalamus of C3H mice fed 8 weeks of either LFD or HFD. Data are mean ± SEM. *difference from LFD control, $P < 0.05$.

In addition to PCR data, serum Resistin, represented in **Figure 20**, was also measured through ELISA (R & D systems) as previously described in Chapter 2.

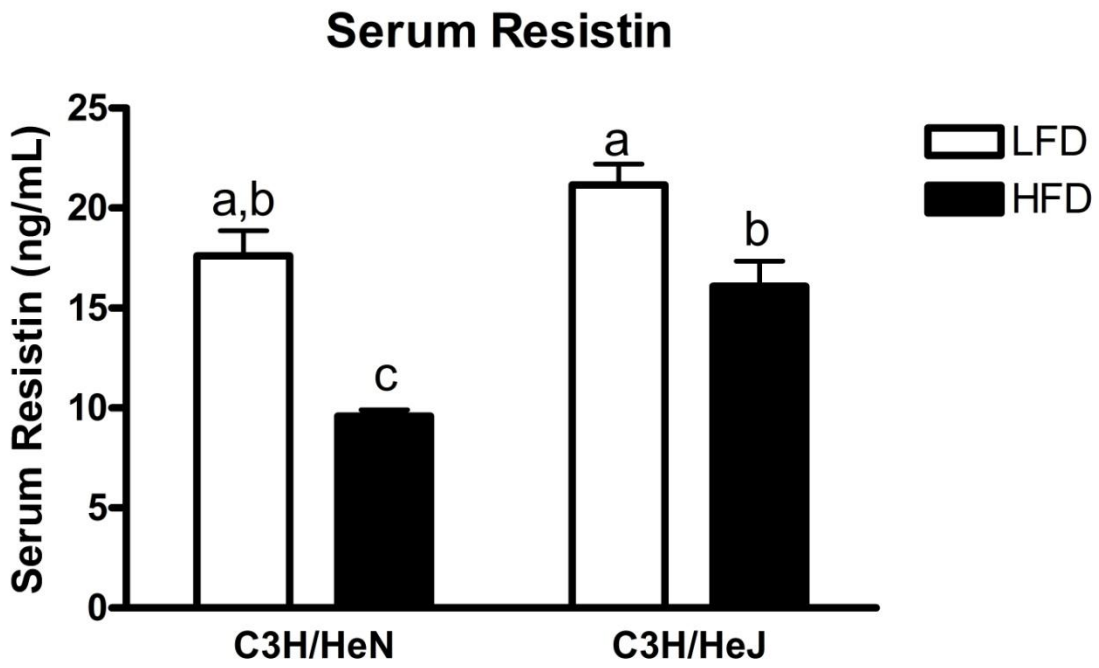

Figure 20. Serum Resistin of fed C3H mice after 8 weeks of either LFD or HFD feeding. Data are mean ± SEM. Labeled means without a common letter differ, $P < 0.05$.

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