Diet induced neuroinflammation and cognitive decline

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Abstract

The prevalence of obesity in the U.S. is over 50%, and midlife obesity is a clinical risk factor for cognitive impairment and the onset of neurodegenerative diseases such as Alzheimer's disease (1-3). Inflammation of the brain (neuroinflammation), a state associated with progressive neuronal loss, is heightened in cognitive decline and obesity (4-7). Consumption of high fat diets (HFD), specifically those high in the saturated fatty acid palmitic acid (C16:0; PA), exacerbate neuroinflammation, neurodegeneration, and cognitive impairment (8-16).

The research presented within this thesis seeks to define the role of microglia in the context of obesity and cognition. The central hypothesis of my thesis is that high fat diets induce microglial activation resulting in altered immunometabolic response, neuroinflammation, and subsequent cognitive decline. While neuroinflammation normally increases with age, risk of neuroinflammation and cognitive impairment is exacerbated by obesogenic diets (14). The findings from this work will provide a deeper understanding of diet-induced neuroinflammation, and will facilitate the development of novel therapeutics for cognitive disorders. This research has focused on utilizing basic science approaches to understand the effects of high fat diets on the central nervous system. Highlighted below are the major findings from my dissertation research.

1) Orexin A-induced neuroprotection: Excess intake of dietary PA increases the risk for developing obesity (17, 18). PA is known to induce neuronal cell death in the hypothalamus, a region of the brain important in regulating feeding behaviors (11, 19-21). One potential target to prevent this is orexin A, a hypothalamic signaling protein important in promoting obesity resistance that also has neuroprotective properties (22). I hypothesized that orexin A would protect against PA-induced hypothalamic cell death. To test this, I evaluated the response of hypothalamic neurons to orexin A and PA. I demonstrated that orexin A decreases

PA-induced programmed cell death and stabilizes expression of the pro-survival gene Bcl-2. I also demonstrated that orexin A protects against PA-induced damage to the mitochondria (measured via changes in reactive oxygen species (ROS) and mitochondrial respiration). These data support that orexin A protects against PA-induced hypothalamic cell death.

2) Orexin A signaling in PA-activated microglia: Obesity is associated with chronic low-grade inflammation, characterized by increased circulating pro-inflammatory signals and immune cell activation (8, 11, 23). Microglia are highly responsive to changes throughout the brain, and communication between neurons and microglia depends in part on pro- or anti-inflammatory secreted signals (cytokines and chemokines). Furthermore, PA promotes microglia to release pro-inflammatory signaling cascades (9, 24-26). My next goal was to determine how PA and orexin A treatment influences microglial secretion and activation states. To test this, I exposed microglial cells to orexin A and PA and measured changes in secreted signals. I demonstrated that orexin A treatment reduces PA-induced upregulation of pro-inflammatory markers and increases anti-inflammatory markers in microglia. Next, I sought to determine if the factors secreted by the activated microglia influenced neuronal survival. To test this, I filtered the microglial cell culture media to remove excess orexin A and PA while retaining the secreted cytokines, exposed neurons to this filtered supernatant, and determined neuronal cell death. I found that neurons exposed to media from orexin A-treated microglia have increased cell survival compared to those treated with media from PA-activated microglia. This result demonstrated that microglia exposed to orexin secreted protective signals that protected neurons, whereas microglia exposed to PA alone secreted harmful cytokines that resulted in neuronal cell death. My findings are the first to demonstrate that orexin A modulates PA-activated microglial cells.

3) Loss of orexin and high fat diet increases cognitive impairment: Obesity is recognized as a risk factor for development of cognitive disorders such as Alzheimer's disease (3). Moreover, deficiencies in orexin signaling have been linked to neurodegenerative diseases. My overall hypothesis was that reduced orexin signaling will increase diet-induced cognitive decline through a microglial-mediated pathway. To test this, I used wild type (WT) mice or a mouse model of orexin loss to determine differences in a cognitive task. I found that mice lacking orexin showed significant impairments in cognition vs. WT mice. Next, to determine the effects of HFD on microglia and cognition, mice were placed on a HFD or remained on normal chow, and the cognitive task was retested at 2 and 4 weeks. I demonstrated that cognition was impaired and microglial activation was increased in mice lacking orexin given a HFD vs. WT mice. Collectively, my results show that orexin loss impairs cognition, and that HFD accelerate cognitive deficits and the onset of neuroinflammation in orexin-deficient mice.

4) Fatty acid binding protein 4-uncoupling protein 2 axis in modulating microglia and cognition: Fatty acid binding proteins (FABP) are lipid chaperones regulating metabolic and inflammatory pathways in response to fatty acids (27, 28). To further define a mechanism for diet-induced microglial activation and cognitive decline, I sought to determine if the FABP4-UCP2 (uncoupling protein 2) axis is involved in neuroinflammation. I hypothesized that inhibition of microglial FABP4 would upregulate UCP2 and attenuate PA-induced inflammation. To test this, I measured hypothalamic gene expression changes in WT mice and mice lacking FABP4 (AKO mice) fed a HFD. I found hypothalamic tissue from AKO mice exhibit increased UCP2 expression and reduced pro-inflammatory makers compared to WT mice. Next, I pharmacologically inhibited FABP4 in microglia and demonstrated increased UCP2 expression and reduced PA-induced pro-inflammatory response and ROS production. Further, this effect is negated in microglia lacking UCP2, indicating the FABP4-UCP2 axis is pivotal in obesity-

induced neuroinflammation. Finally, to determine if the FABP4-UCP2 axis was involved in attenuating diet-induced cognitive decline, WT and AKO mice were fed a HFD for 12 weeks and tested in a panel of cognitive tasks. I found that mice maintained on a HFD had reduced locomotor activity. Further, WT mice maintained on HFD had impaired memory, and AKO had attenuated HFD-induced memory impairment. Collectively, these results indicate that the FABP4-UCP2 axis is a link between HFD, neuroinflammation, and cognitive impairment.

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control (C), p<0.0001 PA vs. TAK-242, and TAK-242+PA, p<0.001 C vs. TAK-242 and TAK-242+PA). (B-C) Palmitic acid increases orexin 1 receptor expression. Microglia exposed to PA or LPS show increased OX1R expression (B) but not OX2R expression (C). Pretreatment of microglia with OXA before PA challenge increased OX1R expression and reduced OX2R expression. Different letters above bars represent statistical significance at p<0.05 OXA vs. OXA/PA. (D) Orexin A suppresses pro-inflammatory IL-6 expression in microglial cells. Pro-inflammatory marker IL-6 expression is increased in BV2 microglia following PA and LPS exposure. Pretreatment of microglia with OXA before PA challenge reduces IL-6 expression compared to microglia exposed to PA only, but remains increased relative to vehicle (p<0.05 C vs. OXA, p<0.001 LPS vs. OXA and OXA/PA, p<0.001 PA vs. OXA and OXA/PA p<0.0001 C vs. LPS, PA, and OXA/PA). (E) Orexin A suppresses pro-inflammatory iNOS expression. Palmitic acid and LPS increase microglial iNOS expression, while OXA pretreatment attenuates PA-induced iNOS expression (PA and LPS p<0.001 vs control, p<0.05 vs OXA and OXA/PA). (F) Orexin A reduces TNF-α secretion from BV2 microglial cells. PA and LPS treatment increase TNF-α secretion compared to vehicle– or OXA only–treated microglia. Pretreatment of microglia with OXA before PA challenge reduces TNF-α secretion compared to PA only– but not vehicle–treated microglia. Different letters above bars represent statistical significance at p<0.0001. (G) Orexin A increases M2 marker arginase-1 gene expression. Pretreatment with OXA prior to PA challenge increases arginase-1 gene expression (OXA/PA p<0.001 vs. control, p <0.05 vs. PA and LPS). OXA alone increases arginase-1 expression in microglial cells (p <0.001 OXA vs. control, PA, and LPS). (H) Orexin A attenuates hypothalamic neuronal cell death in microglial-conditioned media. Adult hypothalamic cells have increased cell death following 24 h exposure to conditioned media from microglia stimulated with PA. Hypothalamic cells exposed to conditioned media from microglia pretreated with OXA before PA challenge, or vehicle-treated microglia, have reduced cell death compared to those exposed to media from PA-challenged microglia. p<0.0001 Vehicle vs. PA, p<0.001 PA vs. OXA/PA, p<0.0001 PA vs. OXA. (I) Hypothesized Orexin A microglial immunomodulation pathway. We hypothesize that OXA reduces M1 microglial activation and increase M2 microglial activation to maintain neuronal survival. Saturated fatty acid challenge (PA) induces an M1 microglial phenotype and the release of pro-inflammatory cytokines, contributing to neurodegeneration. Orexin A may influence proportion of M1 microglia by reducing rate of activation or potentially by aiding in conversion between M1 and M2 states. 48

Figure 4.1. High fat diet increases body weight gain and fat mass. WT and O/A3 mice were given HFD or NC for 28 d. Cumulative food intake was increased after 6, 8, and 10 d HFD exposure regardless of genotype (A, *p<0.05). Mice fed HFD had significantly increased body weight gain days 10-28 compared to mice fed NC

Chapter 1

Obesity and Brain Health

Obesity and Public Health:

The prevalence of obesity within the US is currently greater than 50%, with an estimated economic impact of over $147 billion per year in associated health care costs (1, 2). As obesity rates rise, so do indirect costs associated with obesity, including reduced years of disability-free life, increased mortality prior to retirement, early retirement, disability, and decreased productivity at work (29). Additionally, obese employees are more likely to take sick leave and obese men (body mass index; BMI $\geq$ 40 kg/m^2) are reported to have one month of lost productivity and cost employers $3,792 annually compared to their lean counterparts (29). The risk for developing comorbidities associated with obesity (type 2 diabetes, cardiovascular disease, cancer, and cognitive disorders), poor quality of life, and increased cost of health care provide reasons why it is essential to fully understand the underlying mechanisms driving development of obesity (3, 30, 31). Both genetic and environmental factors contribute to the predisposition for obesity. Rodent and human genome-wide association studies have identified loci contributing to obesity and its associated comorbidities, including neuronal regulators of energy balance (32-34). Environmental factors including caloric intake, dietary composition, physical activity, and non-exercise related energy expenditure also contribute to the development of obesity (17, 18). Furthermore, once obesity is established, weight loss and maintenance is more difficult than would be predicted for a lean body weight that has never been obese (35, 36). Identifying therapeutic targets to treat and prevent obesity would be beneficial for maintaining quality of life and overall health of individuals, and would also have a positive economic impact through reduction in obesity rate and associated costs.

Obesity and Neuroinflammation:

Midlife obesity is a clinical risk factor for cognitive impairment and the onset of neurodegenerative diseases such as Alzheimer's disease (AD) (3). BMI is negatively correlated with cognitive performance and brain morphology (37, 38). A common link between obesity and poor brain health is chronic inflammation. Obesity is characterized by chronic low-level inflammation, paralleling the prevalence of metabolic disorders such as type-2 diabetes (8, 11, 23). Prolonged overnutrition and excess intake of saturated fatty acids (SFA) increases the amount of fatty acids in the brain and induces neuroinflammation (8-11, 39). Neuroinflammation is a characteristic of many neurodegenerative diseases and metabolic related disorders (4-7). The dietary SFA palmitic acid (PA) increases activation of a neurotoxic phenotype in microglia (brain immune cells), resulting in release of pro-inflammatory cytokines and factors that may contribute to cognitive decline (11, 25, 26). PA promotes the switch to a neurotoxic phenotype, triggering release of pro-inflammatory cytokines such as TNF-α (tumor necrosis factor-alpha) (9, 11), and activation of the TLR-4/NFκB pathway (toll like receptor 4/ nuclear factor kappa-light-chain-enhancer of activated B cells), the central regulatory pathway of inflammation (26). The transcriptional regulator NFκB is constitutively inhibited by IκB (inhibitor of NFκB). Upon stimulation of TLR-4 receptor, IκB is marked for degradation, allowing NFκB to initiate transcription of TNF-α. Chronic activation of microglia to a pro-inflammatory state can lead to reduced hippocampal cognitive function and neurogenesis (40-42). While neuroinflammation and cognitive impairment are correlated, few studies clearly define a mechanism that explains describes the relationship between diet-induced obesity, cognitive impairment, and the onset of neuroinflammation. Despite demonstration that SFA induces activation of microglia to a neurotoxic phenotype, the underlying mechanisms driving microglial activation and response to dietary metabolites remains undefined.

History of Microglia:

Through the 20th century, the central nervous system was thought to be composed of two cell types, nerve cells and neuroglia, which were differentiated by their function. Rudolf Virchow introduced the term neuroglia (translated from the term nevernkitt meaning nerve-glue) to refer to non-neuronal cell types that were thought to be a matrix embedded with nuclei (43). Work by Santiago Ramón y Cajal and Pio del Río-Hortega would further redefine the term neuroglia, to the "third element of the nervous system", a distinct subtype of non-neuronal cells (43). Upon further investigation, del Río-Hortega identified microglia, cells that were non-neuronal and non-astrocytic, distinct from oligodendrocytes, and had phagocytic and migratory capacity (43). Del Río-Hortega's seminal work indicated that microglia initially arise from mesodermal cells of the pia mater as well as blood mononuclear cells based on their parallel behavior and morphology to monocytes (43). This theory was disputed for many decades until recent work confirmed del Río-Hortega's theories that microglia are derived from yolk-sac macrophages early in development (43, 44). As early as murine embryonic day 16 (E16), macrophage-like cells develop "hot-spots" in the brain parenchyma, where they eventually differentiate and become ramified microglia (45). In humans, similar events occur as microglial influx into the brain is detected around 22 weeks, and well-differentiated microglial populations can be identified at 35 weeks (46). During development, microglial population expands rapidly, and contributes to neurodevelopment via secretion of neurotransmitters, cytokines, and chemokines (47, 48).

Microglia constitute 5-20% of the cell population in the CNS, and are widely distributed throughout the brain and spinal cord (49). Until recently, microglia were thought to be passive, resting cells (50). Current evidence shows that microglia are in fact highly dynamic cells that mediate immune response, maintain synaptic terminals, remove debris, and secrete factors for neuronal survival (51). Microglial

activation can in turn activate astrocytes (CNS support cells involved in injury repair, synaptic transmission, and brain metabolism) to neurotoxic states that further increase CNS injury and promote neurodegeneration (52). Both macrophages and microglia share similar functions, including phagocytosis activity, production of ROS (reactive oxygen species), chemokines, and cytokines (53). Seminal classifications of microglial phenotypes were based on the macrophage polarization concept that "resting" microglia should be characterized into dichotomous phenotypes upon activation, either "M1-like" classical/neurotoxic and "M2-like" alternative/neuroprotective phenotypes. The M2 phenotype has been further sub-classified into two phenotypes: 1) M2a repair and regeneration, and 2) M2b immunoregulatory (54). Microglial phenotypes have been further classified by cell surface receptor expression and the release of cytokines and chemokines (**Table 1.1, below**). Despite the similarities and rationale for using macrophages to define microglial function and activation states, recent research has identified distinct differences in the transcriptomes of microglia and macrophages, mostly of proteins that sense endogenous ligands and microbes (53). These differences may allow microglia to behave uniquely in the CNS microenvironment.

Recent shifts in perspective have appreciated microglial populations to be heterogeneous and therefore have begun to diverge from an "M1-M2" description. With advances in "omics" technologies, we are able to profile cells using high-throughput genomic, proteomic, and computational approaches. As a result of the new technologies, new considerations of microglial characteristics are being taking into account, including genomic and proteomic profiles, brain region differences, continuum of aging, and patterns of response to stressors (13, 53). It should also be noted that microglia are self-renewing cells, and in humans have a turnover rate of ~4.2 years (55). Methylation, acetylation, and phosphorylation of histones or

Table 1.1. Proposed microglial phenotype classifications*

	Proposed role in inflammation				
	Inflammotoxic	**Pro-inflammatory**	**Anti-inflammatory**	**Repair**	**Immunoregulatory**
M1 cytotoxic	TNF-α iNOS	IL-1β IL-6 IL-12 INF-γ CXCL1 (KC) IL-1α IL-2 CCL5 (RANTES) CX3CR1			
M2a repair and regeneration			COX-2 IL-4 IL1-RA CX3CR1 TGF-β	YM1 FIZZ1 IGF-1 Arg1 G-CSF GM-CSF	CD206 Gal-3 CCL2 (MCP-1) CCR2
M2b immunoregulatory			IL-10 COX-2 SphK1/2 SOCS3 IL-4Rα		

*Table modified from Chhor et al 2013 (54).

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DNA binding proteins are influenced by pathology of various disease states including obesity, neuroinflammation, and AD (56-59). Given this, epigenetic traits should also be considered, and could potentially pass down ramified/refractory traits to microglia, making them less responsive to stimuli. As more data is generated and classified, further delineation of microglial phenotypes and activation characteristics will occur. Nonetheless, neuroinflammation and microglial activation is understood to be both beneficial or detrimental, as factors released from microglia can be neuroprotective or neurotoxic (9, 24, 25, 39). Therefore, use of rigid nomenclature and classification systems may not be the best approach. The research presented in this thesis has utilized current and available technology to survey microglial activation and phenotypes using genomic and proteomic approaches. For the purposes of this proposal, we reserve the "classical" dichotomous breakdown terminology of "M1-like" vs. "M2-like" microglial states to refer to those diverse microglial phenotypes that are broadly pro- or anti-inflammatory, respectively, for the sake of simplicity only, with the full recognition that these descriptive groupings do not imply uniformity of phenotype within the classes described.

Microglial-Neuronal Crosstalk and Cognition:

During the normal aging process, hippocampal neuroinflammation is known to be increased (4). Additionally, neuroinflammation, accompanied with progressive neuronal loss, is heightened in cognitive decline and obesity (4). While hippocampal neuroinflammation normally increases with age, risk of neuroinflammation and cognitive impairment is exacerbated by chronic consumption of diets high in SFAs such as PA (14). Microglia have recently been appreciated to have an important role in cognition (60). In the healthy brain, microglia are constantly surveying the surrounding microenvironment to promote structural formation and elimination of neuronal synapses (60). This process is especially important in the hippocampus. Neuronal circuits are constantly being

remodeled either by establishing new synapses or through elimination of selected synapses (defined as synaptic plasticity). In adult brains, reduced activity at synapses results in elimination of these connections. The events of synaptic plasticity are thought to underlie cognitive functions including memory formation and learning (61-63). Moreover, microglial/neuronal communication has been identified as a key mediator in this process, through internalization of synaptic terminals, release of signals to recruit microglia to inactivate synapses, and release of neurotrophic factors (64, 65).

Increased evidence indicates that mid-life obesity is a predictor of mild cognitive impairment later in life (3, 31). Although cognitive decline is part of the normal aging process, a negative correlation between BMI and cognitive performance is evident when controlling for aging (66). Neuroinflammation represents a key link between development of mild cognitive impairment and more serious neurodegenerative diseases such as AD. Identification of risk factors associated with mild cognitive impairment and AD, including neuroinflammation, are of importance to develop therapeutic treatments. As such, increased circulating pro-inflammatory cytokines such as TNF-α are associated with cognitive decline and increase the risk of progressively developing AD (67). Aged microglia have distinct transcriptome changes. Specifically, genes associated with the Stat3 and Neuregulin-1 pathway are upregulated, and those associated with oxidative phosphorylation are downregulated (53). Moreover, differences between young (5 mo) and aged (24 mo) microglia have differences in gene expression when stimulated with the pro-inflammatory stimulus interferon-γ (53). These data indicate that age is associated with shifts in basal microglial phenotypes and response to environmental stimuli. Seminal work has begun to describe dietary contributions to neuroinflammation, microglial phenotypes, and increased risk of developing cognitive impairments such as AD. In a rodent model of obesity, impaired cognition and altered hippocampal synaptic plasticity is evident following

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exposure to HFD (15). Interestingly, these effects are reversed when animals are returned to a low fat diet (15). In a separate study, a mouse model of AD (APP23) fed HFD had increased AD pathology, worsened cognitive performance, and increased expression of genes related to immune response compared to mice fed normal chow (13). Moreover, in a younger cohort of HFD-fed AD mice, increased cognitive impairments and markers of microglial activation were evident (68). These data indicate that dietary effects of cognition rely in part upon microglia, and chronic HFD-induced microglial activation may precipitate the onset of mild cognitive impairment and spur a faster transition to worse outcomes such as AD. To better understand how diet influences cognition, a comprehensive panel of tests is needed. These tests examine different aspects of memory and cognition that utilize various brain regions. For the purpose of this thesis, the following tests were performed as outlined in **Table 1.2.**

Neuropeptides and microglia:

Neurons in specific hypothalamic nuclei are integral in maintaining body homeostasis (69). Caloric overconsumption, HFD, and obesity perturb normal hypothalamic function and induce hypothalamic inflammation (5, 9, 11, 24, 25). Recent evidence shows that the hypothalamus also has an important role in aging (70). One link between diet and cognitive decline could be through increased neuroinflammation and disrupted hypothalamic signaling. Neuropeptides activate neuronal and glial cells, as well as mediate multiple behaviors including eating, sleep/wake, reward, metabolism, reproduction, and learning and memory (5, 71-73). Many feeding-related peptides that primarily act in the hypothalamus also mediate hippocampal dependent behaviors, as their receptors are highly expressed throughout the hippocampus (5, 71-73). Microglia can be activated via neuropeptides and neurotransmitters, upon activation of a receptor. Recent research has focused on the role of neuropeptides and microglial response in the context of neuroinflammation and neurodegeneration. Here, I will review the role

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Table 1.2. Behavior testing in models of HFD-induced cognitive decline

Behavior test	Type of memory/cognition	Test overview	Brain Region involved
Barnes Maze (74-76)	**Spatial reference memory:** memory that is based on the constant spatial, contextual, and factual components of a task	Mice learn to escape from a brightly lit, circular open platform to an escape box located under one of the 20 holes around the perimeter of the platform using spatial reference points surrounding the maze.	Hippocampus
Spontaneous alteration T-Maze (77)	**Short term working reference memory:** response is based on previous actions	Mice are placed in the maze which is in a T-shape. Mice will spontaneously alternate between sides of T-arm that is explored.	Hippocampus Prefrontal cortex
TWAA (78)	**Associative learning:** learn and remember a relationship between unrelated items (CS+UC+avoidance)	learn to cross to the opposite side of a conditioning chamber to avoid a tone-signaled foot shock.	Amygdala, hippocampus
Open field	General locomotor activity and anxiety	Animals are put in large open chamber with bright light to determine general movement.	

of a few key neuropeptides in modulation of microglial activity. The neuropeptides selected are those known to have receptors expressed on microglial cells, indicating a potential role in inflammation, neurodegeneration, and cognition.

Neuropeptide Y (NPY)

NPY is a hypothalamic neuropeptide with pleotropic effects in the brain including strong orexigenic effects, influence on learning and memory, and anti-inflammatory properties (79). NPY release is stimulated under fasting conditions, as the NPY neurons directly sense peripheral signals including blood glucose and leptin. In [this animal], delivery of NPY to the third ventricle improves memory retention in cognitive tasks (80). Conversely, in [what other animal], inhibition of NPY receptor Y2 and NPY knockout impair performance in cognitive tasks (81, 82). Microglial activation is one potential mechanism through which NPY influences cognitive function. Recent data support that NPY modulates microglial response. For example, microglia treated with NPY show attenuated LPS-induced iNOS and IL-1β release and alters phagocytic response (83). Suppressing inflammation and altering phagocytic response are important behaviors in maintaining memory formation and preventing cognitive impairments.

Leptin

Leptin is an adipocyte-derived peptide that has an integral role in regulating appetite and energy expenditure, mediated in part via activation of hypothalamic neurons (84, 85). Under physiological conditions, the brain responds to increased leptin signaling by suppressing food intake and increasing energy expenditure (86, 87). Overconsumption of a high fat diet is thought to induce leptin resistance and contribute to obesity (86, 87). Microglial activation is known to be heightened following high fat feeding (9, 24). Leptin receptors (LepA and LepB) are expressed on microglia, and once activated, these receptors initiate a pro-inflammatory response (88). Leptin resistance is present in obese individuals and may contribute

to increased neuroinflammatory response (89). Additionally, leptin levels are increased in the hippocampus of patients with AD while leptin receptor expression is suppressed, indicating a link between leptin, inflammation, and neurodegenerative disorders (90).

Orexin

The orexins (hypocretins) are hypothalamic peptides, orexin A (OXA; hypocretin 1) and orexin B (OXB; hypocretin 2), proteolytically derived from the same precursor protein, preproorexin (91, 92). Orexin A and B are ligands for the G-protein coupled receptors orexin receptor 1 and 2 (OX1R and OX2R respectively). Upon activation of the orexin receptors, rapid increased intracellular Ca^{2+} influx occurs. While OX1R has greater affinity for OXA over OXB (30- to 100-fold higher), OX2R is nonselective (92). Orexin neurons are found only in the lateral hypothalamus, but project throughout the central nervous system (CNS), including the hippocampus (93). Although orexins are more commonly recognized for their role in regulation of sleep/wake, arousal, feeding, and energy expenditure (94, 95), orexins also mediate cognitive processes, operant tasks, and fear conditioning (96-100). Additionally, orexin signaling is altered in AD patients (101, 102). We and others have demonstrated that orexin loss impairs memory, supporting that orexin mediates cognitive function (103, 104).

Recent studies highlight novel functions of OXA, including neuroprotection and decrease of apoptosis and neuroinflammation via activation of orexin receptors (22, 105-108). Data indicate orexin-induced neuroprotection could rely on microglial modulation (25, 107). In cerebral ischemia models, pretreatment with OXA reduces infarct size through a microglial-mediated pathway (107). Additionally, chronic exposure to the potent pro-inflammatory agonist lipopolysaccharide (LPS) reduced orexin signaling, indicating orexin has a role in the inflammatory response (109). Microglia may also become more sensitive to orexin signaling after activation (25). LPS increases TNF-α in microglia, but also

increases OX1R expression, and OXA treatment prior to LPS exposure reduces TNF-α in microglia (107). We have also demonstrated PA increases OX1R and PA treatment causes microglial shift to an M1-like activation state, indicated by increased TNF-α, IL-6, and iNOS (25). However, when microglia are pretreated with OXA prior to PA exposure, the PA-induced pro-inflammatory response is attenuated (25). These data indicate increased sensitivity to OXA may allow orexin-mediated attenuation of M1-like microglial phenotype to an M2-like state.

Microglia and ROS:

ROS, radical or non-radical oxygen containing molecules, react with lipids, proteins, and nucleic acids. The generation of mitochondrial ATP is coupled with the production and accumulation of ROS. Because of the large amount of oxygen consumption and ATP utilization, the brain is highly susceptible to oxidative stress (110). Although modest amounts of ROS act as cell signaling molecules and are important in nutrient sensing, chronic and sudden increases in ROS, such as those observed after HFD exposure, alter cell physiology and induce pathways of cell death (111). Moreover, oxidative stress in the brain is associated with the onset of neurodegenerative diseases and aging (110). In rodent models of obesity, HFDs increase ROS in the hippocampus and contribute to cognitive decline (112). Chronic exposure to pro-inflammatory stimuli including HFDs causes microglia to become primed and refractory, worsening the pathogenesis in neurodegenerative diseases, resulting in reduced hippocampal dependent cognitive function, and decreased neurogenesis (42, 113). Additionally, increased ROS production is responsible for controlling transcription of pro-inflammatory cytokines and phagocytosis (114). Uncoupling protein 2 (UCP2), a non-thermogenic inner mitochondrial membrane, dissipates the proton gradient, uncoupling mitochondrial metabolism from ATP production (115, 116) UCP2 also regulates the oxidative stress response (117). Genetic ablation of UCP2 results in increased ROS and inflammatory response centrally, and exacerbates neuronal cell death (118).

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Further, UCP2 has been demonstrated to regulate microglial polarization (117).

Neuroinflammation and Lipid Metabolism:

Lipids are appreciated to have more than just structural properties in cells, and can act as vital signaling molecules. Exploration of molecular and neuroendocrine mechanisms within the CNS has begun to identify interactions between genetic and environmental inputs that regulate lipid metabolism. In model systems, neuropeptides have proven to have a key role in regulating total body lipid metabolism, however the individual cellular components are poorly defined (119-123). In both macrophages and microglia there is a shift in energy metabolism depending on the stimuli. The link between inflammation and lipid metabolism indicates a key unexplored role in microglia, and a key role for FABP4 (fatty acid binding protein 4; adipocyte protein-2; aP2). Fatty acid binding proteins (FABP) are lipid chaperones regulating metabolic and inflammatory pathways in response to fatty acids (27, 28). In mice that lack FABP4 (known as FABP4/aP2$^{-/-}$ or AKO mice), high saturated fat diet feeding results in a paradoxically obese yet anti-inflammatory phenotype, without development of metabolic syndrome (insulin resistance and glucose intolerance) (24, 124, 125). Prior studies have revealed that the mechanistic basis for the anti-inflammatory phenotype of the AKO mouse is driven by the FABP4-UCP2 axis (24, 125). Without FABP4, increase in UCP2 attenuates the production of ROS, and prevents the switch to a pro-inflammatory state in both peripheral macrophages and microglia (24, 125). Recently, Xu et al. reported that ablation of FABP4 in macrophages improves mitochondrial function and attenuates NFκB signaling via monounsaturated fatty acid induction of UCP2 (125). The loss of FABP4 results in increased intracellular monounsaturated fatty acids, predominately C16:1, that upregulate the expression of UCP2. Therefore, the loss of FABP4 disrupts the switch to a toxic M1-like phenotype.

Metabolism and Immune Cell Polarization:

Diet-induced shift in metabolism (metabolic remodeling) is directly coupled to immune effector functions and can directly impact the regulation of microglial/macrophage transition between M1-like and M2-like phenotypes. For example, activation of macrophages to an M1-like state via LPS stimulation of TLR4 is marked by a robust upregulation of aerobic glycolysis, impaired mitochondrial respiration, and subsequent disruption of the tricarboxylic acid cycle (TCA or citrate cycle) (126, 127). These shifts are directly coupled to the accumulation of TCA intermediates such as itaconate (also known as methylene-succinic acid) and succinate, resulting an increase of lactate and a decrease in intracellular oxygen consumption (126, 127). Recent data suggest this pathway is driven by upregulation of the mitochondrial-associated enzyme immune responsive gene-1 (Irg1), which decarboxylates aconitate to produce itaconate. The accumulation of itaconate results in a series of changes in the TCA cycle that result in M1 polarization via inhibition of succinate dehydrogenase (SDH or electron transport chain complex II), and through elevated succinate that cannot be oxidized to fumarate and malate. This remodeling of the TCA cycle in macrophages provides insight into the direct link between metabolic adaptation and immune function.

Although microglia are known to be capable of similar metabolic shifts, the parallel pathways of metabolic adaptation in microglia have remained largely hypothetical (128), as few studies have been conducted to investigate the details of microglial metabolism and polarization in response to dietary metabolites, and downstream consequences on neuroinflammation and cognition (24).

Overview of Chapters:

Current evidence indicates a direct link between obesity, neuroinflammation, and cognitive decline. However, the pathological mechanisms of microglial immune

response, obesity, and cognitive impairment are not well characterized, therefore limited clinical treatments for individuals are available. While some degree of neuroinflammation is part of normal aging, age-related inflammation is exacerbated by metabolic perturbations and chronic consumption of high saturated fat diet (3, 9, 10). The central hypothesis of my thesis is that high fat diets induce microglial activation resulting in altered immunometabolic response, neuroinflammation, and subsequent cognitive decline (**Figure 1.1**). I will highlight this in the context of the role of dietary fat intake and neuroinflammation in the pathologic progression of neurodegeneration and cognitive function using multiple models of diet induced-obesity. Herein, I present a series of investigations intended to research mechanisms related to the microglial contributions of cognitive decline. The second chapter investigates the role of OXA attenuation of PA-induced hypothalamic cell death. Chapter 3 investigates the role of OXA signaling in PA-activated microglial cells. Chapter 4 investigates the role of OXA in HFD-induced cognitive decline. In a divergence from the topic of OXA signaling, in Chapter 5 I present data showing that the FABP4-UCP2 axis in microglia regulates inflammation. Finally, in Chapter 6 I will discuss the role of the FABP4-UCP2 axis in diet-induced cognitive decline. My research seeks to define the role of microglia in the context of obesity and cognition. The findings from my work will provide a deeper understanding of diet-induced neuroinflammation, and will facilitate the development of novel therapeutics for cognitive disorders. My doctoral work has focused on utilizing basic science approaches to understand the effects of high fat diets on the central nervous system. My work utilizes multidisciplinary approaches including nutrition, biochemistry, pharmacology, and neuroscience to better understand the impact of dietary metabolites on central nervous system function.
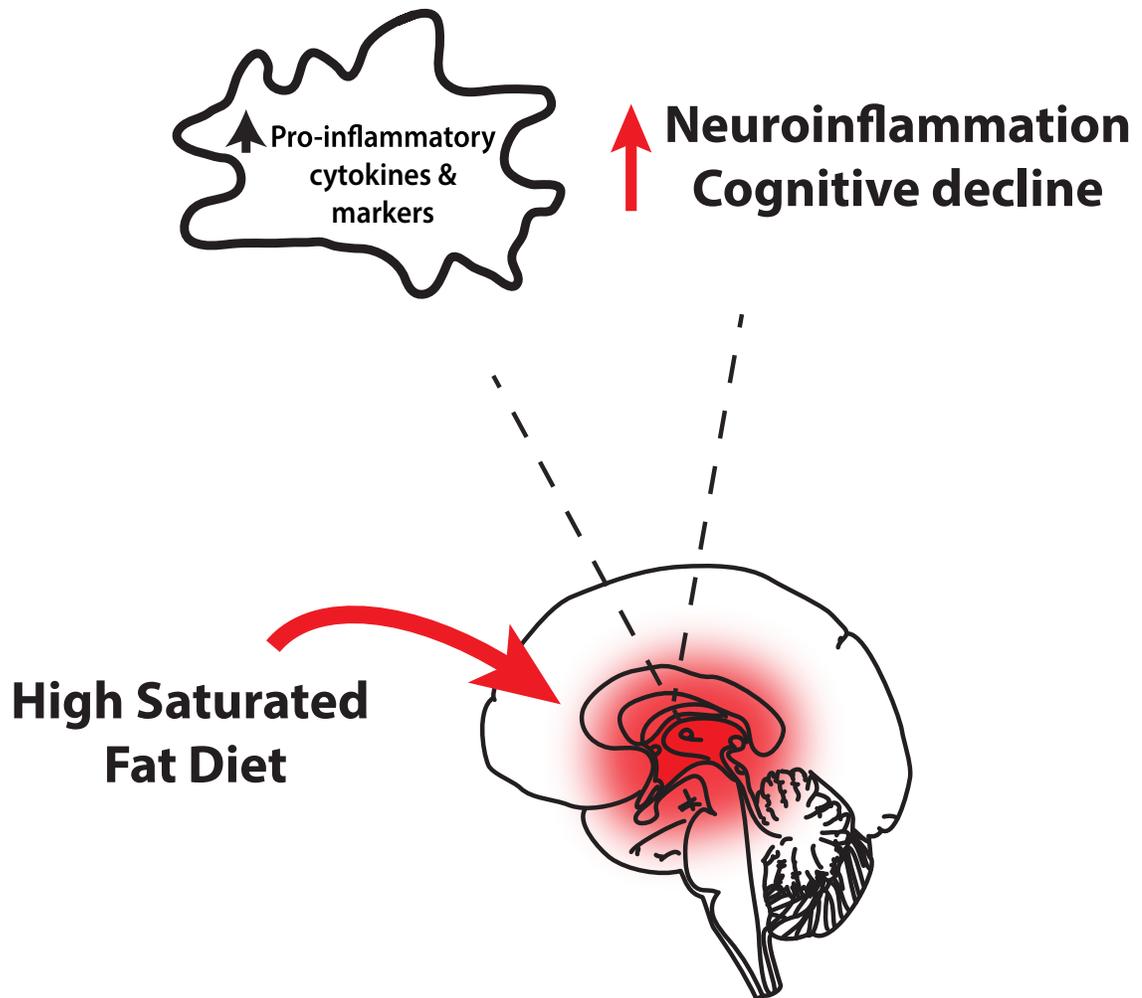
Figure 1.1. Schematic of diet induced cognitive decline. Intake of high saturated fat diet induces microglial activation to a pro-inflammatory phenotype and contributes to subsequent cognitive decline.

Chapter 2

Duffy, C. M., Nixon, J. P. and Butterick, T. A. (2016). "Orexin A attenuates palmitic acid-induced hypothalamic cell death" Mol Cell Neurosci, 2016. 75: p. 93-100.

Chapter 2

Orexin A attenuates palmitic acid-induced hypothalamic cell death

Introduction:

Diets rich in the saturated fatty acid palmitic acid (PA) promote hypothalamic dysregulation and contribute to obesity through disruption of appetite regulating signals (19, 129). Mechanisms triggered by PA exposure include increased oxidative stress, insulin resistance, release of pro-inflammatory cytokines, and apoptosis (11, 19-21). In the hypothalamus, diet-induced obesity also alters genes regulating the apoptotic pathway. For example, hypothalamic expression of anti-apoptotic protein B cell lymphoma 2 (Bcl-2) is decreased, while expression of the pro-apoptotic protein B cell lymphoma 2 associated X protein (Bax) is upregulated (20). These gene expression changes are linked to obesity pathogenesis. High fat diets (HFD) also contribute to the overproduction of reactive oxygen species (ROS) in the brain, resulting in increased oxidative stress and cell damage (130-132). Because production of ROS is a crucial signal resulting in cell death, oxidative stress and apoptosis are closely related pathways. Further, ROS suppresses neuronal anti-apoptotic proteins including Bcl-2 (133).

Because dietary components such as PA can directly and negatively affect brain health, it is likely that protection against obesity involves neural mechanisms capable of ameliorating diet effects on central nervous system signaling. One potential candidate are the orexins (hypocretins), hypothalamic peptides important in maintaining energy metabolism, sleep/wake cycles, and promoting obesity resistance (94, 134). Orexin A (OXA) and orexin B (OXB) act through two G-protein coupled receptors (orexin receptors 1 and 2; OX1R and OX2R, respectively) to alter intracellular metabolic functions (135-137) and promote cell survival against

oxidative stress and ischemic events (22, 138) in part through activation of Akt (protein kinase B) (105). Here we focus on elucidating neuroprotective pathways through which orexin alters brain response to PA, a major component of obesogenic diets.

Work from our lab has demonstrated that OXA attenuates caspase-3/7 mediated apoptosis in response to H_2O_2, an initiator of oxidative stress (22). Because HFDs are known to induce oxidative stress and neuronal cell death, (20, 139) we sought to determine whether OXA protects against PA-induced cell death (**Figure 2.1**). To test this, we evaluated the response of an immortalized murine hypothalamic cell line (designated mHypoA-1/2) (140) to OXA. We demonstrate here that OXA attenuates PA-induced apoptosis via reducing caspase-3/7 activity, increasing Akt activation, stabilizing expression of the pro-survival gene Bcl-2, and inhibiting ROS production. Finally, we show that OXA alters intracellular metabolic function in mHypoA-1/2 cells in real-time via increased basal respiration, maximum respiration, ATP production, and reserve capacity.

Methods:

Cell culture and reagents:

Differentiated immortalized adult mouse hypothalamic (mHypoA-1/2, cited elsewhere as CLU172; CELLutions-Cedarlane, Burlington ON CAN) (140, 141) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad CA USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic cocktail (penicillin, streptomycin and neomycin) at 37°C with 5% CO_2. The mHypoA-1/2 cells do not express endogenous orexin (142), independently confirmed by our laboratory (data not shown). Orexin A peptide (ThermoFisher Scientific, Waltham MA USA) was suspended in phosphate buffered saline (PBS; Invitrogen) and diluted to a final concentration of 300 nM in DMEM. Palmitic acid (Sigma-Aldrich, St Louis, MO USA) was suspended in dimethyl sulfoxide (DMSO)

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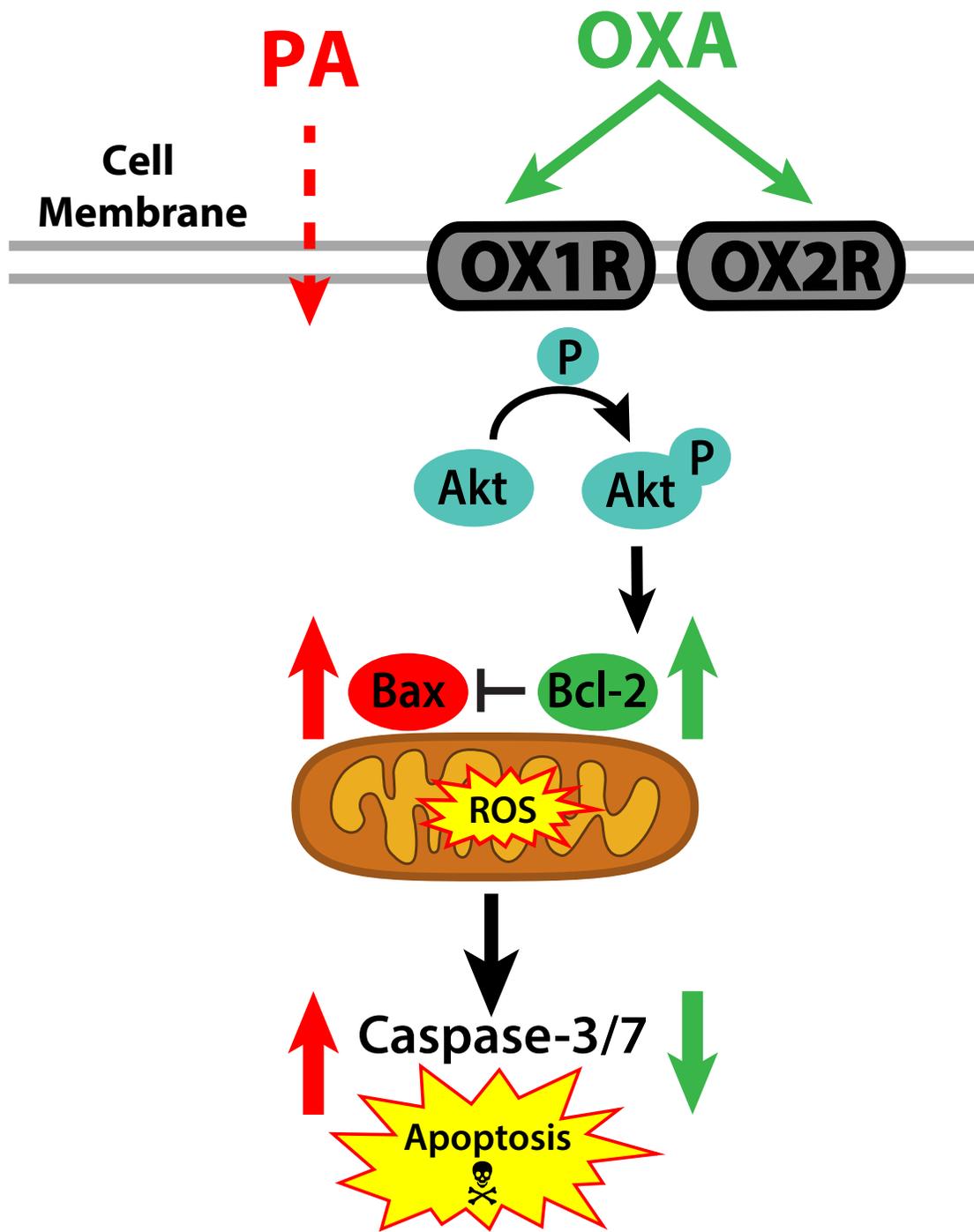
Figure 2.1. Hypothesized mechanism of orexin A induced neuroprotection

and diluted to a final concentration 0.1 mM in DMEM. Dual orexin receptor antagonist (DORA; TCS 1102; Tocris Bioscience, Avonmouth GBR) was reconstituted in DMSO and diluted to a final concentration of 1.16 nM in DMEM (143). The dual orexin receptor antagonist used in this study binds similarly to both OX1R and OX2R rapidly with high affinity (143).

Treatments:

In general for treatment assays, cells underwent two steps: First, a 24 h pretreatment with a test substance (e.g. orexin or vehicle); and second, a 2 to 24 h challenge with palmitic acid or vehicle, with or without the test substance used for 24 h pretreatment. Specifically, for cell viability assays, neurons were treated with DORA or DMSO vehicle for 20 minutes to block orexin receptors, subjected to a 24 h treatment with OXA or PBS vehicle, and finally challenged with vehicle (PBS+DMSO), PA, DORA, or OXA for 24 h. For the caspase-3/7, ROS, and gene expression experiments, cells underwent 24 h pretreatment with OXA or PBS vehicle, followed by 2 h challenge with PA or DMSO vehicle in the presence of OXA or PBS vehicle. For the mitochondrial respiration assays without PA challenge, cells were exposed to vehicle (PBS+DMSO), OXA (50, 150, or 300 nM) or DORA for 2 h. For the mitochondrial assay with PA challenge, cells were first pretreated with OXA (300 nM) or vehicle control (PBS) for 24 h followed by a 6 h challenge with vehicle (PBS+DMSO), PA, or OXA. All concentrations and time points were based on previous studies (22, 125, 143, 144).

Cell viability assay:

Cell survival was determined using a resazurin-based assay (Presto Blue, Invitrogen) as previously described (22). Viable cells produce a fluorescent signal ($560_{EX}/590_{EM}$) as determined using a spectrophotometer (SpectraMax-M5; Molecular Devices, Sunnyvale CA USA). Data are reported as percent relative fluorescence units (RFU) change vs. control.

Caspase Activity:

Caspase 3/7 activity was determined as previously described (22, 144). Briefly, caspase-3/7 activity was determined by the addition of a luminogenic caspase substrate DEVD based assay (Caspase-Glo 3/7, Promega, Madison WI USA). Changes in relative luminance units (RLU; 650 nm) were analyzed using a microplate spectrometer reader (SpectraMax-M5). Data are presented as fold increase in caspase activity normalized to cell number.

Reactive Oxygen Species assay:

Intracellular ROS (superoxide and hydroxyl radical) production was determined using a commercially available deep red fluorescence kit following manufacturer's protocol (Abcam, Cambridge GBR). Cells were pretreated with OXA (or vehicle) for 24 h, then challenged with PA (or vehicle) plus OXA (or vehicle) for 2 h. During the last hour of challenge, the cell-permeable deep red dye was added to cells and allowed to incubate at 37°C and 5% CO_2. Intracellular superoxide and hydroxyl radicals oxidize the deep red dye producing a fluorescent signal (145). Fluorescence was measured at $650_{EX}/675_{EM}$ using a spectrophotometer (SpectraMax-M5). Data are presented as fold change vs. control.

Real time RT-PCR:

Total RNA was extracted from cultured mHypoA-1/2 cells with the aid of Trizol reagent (Invitrogen). A final concentration of mRNA (100 ng/µl) was determined using 260 and 280 nm readings on a spectrophotometer (Nanodrop ND-8000; ThermoFisher). Primers were designed using MacVector 12 (MacVector Inc, Cary NC USA) and sequences are listed in table 1. Relative expression of target genes was determined with SYBR Green using the $2^{-\Delta\Delta CT}$ method, normalized to GAPDH (146).

Table 2.1. Real-time qPCR primer sequences

Target and accession number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Bcl-2 (NM_177410.2)	CACCGCGAGGGGACGCTTTG	AGGTCGCATGCTGGGGCCATA
Bax (NM_007527.3)	GCTGAGCGAGTGTCTCCGGC	ACGCGGCCCCAGTTGAAGTT
GAPDH (NM_017008)	GACATCAAGAAGGTGGTGAAGCAG	AAGGTGGAAGAGTGGGAGTTGC

Cell-based enzyme-linked immunosorbent assay (ELISA):

Changes in Akt activation were determined using a commercially available kit (R&D Systems, Minneapolis, MN). Briefly, cells were pretreated with OXA (or vehicle) for 24 h and challenged with PA (or vehicle) and an additional dose of OXA (or vehicle) for 1 h. Time point was based on Sokolowska *et al* 2014 (105). Cells were fixed with 4% formaldehyde, blocked, and incubated with primary antibodies (anti-phospho-Akt (S473) and anti-total Akt) overnight. Unbound primary antibodies were washed away and secondary antibodies (HRP-conjugated or AP-conjugated) were added. Unbound secondary antibodies were washed away and fluorogenic substrates were added. Fluorescence was measured on a spectrophotometer ($540_{EX}/600_{EM}$ and $360_{EX}/450_{EM}$). Data are reported as the ratio of phospho-Akt normalized to total Akt in each well.

Mitochondrial respiration assay:

Mitochondrial function was assessed using either the XF24 or XF^e96 extracellular flux analyzer (Seahorse Biosciences, North Billerica MA USA). Cell number and inhibitor titration experiments were performed for optimal response based on manufacturer instructions. For dose response assay, mHypoA-1/2 cells were plated on V7 microplates (Seahorse Biosciences) at $2x10^4$ cells per well in DMEM supplemented with 10% FBS and 1% PSN and incubated overnight at 37°C in 5% CO_2. Cells were treated with increasing concentrations of OXA (50, 150, 300 nM) or vehicle for 2 h (n=4-5/treatment group). Following incubation, DMEM was removed and replaced with XF Assay media (Seahorse Biosciences, supplemented with pyruvate (1 mM), L-glutamine (2 mM), and glucose (10 mM) containing respective treatment groups described above (147). During the assay, cells were treated with compounds in the following order: oligomycin (2 µM; Sigma) to determine mitochondrial ATP production, an uncoupler FCCP (carbonyl

cyanide-4-phenylhydrazone; 1.2 µM; Sigma) to determine the maximal respiration, and antimycin A (4 µM; Sigma) to determine the spare capacity.

For DORA studies, cells were seeded at a density of 1×10^4 cells/well on XF96 microwell plates and incubated overnight at 37°C in 5% CO_2. Cells were treated with either vehicle control (PBS and DMSO), OXA only (300 nM), DORA only (1.16 nM), or DORA plus OXA for 2 h (n=10-12/treatment group). Following incubation, DMEM was removed and replaced with XF Assay media (supplemented with pyruvate, L-glutamine, and glucose) containing respective treatment groups described above. During the assay, cells were treated with oligomycin (1 µM; Seahorse Biosciences), FCCP (0.5 µM; Seahorse Biosciences), and antimycin A (4 µM; Seahorse Biosciences).

For OXA and PA studies, cells were seeded at a density of 2×10^3 cells/well on XF96 microwell plates and incubated overnight at 37°C in 5% CO_2. Cells were then pretreated with vehicle (PBS) or OXA (300 nM) for 24 h and challenged with PA (or vehicle) and an additional dose of OXA (or vehicle) for 6 h (n=10-12/treatment). Time points based were on (148). Following incubation, DMEM was removed and replaced with XF Assay media as described above. During the assay cells were exposed to compounds in the following order oligomycin (1 µM; Seahorse Biosciences), FCCP (0.5 µM; Seahorse Biosciences), and antimycin A (4 µM; Seahorse Biosciences).

The oxygen consumption rate (OCR) was automatically calculated and recorded by the Seahorse XFe software, respiration rates were manually calculated and normalized to cell number as described by Brand and Nicholls (149). The non-mitochondrial respiration rate was calculated as the minimum rate following antimycin A injection. Basal respiration was determined as the last rate measurement before oligomycin injection minus the non-mitochondrial respiration rate. ATP turnover was calculated as the last rate measurement before oligomycin injection minus the minimum rate measurement after oligomycin injection.

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Maximum respiration was determined by subtracting non-mitochondrial respiration from maximum rate measurement following FCCP injection. The reserve capacity was determined by subtracting the basal respiration from the maximal respiration.

Statistical methods:

Statistical differences were determined using a one-way ANOVA followed by Tukey's post hoc test, using GraphPad Prism 5 (GraphPad Software, San Diego CA USA). Letters indicate significant differences between treatment groups (e.g. columns with the same letters do not differ from each other, while columns with different letters are significantly different).

Results:

Figure 2.2: OXA attenuates PA induced cell death:

To determine whether OXA inhibits PA-induced neuronal cell death, mHypoA-1/2 cells were pretreated with or without OXA (300 nM) for 24 h, and then exposed to PA in the presence or absence of OXA for an additional 24 h. As expected, PA significantly reduced cell survival (Fig 2.2A; $p<0.001$ vs. vehicle control; C). Conversely, OXA pretreatment attenuated PA-induced cell death (Fig 2.2A; $p<0.05$ vs. OXA only and OXA+PA). Further, treating the cells in the presence of DORA negates the effects of OXA, preventing attenuation of PA-induced cell death (Fig 2.2A; $p<0.001$ vs. OXA only and OXA+PA). To determine if cell death occurred via apoptosis, caspase-3/7 activity was measured. Hypothalamic neurons were pretreated with or without OXA for 24 h, and then challenged with PA in the presence or absence of OXA for an additional 2 h. As predicted, PA significantly increased caspase-3/7 activity (Fig 2.2B; $p<0.0001$ vs. C). However, OXA attenuated PA-induced caspase-3/7 activity (Fig 2.2B; $p<0.0001$ vs. OXA only, $p<0.001$ vs. OXA+PA).
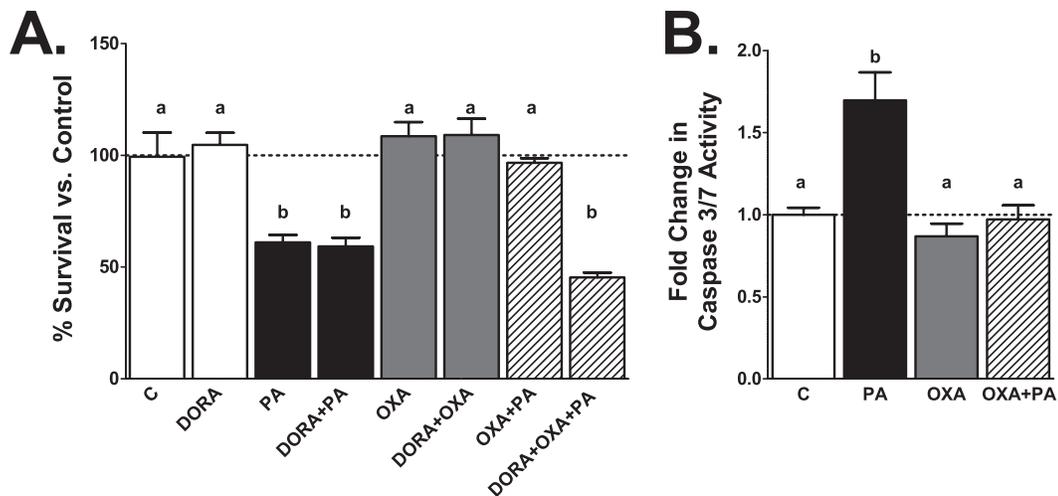
Figure 2.2. Orexin A attenuates PA-induced hypothalamic cell death. A) mHypoA-1/2 cells were pretreated with OXA for 24 h and then incubated with PA in the presence or absence of OXA or DORA for an additional 24 h. PA significantly reduces cell viability ($p < 0.001$ vs. vehicle control; C). OXA attenuates PA induced cell death ($p < 0.05$ vs. OXA only and OXA+PA). DORA negates OXA induced cell survival ($p < 0.001$ vs. OXA only and OXA plus PA). B) PA significantly increases caspase-3/7 activity ($p < 0.0001$ vs. C). OXA reduces caspase 3/7 induced apoptosis ($p < 0.0001$ vs. OXA only, $p < 0.001$ vs. OXA+PA).

Figure 2.3. Orexin A reduces reactive oxygen species in mHypoA-1/2 cells.

Prior data indicate OXA is protective against oxidative stress (22, 105, 150). To determine if OXA protects against PA-induced ROS production, mHypoA-1/2 cells were pretreated with OXA for 24 h, and then challenged with or without PA for an additional 2 h. As expected, PA significantly increased ROS production compared to control (Fig 2.3; $p<0.05$). Further, in the presence of OXA, PA-induced ROS production was attenuated (Fig 2.3; $p<0.05$ PA vs. OXA+PA).

Figure 2.4-2.6: OXA increases Akt activation and stabilizes expression of anti-apoptotic gene Bcl-2

Prior reports indicate OXA activates Akt phosphorylation (105). Likewise, we demonstrate OXA significantly increases Akt phosphorylation with or without PA exposure (Fig 2.4; $p<0.05$ vs. C, $p<0.001$ vs. PA). To determine if apoptotic gene expression is altered following exposure to PA and OXA, changes in the anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax were assessed. Following 2h PA exposure, Bcl-2 expression was significantly reduced (Fig 2.5A; $p<0.05$ vs. C). Cells treated with OXA in the presence or absence of PA had stabilized Bcl-2 gene expression compared to those treated with PA only (Fig 2.5A; $p<0.05$ vs. OXA only and OXA plus PA). The ratio of Bax/Bcl-2 expression was significantly upregulated in mHypoA-1/2 cells treated with PA only (Fig 2.5B; $p<0.05$ vs. C). In contrast, cells treated with OXA or OXA plus PA had stabilized Bax/Bcl-2 ratios (Fig 2.5B; $p<0.001$ OXA vs. PA, $p<0.05$ OXA+PA vs. PA). Additionally, orexin receptors (OX1R and OX2R) gene expression in mHypoA-1/2 cells is unchanged following acute 2 h PA exposure (Fig 2.6A-B).
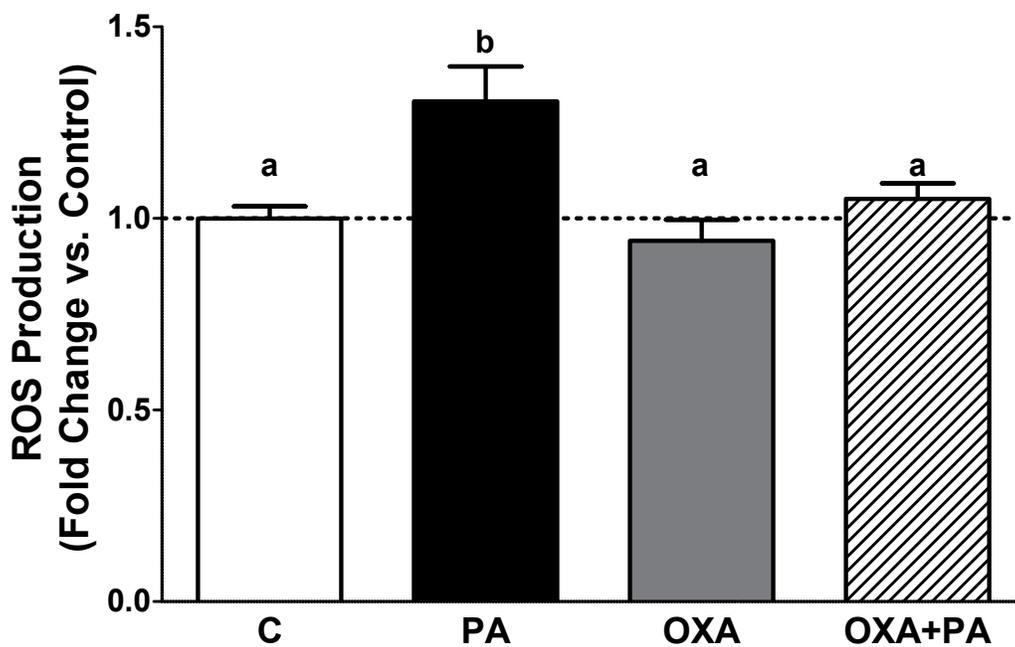
Figure 2.3. Orexin A reduces reactive oxygen species in mHypoA-1/2 cells.
mHypoA-1/2 cells were pretreated with OXA for 24 h and then incubated with PA
in the presence or absence of OXA for an additional 2 h. ROS is significantly
increased following 2 h PA exposure ($p < 0.05$ vs. C). OXA treatment attenuates
PA-induced ROS production ($p < 0.001$ vs. OXA only, $p < 0.05$ vs. OXA+PA).
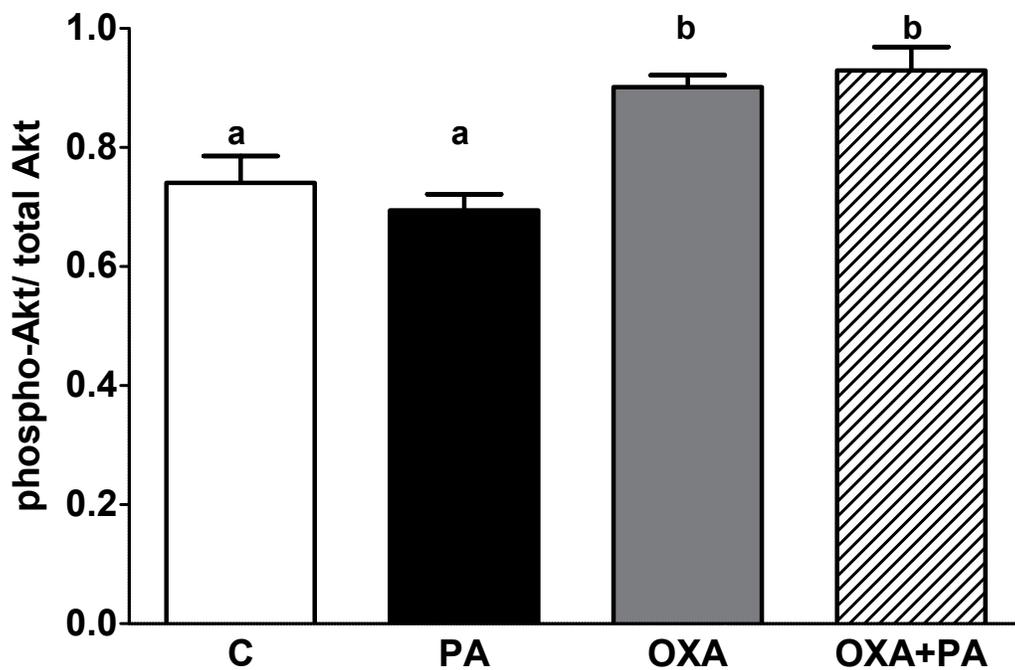
Figure 2.4. Orexin A increases Akt phosphorylation. mHypo-A1/2 cells were pretreated with OXA for 24 h and then exposed to PA in the presence or absence of OXA for an additional 1 h. Phosphorylated Akt is significantly increased following exposure to OXA in the presence or absence of PA ($p < 0.05$ vs. C, $p < 0.001$ vs. PA).
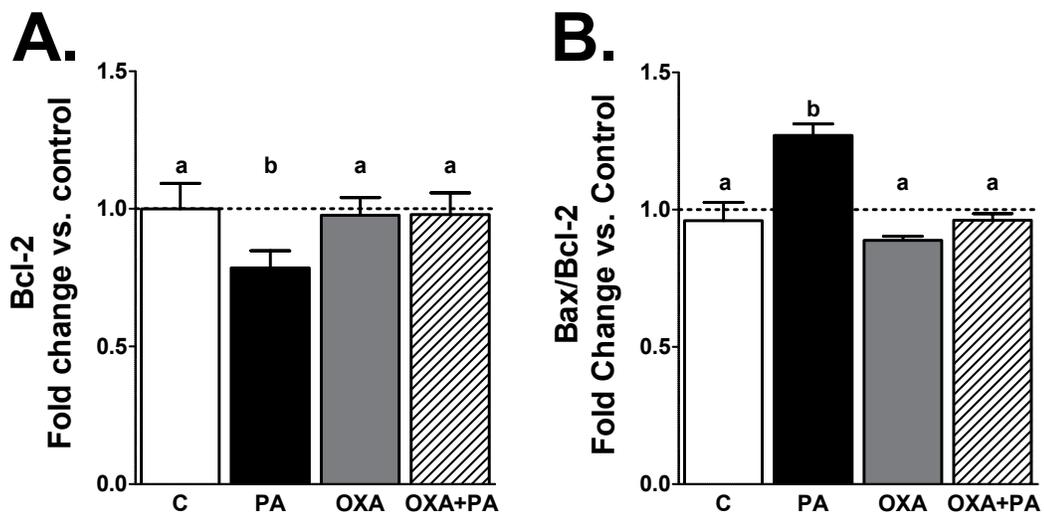
Figure 2.5. OXA stabilizes expression of anti-apoptotic gene Bcl-2. mHypoA-1/2 cells were pretreated with OXA for 24 h and then challenged with PA in the presence or absence of OXA for an additional 2 h. A) PA significantly reduces Bcl-2 expression (p<0.05 vs. C). OXA stabilizes Bcl-2 expression (p<0.05 vs. OXA only and OXA+PA). B) The Bax/Bcl-2 ratio is significantly increased following PA challenge (p<0.05 vs. C). However, OXA stabilizes Bax/Bcl-2 ratio (p<0.001 vs. OXA only, p<0.05 vs. OXA+PA).
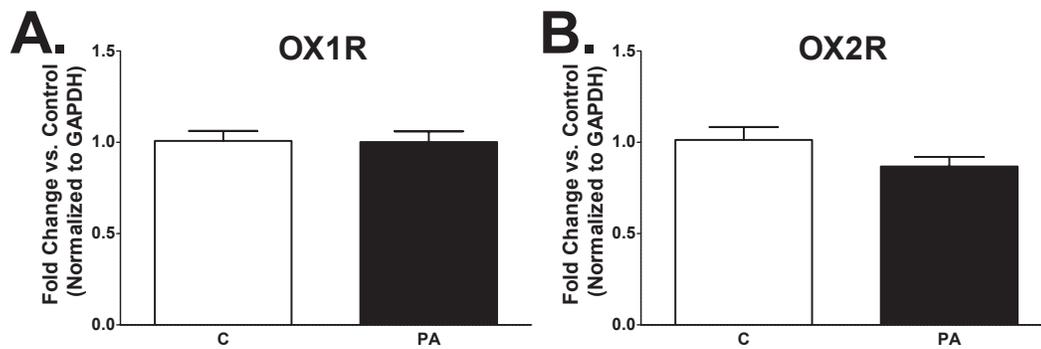
Figure 2.6. Expression of OX1R and OX2R is unchanged following acute PA exposure. mHypoA-1/2 cells exposed to vehicle (C) or PA (0.1 mM) for 2h. Changes in gene expression was determined using qRT-PCR. The following sequences were used OX1R (NM_198959) Forward: GCGATTATCTCTACCCGAAGC, Reverse: CAGGGACAGGTTGACAATG and OX2R (NM_198962): Forward: AATCCCACGGACTATGACGACG, Reverse: GAGAGCCACAACGAACACGATG.

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Figure 2.7-2.9. OXA alters metabolic respiration.

To determine the effects of OXA on metabolic respiration, mHypoA-1/2 cells were treated with increasing concentrations of OXA (50, 150, and 300 nM). We show that OXA increased basal respiration ($p < 0.05$ vs. C), ATP turnover ($p < 0.05$ 50, 300 nM OXA vs. C, $p < 0.001$ 150 nM OXA vs. C) maximum respiration ($p < 0.05$ 150, 300 nM OXA vs. C), and reserve capacity ($p < 0.05$ 150, 300 nM OXA vs. C) in a dose-dependent manner (Fig. 2.7A-E). This effect is directly mediated by orexin receptors, as treatment with DORA attenuated OXA-increased basal respiration ($p < 0.0001$ DORA, DORA+OXA vs. OXA), ATP turnover ($p < 0.0001$ DORA, DORA+OXA vs. OXA), maximum respiration ($p < 0.0001$ DORA, DORA+OXA vs. OXA), and reserve capacity ($p < 0.0001$ DORA vs. OXA, $p < 0.001$ DORA+OXA vs. OXA; Fig. 2.8A-E). We next sought to determine if PA alters metabolic respiration and if OXA treatment would negate this effect. We demonstrate PA significantly reduced basal respiration ($p < 0.0001$ vs. C, OXA, OXA+PA), ATP turnover ($p < 0.0001$ vs. C, OXA, $p < 0.001$ vs. OXA+PA), maximum respiration ($p < 0.0001$ vs. OXA, $p < 0.001$ vs. C, OXA+PA), and reserve capacity ($p < 0.05$ vs. C, OXA+PA, $p < 0.0001$ vs. OXA) (Fig 2.9A-E). Importantly, OXA attenuated PA-induced reduction in metabolic respiration (Fig 2.9A-E).

Discussion:

The hypothalamus is vital in sensing changes in fuel availability and maintaining energy balance. High fat diets contribute to hypothalamic dysregulation and obesity in part via increased inflammation, oxidative stress, and apoptosis (9, 20, 21, 25). Therefore, identifying mechanisms to prevent HFD-induced hypothalamic dysregulation is of major importance to treating obesity. Data support that OXA is a mediator of obesity resistance through increased spontaneous physical activity (94, 134), yet the intracellular components of how this occurs are unclear.
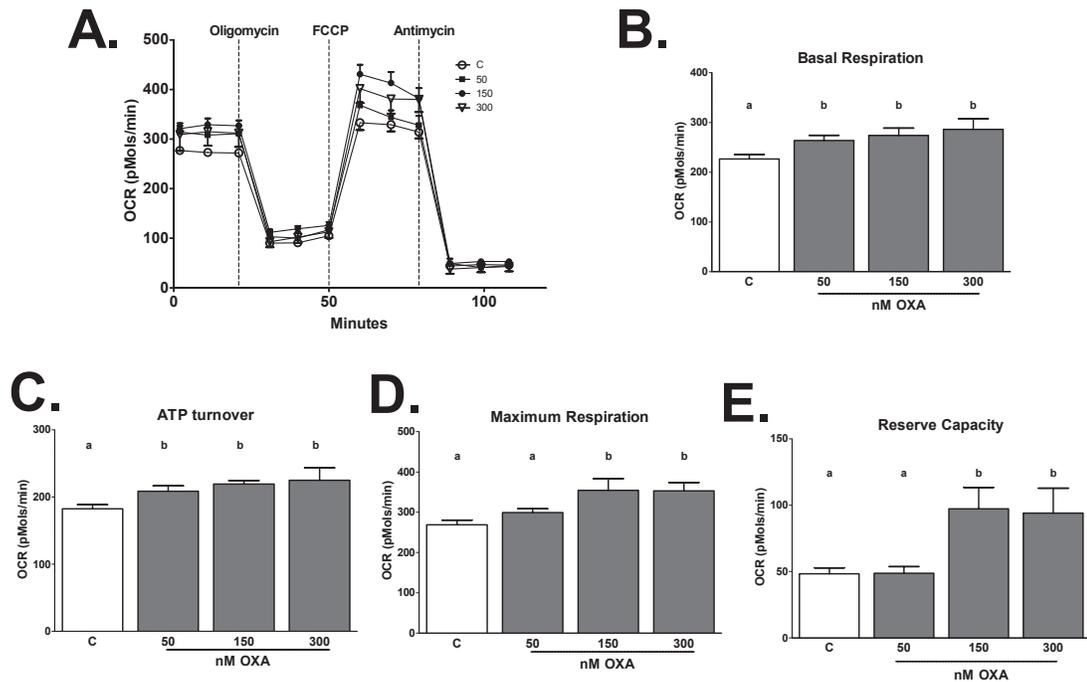
Figure 2.7. OXA alters metabolic respiration in a dose dependent manner.
Oxygen consumption rates (OCRs) were determined in mHypoA-1/2 cells treated
with increasing concentrations of OXA (50, 150, 300 nM) for 2 h. OXA increases
B) basal respiration ($p<0.05$ vs. C), C) ATP turnover ($p<0.05$ 50, 300 nM OXA vs.
C, $p<0.001$ 150 nM OXA vs. C), D) maximum respiration ($p<0.05$ 150, 300 nM
OXA vs. C), and E) reserve capacity ($p<0.05$ 150, 300 nM OXA vs. C).
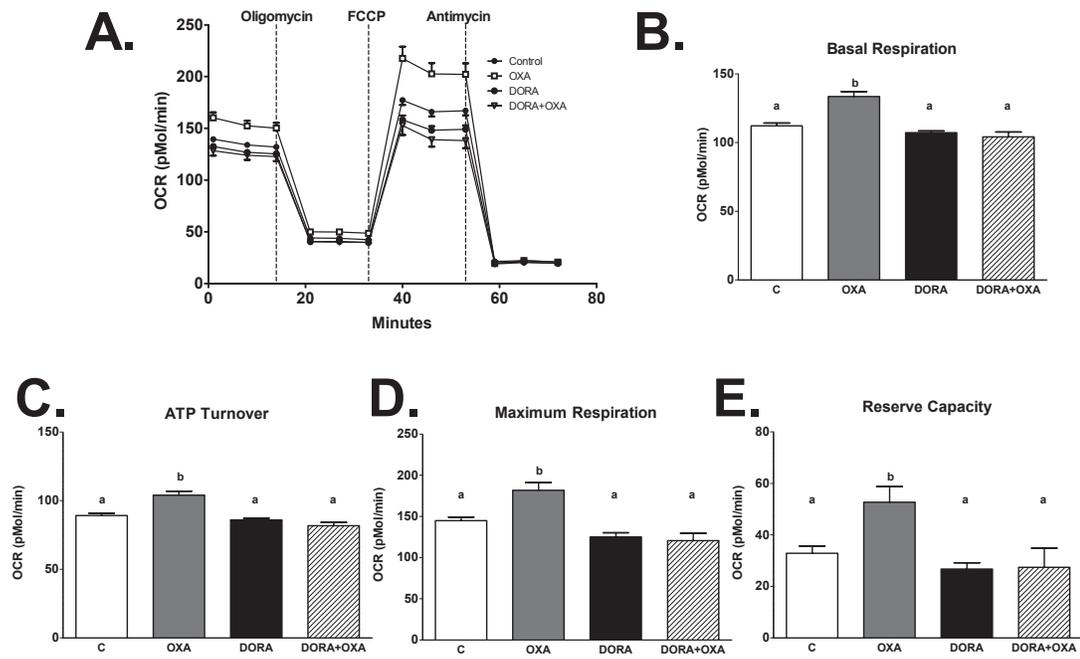
Figure 2.8. Orexin induced metabolic respiration is directly mediated by orexin receptors. Oxygen consumption rates (OCRs) were determined in mHypoA-1/2 cells treated with OXA (300 nM) and/or DORA (1.16 nM) for 2 h. OXA increases B) basal respiration ($p < 0.0001$ vs. C), C) ATP turnover ($p > 0.001$ vs. C), D) maximum respiration ($p < 0.0001$ vs. C), and E) reserve capacity ($p < 0.05$ vs. C). Treatment with DORA attenuates OXA-increased B) basal respiration ($p < 0.0001$ vs. OXA), C) ATP turnover ($p < 0.0001$ vs. OXA), D) maximum respiration ($p < 0.0001$ vs. OXA), and E) reserve capacity ($p < 0.0001$ DORA vs. OXA, $p < 0.001$ OXA+DORA vs. OXA).

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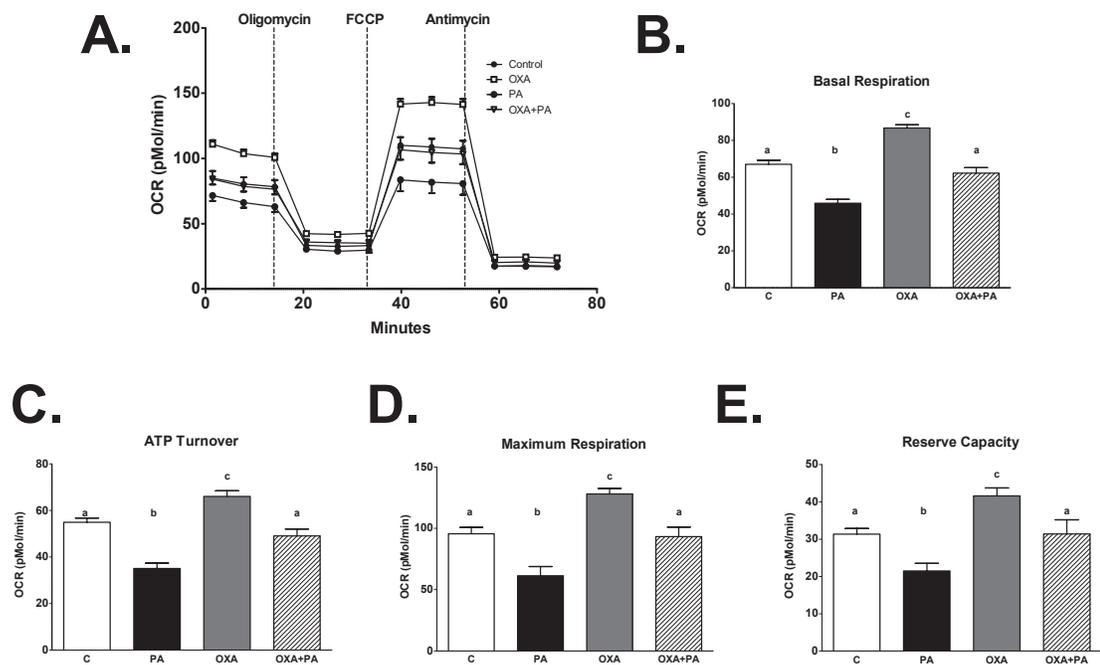
Figure 2.9. Orexin stabilizes metabolic respiration. Oxygen consumption rates (OCRs) were determined in mHypoA-1/2 cells pretreated with OXA (300 nM) for 24 h and challenged with PA in the presence or absence of OXA for 6 h. PA significantly reduces B) basal respiration ($p < 0.0001$ vs. C, OXA, OXA+PA), C) ATP turnover ($p < 0.0001$ vs. C, OXA, $p < 0.001$ vs. OXA+PA), D) maximum respiration ($p < 0.0001$ vs. OXA, $p < 0.001$ vs. C, OXA+PA), and E) reserve capacity ($p < 0.05$ vs. C, OXA+PA, $p < 0.0001$ vs. OXA). Treatment with OXA stabilizes PA-reduced B) basal respiration, C) ATP turnover, D) maximum respiration, and E) reserve capacity.

Prior reports demonstrate that OXA is neuroprotective against oxidative stress in hypothalamic and cortical neurons (22, 105). Likewise, we show that OXA prevents PA-induced apoptosis through inhibition of caspase-3/7 activity (Fig 2.2A-B). Our data show that PA increases ROS in hypothalamic cells (Fig 2.3), and notably, we show that OXA attenuates PA-induced ROS production (Fig 2.3). We also demonstrate that OXA increases Akt phosphorylation and stabilizes both Bcl-2 expression and the Bax/Bcl-2 ratio following PA challenge (Fig 2.5-2.7). These data indicate that neuroprotective mechanisms of OXA rely in part on attenuation of ROS signaling and caspase-dependent apoptosis. To the best of our knowledge, this is the first report demonstrating that OXA attenuates PA-induced hypothalamic cell death. Diets rich in PA exacerbate the production of ROS throughout the brain (130, 131, 151). Further, PA induces neuronal cell death in part via increased ROS production (152). Although modest amounts of ROS act as cell signaling molecules and are important in nutrient sensing, chronic and sudden increases in ROS, such as those observed after HFD exposure, alter cell physiology and induce pathways of cell death (111). Hypothalamic neuropeptide Y (NPY) and pro-melanocortin (POMC) neurons are in part regulated by ROS signaling to control food intake (111). Further, orexin neurons directly interact with POMC and NPY neurons (153), suggesting one mechanism through which OXA suppression of ROS could contribute to obesity resistance.

Activation of protein kinase phosphatidylinositol 3-kinase (PI3K/Akt) is required for stabilization of Bcl-2 expression and suppression of apoptosis in part through accumulation of Akt in the mitochondria (154-156). Others have demonstrated OXA is neuroprotective against oxidative stress via involvement of the PI3K/Akt signaling pathway (105, 157). Moreover, OXA may rely on increase mitochondrial accumulation of Akt to inhibit the onset of apoptosis (156). Under oxidative stress conditions, Akt accumulation in the mitochondria is inhibited (156) When PI3K/Akt activity is downregulated, neurons are more susceptible to

oxidative stress-induced cell death (158). Likewise, increased ROS leads to dephosphorylation of PI3K/Akt (159), indicating ROS levels play a crucial role in apoptosis. Phosphorylated PI3K/Akt upregulates Bcl-2 expression; conversely, oxidative stress downregulates Bcl-2 expression (133, 160, 161), therefore it is logical that OXA not only influences Bcl-2 expression but also alters mitochondrial function to inhibit apoptosis (156). Although others demonstrate OXA is neuroprotective in cortical neurons without any pretreatment (105, 106), our prior work (22) and data in the current study demonstrate that pretreating hypothalamic neurons with OXA for 24 h prior to challenge produces the most robust neuroprotective response (Fig 2.10). Orexin pretreatment prior to insults such as PA could lead to heightened activation of neuroprotective pathways, including increased Akt, mitogen-activated protein kinase, and hypoxia-inducible factor 1 alpha (HIF-1α) (94, 137, 138).

Mitochondria play an essential role in modulating ATP production, ROS, and apoptosis (162). Because the brain is a highly metabolically active organ, maintaining mitochondrial function and integrity is critically important. Mutations in mitochondrial proteins, impaired mitochondrial dynamics and respiration, and increased ROS production are associated with neurodegenerative diseases such as obesity, Alzheimer's disease, and Parkinson's disease (163-167). Therefore, understanding mechanisms to prevent mitochondrial disturbances are important for developing therapeutics for neurodegenerative diseases. Prior data indicate that OXA increases ATP production, indicating that OXA alters mitochondrial function (137). We demonstrate for the first time that OXA increases basal respiration, maximum respiration, ATP turnover, and reserve capacity in a dose-dependent manner in real time (Fig 2.7A-E). Additionally, we show OXA mediated changes of metabolic function are attenuated in the presence of DORA (Fig 2.8A-E). Increased reserve capacity is linked to promoting neuronal survival in models of oxidative stress (168, 169). Thus, the increased reserve capacity observed after
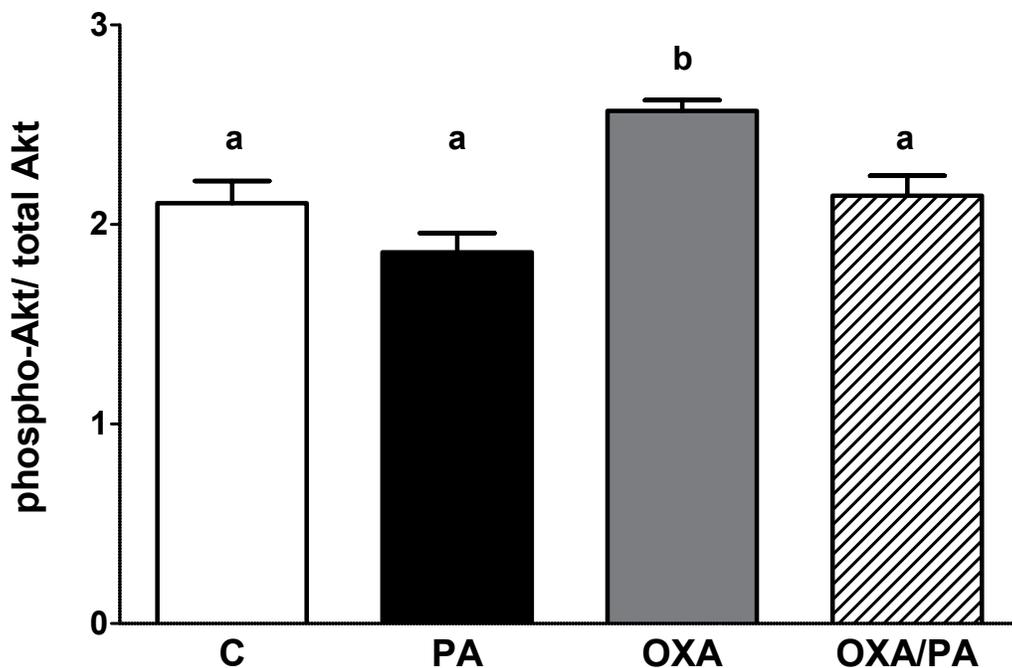
Figure 2.10. OXA pretreatment is necessary to increase Akt phosphorylation prior to simultaneously exposing mHypoA-1/2 neurons to PA and OXA. Using an in-cell ELISA, changes in phosphorylated Akt normalized to total Akt was determined following simultaneous exposure to OXA (300 nM), PA (0.1 mM), and/or vehicle (PBS/DMSO) control for 1 h. As expected, OXA significantly increases phosphorylated Akt ($p < 0.05$ vs. C, PA, OXA+PA). When cells are exposed to OXA and PA simultaneously, phosphorylated Akt is not increased indicating 24 h pretreatment with OXA is necessary for optimal neuroprotection.

OXA exposure could represent a bioenergetic advantage for neuronal survival during PA challenge. To the best of our knowledge, we demonstrate for the first time that OXA stabilizes basal respiration, maximum respiration, ATP turnover, and reserve capacity in the presence of PA (Fig 2.9A-E). These data indicate increasing mitochondrial bioenergetics as a potential mechanism through which OXA protects against insults such as HFDs. Maintaining hypothalamic circuitry would be beneficial in preventing the pathophysiology of neurodegenerative diseases such as obesity. Mitofusin 2, a mitochondrial membrane protein, participates in mitochondrial fusion and has recently been implicated in obesity pathogenesis. Diaz *et al* demonstrated rodents fed a high saturated fat diet had impaired hypothalamic mitochondrial function and reduction in mitofusin 2 (170). In addition, mitochondrial dynamics governed by mitofusin 2 are integral in maintaining hypothalamic neurons that dictate metabolic status (170-172). As such, OXA-induced neuroprotective mechanisms may rely in part on altering proteins involved in mitochondrial dynamics. Future studies aimed at targeting the effects of OXA on mitochondrial dynamics and a potential role of mitofusin 2 should be considered in the context of obesity and neurodegenerative diseases. Additionally, data suggest the neuroprotective and obesity resistant mechanisms triggered by OXA rely on a HIF-1α-dependent pathway (138). OXA upregulates HIF-1α, increasing ATP production and oxidative phosphorylation by shunting pyruvate into the tricarboxylic acid (TCA) cycle (22, 94, 137, 173). This HIF-1α-triggered increase in ATP could represent a biochemical mechanism through which OXA-producing neurons could prompt increases in cellular activity in target cell populations, subsequently stimulating wakefulness and other behavioral and physiological processes (137, 173).

In summary, these data support that OXA is neuroprotective against PA-induced neuronal insult in part through a ROS/caspase-dependent mechanism. Although *in vitro* studies have limitations, this work demonstrates an important

mechanism in which OXA alters mitochondrial function and promotes neuronal survival following PA challenge. Future work will explore the effects of OXA signaling and the relationship of long and short-term changes in mitochondrial function and proteins in response to HFD. Understanding the role of OXA in maintaining neuronal survival will provide therapeutic targets for prevention or treatment of neurodegenerative diseases such as obesity.

Chapter 3

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Chapter 3

Role of orexin A signaling in dietary palmitic acid-activated microglial cells

Introduction

The orexins (orexins A and B; hypocretin 1 and 2) are hypothalamic peptides produced in lateral hypothalamic neurons and released widely throughout the CNS (91, 92). Orexin A (OXA) and B (OXB) regulate homeostatic mechanisms of energy balance and metabolism (94) through activation of two G-protein coupled receptors, orexin receptors 1 and 2 (OX1R and OX2R, respectively) (92). Recent studies have shown that orexin plays a role in neuroprotection (94, 134), in part by reducing lipid peroxidation, apoptosis, and inflammation (22, 105-107). Data suggest that the neuroprotective effects of orexin could rely on modulation of microglia, the resident immune cells of the brain.

Microglia are initiators of the neuroinflammatory response and are highly reactive to endogenous signaling. Microglia are highly dynamic, transitioning between neurotoxic pro-inflammatory (M1) and neuroprotective (M2) phenotypes. For example, following cerebral ischemic events, microglia are first activated to a neuroprotective M2 phenotype as oxygen levels decrease, and then switch to a pro-inflammatory M1 phenotype, inducing cell death (174). While inflammation is a component of a normal immune response, chronic activation to M1 pro-inflammatory phenotypes can cause microglia to become refractory and contribute to subsequent neuronal dysfunction (42).

Several lines of evidence suggest a role for orexin in modulation of microglia. In cerebral ischemia models, pretreatment with OXA reduces infarct size through a microglial-mediated pathway (107). Microglia may also become more sensitive to orexin signaling after activation. The potent pro-inflammatory agonist

lipopolysaccharide (LPS) increases tumor necrosis factor alpha (TNF-α) in microglia, but also increases OX1R expression, and OXA treatment prior to LPS exposure reduces TNF-α in microglia (107). These data indicate that increased microglial OX1R could enhance responsiveness to orexin, thus enhancing capability to counter an inflammatory insult. We are especially interested in how these orexin-microglia dynamics might impact brain health in the context of diet-induced obesity.

Dietary intake influences neuronal function, overall brain health, and cognition (175). High fat diet increases circulating pro-inflammatory cytokines released from microglial cells, resulting in hypothalamic neuroinflammation and neurodegeneration (8, 11). Chronic intake of saturated fatty acids (SFA) such as palmitic acid (PA, C16:0), activate microglia to an M1 phenotype, eliciting the release of pro-inflammatory cytokines (9, 26). High fat diets cause this response by activating nuclear translocation of microglial nuclear factor-kappa B (NFκB), initiating release of pro-inflammatory cytokines such as TNF-α and interleukin-6 (IL-6) (9, 11, 26). Palmitic acid activates microglia through a toll like receptor 4 (TLR-4)-dependent pathway, inducing the release of TNF-α and IL-6 (26). Further, microglia activated by SFA via TLR-4 induce neuronal cell death (26). Given the above findings on orexin action in microglia, orexin signaling might promote microglia switching to a protective M2 phenotype, protecting against palmitic acid induced inflammation.

The objective of these studies was to determine if orexin reduces PA-induced neuroinflammation by altering microglial M1/M2 phenotype dynamics. To test whether orexin treatment influences microglial phenotype, we evaluated the effect of OXA on PA-induced release of pro-inflammatory cytokines in an immortalized murine microglial cell line (designated BV2). We first validated that PA activates microglia to an M1 state via TLR-4. In our next set of experiments, we tested whether OXA pretreatment influenced levels of the M1 pro-inflammatory

markers IL-6, TNF-α; inducible nitric oxide synthase (iNOS); and the M2 anti-inflammatory marker arginase-1 in microglia. Finally, we performed a series of studies to determine how conditioned media from these prior tests altered hypothalamic neuronal survival.

Materials and Methods

Cell culture and reagents:

Immortalized murine microglial cells (BV2) and adult murine hypothalamic cells (mHypoA-1/2, cited elsewhere as CLU172; CELLutions Biosystems) (140, 141, 176) were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and 1% penicillin, streptomycin, and neomycin (Invitrogen) and maintained at 37°C with 5% CO_2. Orexin A peptide (American Peptides) was suspended in phosphate buffered saline (PBS, Invitrogen) and diluted to 300 nM in DMEM. Palmitic acid (Sigma Aldrich) was conjugated to fatty acid free bovine serum albumin (BSA) (177) and diluted to 0.1 mM in DMEM. Lipopolysaccharide (Sigma Aldrich) was reconstituted in PBS and diluted to 0.4 μg (100ng/ml) in DMEM. The TLR-4 inhibitor TAK-242 (EMD Millipore) was reconstituted in DMSO and diluted to 100 nM in DMEM.

For microglial experiments with OXA, BV2 cells were seeded in T-25 flasks at $7x10^5$ cells and grown to ~80% confluency. Concentrations of OXA and PA are based on Xiong *et al* and Wang *et al* (26, 107). For all assays, cells were serum starved for 24 h. The experiment was completed in two stages: a 1 h pre-incubation followed by a 4 h challenge. Pre-incubation used either vehicle (PBS) or OXA (300 nM). For challenge, cells were exposed to vehicle (fatty acid free BSA), PA (0.1 mM), or LPS (0.4 µg; 100 ng/ml). There were a total of 5 treatment groups: vehicle-vehicle (control), vehicle-LPS, vehicle-PA, OXA-vehicle, and OXA-PA. After treatment, supernatant and cells were rapidly collected and stored at -20°C.

Supernatant from microglial cultures treated as described here was used as conditioned media for hypothalamic neuronal cultures (described below).

For microglial experiments with TAK-242, cells were seeded in 6 well plates at 3.5×10^5 cells per well and grown to ~80% confluency. Concentrations and time points were based on Matsunaga *et al* and Takashima *et al* (178, 179). Cells were serum starved for 24 h. TAK-242 (100 nM) or vehicle was added 20 minutes prior to incubation with PA (0.1mM) or vehicle for 4 h.

Real-time RT-PCR:

Total RNA was extracted from BV2 cells with Trizol (Invitrogen) as previously described (22, 180). Concentrations were determined using spectrophotometric readings at 260 and 280 nm (Nanodrop 8000, Thermo Fisher Scientific) and 2.5 µg RNA was used for each reaction. Primer sequences were generated using MacVector 15 for OX1R (NM_198959), OX2R (NM_198962), IL-6 (NM_031168), iNOS (NM_010927), arginase-1 (NM_007482) and GAPDH (NM_017008). Relative mRNA expression of target genes was determined using SYBR Green detection normalized to GAPDH using the Δ-ΔCT method (146).

Enzyme-linked immunosorbent assay (ELISA):

TNF-α level in culture media was determined using an ELISA kit (BioLegend Inc.). Concentrations were determined using a spectrophotometer (SpectraMax-M5; Molecular Probes). Data are presented as picograms of TNF-α/ml.

Cell Viability Assay for Hypothalamic Cells:

mHypoA-1/2 cells were seeded in a 96 well plate at 5×10^3 cells per well overnight. Amicon Filters (Millipore) were used to remove PA and OXA and concentrate conditioned media (supernatant from microglial cultures described above) (181). Concentrated conditioned media was used at a six-fold concentration and added to mHypoA-1/2 cells for 24 h. Time points were based on previously described

studies (26, 182, 183). Cell survival was determined using a resazurin-based assay (Presto Blue, Invitrogen) producing a fluorescent signal (22). Activity was determined using a spectrophotometer (SpectraMax-M5) and presented as percent relative fluorescence units (RFU) change vs. control.

Statistical Methods:

Significance differences were determined by unpaired, two-tailed *t*-tests using Graph Pad Prism 5.

Results

Palmitic acid activates BV2 microglia via TLR-4.

To verify that PA induces pro-inflammatory cytokine release from microglial cells through activation of TLR-4 receptor, BV2 microglial cells were treated with the TLR-4 inhibitor TAK-242 (or vehicle control; C) in the presence or absence of PA for 4 h. As expected, PA increased TNF-α secretion by 90 percent compared to control, by 320 percent vs. TAK-242 only, and by 400 percent vs. TAK-242 plus PA ($p < 0.001$ vs. C, $p < 0.0001$ vs TAK-242 and TAK-242 plus PA). Treatment with TAK-242 significantly reduced endogenous TNF-α production. Further, TAK-242 attenuated PA induced TNF-α secretion. (Fig. 3.1A)

Palmitic acid increases expression of microglial orexin 1 receptor.

To determine if PA increases microglial OX1R expression in our model, we pretreated BV2 microglia for 1 h with either OXA or vehicle control. Following pretreatment, microglia were exposed to PA, LPS, or vehicle for 4 h. Palmitic acid and LPS increased expression of OX1R (Fig. 3.1B; $p < 0.05$ vs. control and OXA only) by 55 and 75 percent respectively, but not OX2R (Fig. 3.1C) in microglial cells following 4 h exposure.
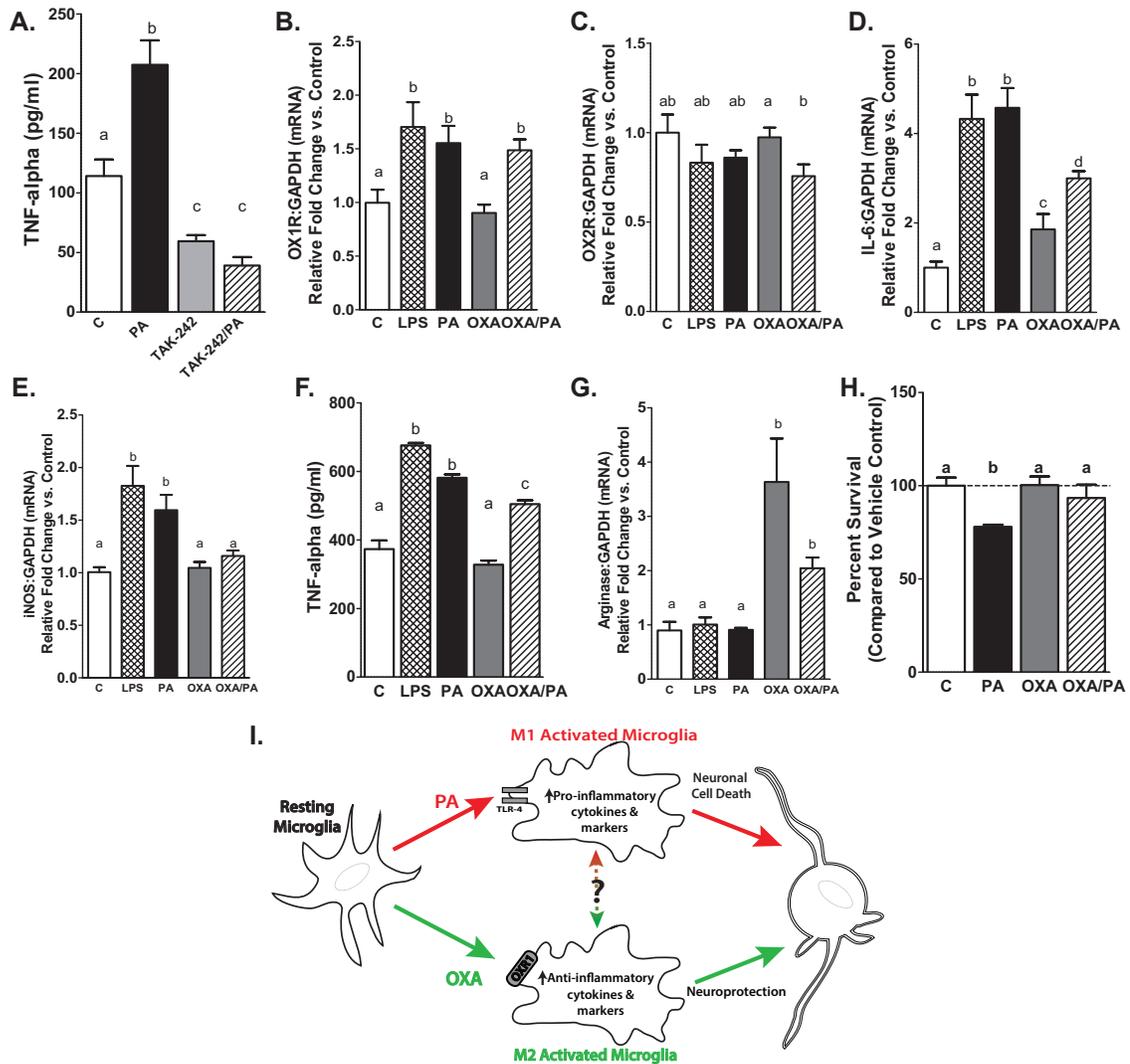
Figure 3.1. (A) Palmitic acid activates microglia via TLR-4. TNF-α secretion from microglial BV2 cells increases following 4 h PA exposure. Inhibiting TLR-4 attenuates PA–induced TNF-α secretion. Microglia treated with TLR-4 inhibitor (TAK-242) have significantly reduced TNF-α secretion ($p<0.001$ PA vs. vehicle control (C), $p<0.0001$ PA vs. TAK-242, and TAK-242+PA, $p<0.001$ C vs. TAK-242 and TAK-242+PA). **(B-C) Palmitic acid increases orexin 1 receptor expression.** Microglia exposed to PA or LPS show increased OX1R expression (B) but not OX2R expression (C). Pretreatment of microglia with OXA before PA challenge increased OX1R expression and reduced OX2R expression. Different

letters above bars represent statistical significance at p<0.05 OXA vs. OXA/PA. **(D) Orexin A suppresses pro-inflammatory IL-6 expression in microglial cells.** Pro-inflammatory marker IL-6 expression is increased in BV2 microglia following PA and LPS exposure. Pretreatment of microglia with OXA before PA challenge reduces IL-6 expression compared to microglia exposed to PA only, but remains increased relative to vehicle (p<0.05 C vs. OXA, p<0.001 LPS vs. OXA and OXA/PA, p<0.001 PA vs. OXA and OXA/PA p<0.0001 C vs. LPS, PA, and OXA/PA). **(E) Orexin A suppresses pro-inflammatory iNOS expression.** Palmitic acid and LPS increase microglial iNOS expression, while OXA pretreatment attenuates PA-induced iNOS expression (PA and LPS p<0.001 vs control, p<0.05 vs OXA and OXA/PA). **(F) Orexin A reduces TNF-α secretion from BV2 microglial cells.** PA and LPS treatment increase TNF-α secretion compared to vehicle– or OXA only–treated microglia. Pretreatment of microglia with OXA before PA challenge reduces TNF-α secretion compared to PA only– but not vehicle–treated microglia. Different letters above bars represent statistical significance at p<0.0001. **(G) Orexin A increases M2 marker arginase-1 gene expression.** Pretreatment with OXA prior to PA challenge increases arginase-1 gene expression (OXA/PA p<0.001 vs. control, p <0.05 vs. PA and LPS). OXA alone increases arginase-1 expression in microglial cells (p <0.001 OXA vs. control, PA, and LPS). **(H) Orexin A attenuates hypothalamic neuronal cell death in microglial-conditioned media.** Adult hypothalamic cells have increased cell death following 24 h exposure to conditioned media from microglia stimulated with PA. Hypothalamic cells exposed to conditioned media from microglia pretreated with OXA before PA challenge, or vehicle-treated microglia, have reduced cell death compared to those exposed to media from PA-challenged microglia. p<0.0001 Vehicle vs. PA, p<0.001 PA vs. OXA/PA, p<0.0001 PA vs. OXA. **(I) Hypothesized Orexin A microglial immunomodulation pathway.** We hypothesize that OXA reduces M1 microglial activation and increase M2 microglial

activation to maintain neuronal survival. Saturated fatty acid challenge (PA) induces an M1 microglial phenotype and the release of pro-inflammatory cytokines, contributing to neurodegeneration. Orexin A may influence proportion of M1 microglia by reducing rate of activation or potentially by aiding in conversion between M1 and M2 states.

Orexin A suppresses pro-inflammatory markers and increases expression of anti-inflammatory M2 marker arginase-1 in microglial cells.

Gene expression of the pro-inflammatory cytokine IL-6 is increased by 3.5 fold following PA and LPS exposure (Fig. 3.1D; $p<0.0001$ vs. control, $p<0.001$ vs. OXA only). Further, OXA pretreatment prior to PA challenge reduced IL-6 expression by 50 percent compared to PA only (Fig. 3.1D; $p<0.0001$ vs. control, $p<0.001$ vs. OXA only). Expression of pro-inflammatory marker iNOS is also increased following PA and LPS exposure by 60 and 75 percent respectively (Fig. 3.1E; $p<0.001$ vs control, $p<0.05$ vs OXA and OXA/PA). Conversely, OXA pretreatment attenuated PA–induced increase of iNOS expression (Fig. 3.1E). To test whether OXA influences TNF-α secretion in microglia, BV2 cells were pretreated with OXA or vehicle and challenged with PA, LPS, or vehicle as described above. An ELISA for TNF-α in the supernatant showed that PA and LPS treatment increased TNF-α secretion by 90 percent compared to vehicle control ($p<0.0001$) or OXA treatment only ($p<0.0001$) (Fig. 3.1F). Microglia treated with OXA plus PA reduced TNF-α secretion by 20 percent compared to PA only, but not to vehicle control (Fig. 1F; $p<0.0001$ vs. control and OXA only). As shown in Fig. 3.1G, OXA increased arginase-1 gene expression by 250 percent ($p<0.001$ vs. control, $p <0.05$ vs. PA and LPS). Notably, OXA pretreatment prior to PA challenge also increased arginase-1 expression by 100 percent ($p<0.001$ vs. control, PA, and LPS).

Orexin A attenuates hypothalamic neuronal cell death in microglial-conditioned media.

Exposure to conditioned media from PA–treated microglia induces neuronal cell death due to the release of pro-inflammatory cytokines and other inflammatory factors (26). We tested whether OXA treatment could affect inflammatory properties of conditioned media from PA-challenged microglia. Hypothalamic cells

exposed to conditioned media from microglia treated with OXA only or pretreated with OXA and challenged with PA showed increased viability compared to hypothalamic cells exposed to conditioned media from PA–activated microglia (Fig. 3.1H; $p < 0.0006$ vs. OXA, $p < 0.05$ vs. OXA/PA). Further, cells exposed to conditioned media from PA–activated microglia had increased cell death compared to vehicle control (Fig. 3.1H; $p = 0.0005$).

Discussion

Microglia are vital to neuronal health by maintaining a favorable microenvironment within the CNS (64, 184). Communication between neurons, microglia, and other CNS cells is highly dynamic and responsive to environmental stimuli. This balance can be disturbed if microglial activation state tips toward a chronic inflammatory phenotype, as is observed in obesity (9, 185, 186). An important unanswered question is whether it is the high fat diet or obesity itself that directly induces microglial activation. High fat diets and obesity, either independently or synergistically, have the same consequences: microglial activation and prolonged circulation of pro-inflammatory cytokines (9, 185, 186). Our data confirm prior reports indicating PA induces microglial cytokine production through a TLR-4 dependent pathway (Fig. 3.1A) (26). We have shown that OX1R expression is increased in microglia challenged with PA or LPS (Fig. 3.1B). To the best of our knowledge, this is the first report demonstrating that PA increases OX1R expression in microglia. Our data also suggest that orexin can alter the activation state of microglia, reducing microglial M1 pro-inflammatory state by promoting the conversion to M2 phenotype, as OXA pretreatment reduced microglial M1 pro-inflammatory response during PA challenge (Fig. 3.1D-F). Consistent with previous reports (107), these data support that OXA can act as an immunomodulatory regulator of microglia. Most importantly, we show for the first time both that hypothalamic cells exposed to conditioned media from OXA and OXA/PA treated microglia have increased cell survival compared to those exposed

to media from PA-activated microglia without orexin, and that OXA pretreatment may shift microglia to an M2 protective phenotype (Fig. 3.1G-H). Our results demonstrate OXA modulates microglial activation states in response to PA exposure by reducing pro-inflammatory cytokine release and increasing anti-inflammatory markers.

In microglia activated by PA, addition of OXA significantly reduced iNOS expression. Nobunaga *et al.* demonstrated that microglial derived iNOS contributed to a loss of orexin producing neurons and subsequent metabolic dysfunction in mice fed a high fat diet (187). Reduction of iNOS expression due to OXA modulation in our study could therefore help in maintaining a favorable environment for surrounding cells. Likewise, conditioned media from PA-activated microglia did not induce hypothalamic cell death when microglia were pretreated with OXA prior to PA challenge. It is plausible that increasing the pretreatment time with OXA prior to PA exposure could further reduce pro-inflammatory cytokine release.

Our findings that PA increases OX1R expression in microglial cells are consistent with previous findings indicating that the pro-inflammatory stimuli LPS also increases OX1R expression (107). Increased microglial OX1R expression in response to a TLR-4 mediated pro-inflammatory stimulus could represent a compensatory response to reduce the release of inflammatory cytokines. Others have demonstrated that astrocytes, a subset of glial support cells, are responsive to OXA through OX1R by increasing migration following OXA exposure, further indicating OXA is modulatory not only through neuronal cells but also glial cells (188). Until recently, microglia were thought to be passive support cells, but are now understood to contribute to fine-tuning neural-glial circuitry through cultivating synapses and altering plasticity in healthy and diseased brains (50, 189). Additionally, microglia maintain the brain microenvironment through phagocytizing debris, pathogens, dead cells, and misfolded proteins. In neurological disorders,

microglia can be chronically activated to M1 phenotypes, resulting in an unfavorable environment for neuronal networks (190, 191). Here we lay the groundwork for understanding how OXA can modulate microglia responses to promote neuronal survival. Performing a more complete profile of other pro– and anti–inflammatory cytokines in future studies could provide insight into how priming microglia with OXA can provide a favorable brain microenvironment and maintain neuronal health. While we do not yet fully know how OXA modulates microglia, one mechanism may be through shifting microglia towards an M2 phenotype, or at least slowing rate of conversion to an M1 state. In other studies of acute neuroinflammatory responses, microglia activated to an M2 protective phenotype, including increased arginase-1 expression, showed reduction in neuronal injury and inflammation (54, 192-194). Promotion of M2 microglial function has been demonstrated to either support or enhance neural-glial cross talk via increases in the microglial CX3CR1 (fractalkine) receptor (195). Fractalkine receptor increase has not been evaluated with respect to OXA stimulation, but merits further research.

In summary, these data support the hypothesis that orexin influences the M1/M2 activation state of microglia during challenge with SFA, reducing pro-inflammatory cytokine release to maintain neuronal survival in the surrounding microenvironment (Fig. 3.1). Future work will examine the implications of orexin on microglial dynamics in the context of diet and obesity. In other inflammatory diseases, microglia have been profiled to gain a better understanding of disease status (41). Chronic microglial activation in response to high fat diet and overnutrition has yet to be fully characterized. Delineating microglial phenotypes in the hypothalamus in response to chronic high fat diet could be useful in appreciating the neuropathology and the development of obesity. Understanding the long and short term effects of PA-activated microglia and OXA-mediated

immunomodulation could lead to therapies for maintaining neuronal networks involved in regulating healthy metabolism.

Chapter 4

High fat diet increases cognitive decline and neuroinflammation

in a model of orexin loss

Introduction

Obesity is coupled with peripheral and central chronic low-grade inflammation (6, 196). Dietary fats increase the risk of developing obesity and cognitive disorders such as Alzheimer's disease (AD) (3, 31, 196). Prolonged overnutrition and excess intake of saturated fatty acids (SFA), specifically palmitic acid (PA) increase neuroinflammation and microglial activation (8-11, 39). Neuroinflammation, accompanied with progressive neuronal loss, is known to be heightened in cognitive decline and obesity (4). Moreover, neuroinflammation and microglial activation represent a common link between age-related neurodegenerative diseases and metabolic related disorders.

Microglia (resident immune cells of the brain) play a dynamic role in responding to changes within the surrounding environment leading to constant transitioning between phenotypes (197). Microglia have recently been appreciated to have an important role in cognition (60). In the healthy brain, microglia are constantly surveying the surrounding microenvironment to promote structural formation and elimination of neuronal synapses, a process especially important in the hippocampus (60). One mechanism in which this could occur is through orexin signaling. Orexin A and B (OXA; hypocretin 1 and OXB; hypocretin 2 respectively) are ligands for the G-protein coupled receptors, orexin receptor 1 and 2 (OX1R and OX2R respectively). Orexin neurons project throughout the central nervous system (CNS), including the hippocampus, and are responsible for mediating cognitive processes, operant tasks, and fear conditioning (93, 96-100). Additionally, orexin signaling is altered in AD patients (101, 102). Moreover, we

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and others have demonstrated that orexin loss impairs memory, supporting that orexin mediates cognitive function (104, 198).

Recent studies highlight novel functions of OXA, including neuroprotection and decreased apoptosis and neuroinflammation via activation of orexin receptors (22, 105-108). Data indicate that orexin-induced neuroprotection could rely upon microglial modulation (25, 107). In a cerebral ischemia model, pretreatment with OXA reduces infarct size through a microglial-mediated pathway (107). Additionally, chronic exposure to the potent pro-inflammatory agonist lipopolysaccharide (LPS), reduced orexin signaling indicating orexin has a role in the inflammatory response (109). Microglia may also become more sensitive to orexin signaling after activation (25). LPS increases TNF-α (tumor necrosis factor-α) in microglia, but also increases OX1R expression, and OXA treatment prior to LPS exposure reduces TNF-α in microglia (107). We have also demonstrated that PA increases OX1R, and that PA treatment causes microglia to shift toward a pro-inflammatory state (25). However, when microglia are pretreated with OXA prior to PA exposure, the PA-induced pro-inflammatory response is attenuated (25). These data indicate increased sensitivity to OXA may allow orexin-mediated attenuation of the toxic microglial phenotype to a protective anti-inflammatory phenotype.

Our overall hypothesis is that orexin effects on memory depend in part upon immunomodulatory control of microglia. To test this, we used orexin/ataxin-3 (O/A3) mice, a rodent model that gradually loses orexin-producing neurons (~84% by 6 mo age) (199). The O/A3 mice were maintained on a HFD for 4 weeks. We then sought to determine if orexin loss alters neuroinflammation and cognition.

Methods:

Animals:

7-8-month-old male wild type (WT) and orexin ataxin-3 (O/A3) mice were obtained from a breeding colony at the Minneapolis VA Health Care System (VAHCS). The colony, maintained by Dr. Catherine Kotz, originated from founder animals obtained from Dr. Masashi Yanagisawa at the University of Texas-Southwestern, bred to wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME USA). Genotype was confirmed using PCR as described in (199). Mice were maintained in a 12:12 hour light/dark cycle in a temperature controlled room(21-22°C), and were group-housed until the beginning of behavioral testing. Normal chow (NC; Teklad 8604 Lab Chow; Harlan, Indianapolis, IN USA) or high fat diet (HFD; Research Diets D12452, 45% fat; New Brunswick, NJ USA) and water were provided *ad libitum*. Body weights and food intake were measured thrice weekly. Body composition was recorded using an EchoMRI 700 (Echo Medical Systems, Houston, TX USA) before HFD exposure and 14 and 28 days following HFD exposure, as previously described (200). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Minneapolis VAHCS.

Two-Way Active Avoidance (TWAA) Task:

Mice were placed in the automated two-way shuttle box (size, 45.7 x 20.3 x 30.5 cm; model 75-FSFX-Fusion; AccuScan Instruments, Columbus, OH USA) and allowed to acclimate for 2 min. Mice were able to move freely throughout the shuttle box during acclimation and training trials. Mice were trained on a massed 20-trial shuttle box two-way active avoidance task. The procedures for the conditioned stimulus and unconditioned stimulus were as follows. A tone (5000 Hz; 45 dB) and a pulsatile light (2.5 Hz) were presented as a conditioned stimulus in the compartment with the animal, paired 5 s later with a 0.3 mA scrambled foot shock (unconditioned stimulus) delivered through the floor grid (steel rods 0.5 cm in

diameter, spaced 1.5 cm between centers). The "scrambled" nature of the shock prevents the animals from finding a "no-shock" position on the floor. To avoid receiving a foot shock the mouse had 5 s to move to the opposite compartment. If the animal did not move to the other compartment, the unconditioned stimulus was delivered for a maximum of 7 s, and the conditioned stimulus ended with the unconditioned stimulus. While receiving the unconditioned stimulus, if the animal moved to the other compartment, both conditioned and unconditioned stimuli ended immediately. The inter-trial interval was variable with a mean of 40 s. A computer using remote monitoring system software (Fusion software) was used to control experimental protocols and data collection (78, 201, 202).

TWAA Data Analysis:

To measure performance on the TWAA task, each trial was analyzed for successful shock avoidances and shock latency. Experimental data were analyzed using two-factor (phenotype x diet) ANOVA, followed by Holm-Sidak post-hoc analysis for multiple comparisons. Significance was considered to be achieved where $p < 0.05$. Analyses were performed to determine, a) the differences in acquisition between phenotypes, and b) differences in retention between treatment groups. The improvement of performance between training trials (first session) and test trials (all sessions thereafter) were analyzed for avoidances (crossing after conditioned stimulus, but before shock delivery) and for escape latency (interval between conditioned stimulus and a response). For all experiments, mice underwent 20 trials for training, 24 h later they were retested for TWAA response. Body composition was determined and mice were randomly divided into 4 groups and given either HFD or remained on NC (WT+NC, n = 8; WT+HFD, n = 8; O/A3+NC, n = 9; O/A3+HFD n = 11). Following 14 d and 28 d of diet exposure mice were retested for TWAA response and body composition. These parameters were also compared between groups during test sessions to see if there was any

specific effect of treatment (NC vs. HFD) on memory retention. Statistics were performed using GraphPad Prism 7 (78, 202).

Locomotor activity:

To measure exploration and general locomotor activity, mice were placed in a square chamber (27.3 cm x 27.3 cm x 20.3 cm; Med-Associates). The chamber is divided into a grid by infrared beams. An automated system monitors beam breaks to determine exploratory activity. Mice were allowed to explore for 2 h. All tests were conducted 5 h after lights on.

Reverse transcription polymerase chain reaction (qRT-PCR):

Hippocampal tissue was rapidly dissected from WT and O/A3 mice. Total RNA was isolated with the aid of TRIzol reagent (Invitrogen). Real-time thermocycling was performed on ABI 7900HT Fast Real Time PCR System (Applied Biosystems) by two-step RT-PCR using SYBR green. Genes analyzed are indicated in Table 4.1. Data analysis was performed using Sequence Detection System software from Applied Biosystems. The experimental Ct (cycle threshold) was calibrated against the endogenous control product GAPDH. Samples were analyzed for relative gene expression by the ΔΔ-Ct method (203)

Results:

High fat diet increases obesity in orexin/ataxin-3 mice.

We first sought to determine the effects of HFD on food intake, body weight gain and body composition. WT and O/A3 mice have increased cumulative food intake on days 6-10 (Fig 4.1A). Body weight gain is significantly increased in O/A3-HFD mice compared to WT-NC beginning at day 6 and continue to be significant through day 28 ($p < 0.01$). WT-HFD mice have significantly increased body weight compared to WT-NC at day 8 through day 28 ($p < 0.05$). Both WT and O/A3 mice have increased body weight compared to O/A3-NC and WT-NC at day 10 through

Table 4.1. qPCR Primers (5'→3')

Gene	Forward Primer	Reverse Primer
OX1R (NM_198959.2)	CTCATAGCCTTGGTGGGCAA	ACCGACACAGCCTGGAGATA
OX2R (NM_198962.3)	CACGGACTATGACGACGAGG	AGAGCCACAACGAACACGAT
CX3CR1 (NM_009987)	CCGCCAACTCCATGAACAAC	GGATGAGTCTGACGGCTCTG
Iba1 (NM_019467.2)	GGAGATTTCAAAAGCTGATGTGGA	CCTCAGACGCTGGTTGTCTT
TNF-α (NM_001278601)	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG
Irg1 (NM_008392.1)	TATGCCAACTACTCCCCCGA	CGGGAAGCTCTTAAAGGCCA
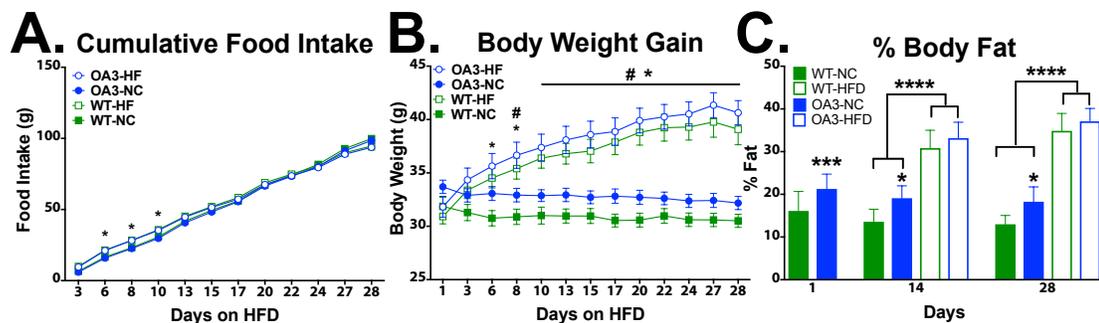
Figure 4.1. High fat diet increases body weight gain and fat mass. WT and O/A3 mice were given HFD or NC for 28 d. Cumulative food intake was increased after 6, 8, and 10 d HFD exposure regardless of genotype (A, *p<0.05). Mice fed HFD had significantly increased body weight gain days 10-28 compared to mice fed NC (B, * p<0.001 vs. WT-NC, # p<0.01 vs. O/A3-NC). O/A3 mice have a higher percent body fat compared to WT mice (***p<0.001), following 14 and 28 d on HFD WT and O/A3 mice have significantly more body fat compared to WT and O/A3 mice on NC (C, *p<0.05, ****p<0.0001).

day 28 (Fig 4.1B). Despite no significant difference in body weight, O/A3-NC mice have increased body fat compared to WT-NC mice on days 1, 14, and 28 (Fig 4.1C). HFD-fed O/A3 and WT mice have increased body fat compared to NC-fed WT and O/A3 mice on days 14 and 28 (Fig 4.1C). High fat diet reduces locomotor activity but does not impair anxiety like behavior.

To determine if changes in body fat and weight were due in part to orexin signaling, we analyzed locomotor activity in mice following 4 weeks HFD or NC. We demonstrate that locomotor activity is lower in O/A3-NC and reduced even further in O/A3-HFD mice (Fig 4.2A; $p<0.001$). To determine if anxiety-like behavior was altered in response to lack of orexin signaling or HFD, the first 5 min of activity was analyzed. We demonstrate that anxiety-like behavior (open field test) is not impaired in WT or O/A3 mice (Fig 4.2B).

High fat diet impairs cognitive function in mice lacking orexin.

Previous studies indicate that orexin is integral in cognitive function (104, 198, 204). We sought to determine if mice lacking orexin maintained on HFD have impaired cognition. We demonstrated that there was no difference in learning ability between WT and O/A3 mice. When tested 24 h later, cognitive impairment was evident as measured via reduced avoidances and increased latency in O/A3 mice (Fig 4.3A-B, $p<0.01$ vs. WT-NC). Mice were then placed on HFD or maintained on NC. Long term memory was tested following 14 and 28 d of HFD exposure. We demonstrate that HFD significantly impairs long term memory in O/A3 mice as indicated by reduced avoidances and increased latency (Fig 4.3A-B; *$p<0.05$ vs. WT-NC, WT-HFD, O/A3-NC; **$p<0.01$ vs. WT-NC, WT-HFD, O/A3-NC).
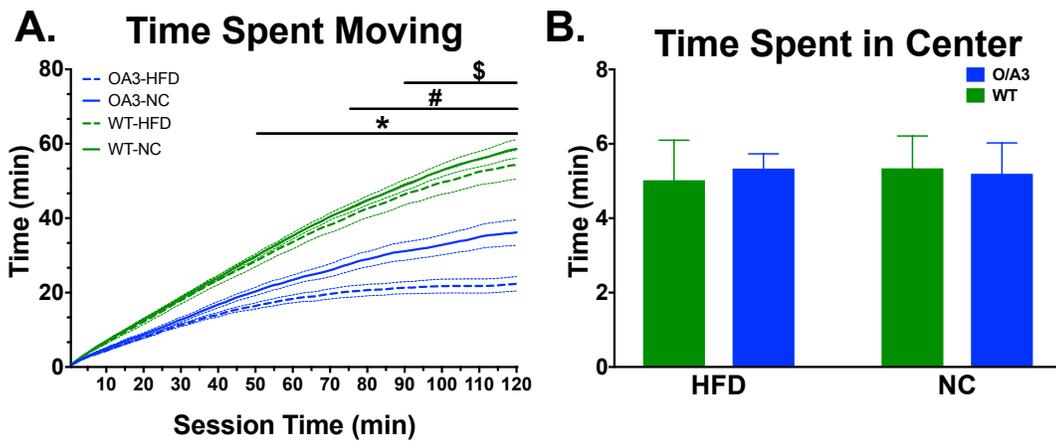
Figure 4.2. High fat diet reduces locomotor activity but does not impair anxiety like behavior. WT mice move significantly more compared to O/A3 mice fed NC or HFD (A). Anxiety-like behavior is not different between strains, as there were no differences in time spent in the chamber center (B). *p<0.05 O/A3-HFD vs. WT #p<0.05 O/A3-NC vs. WT.
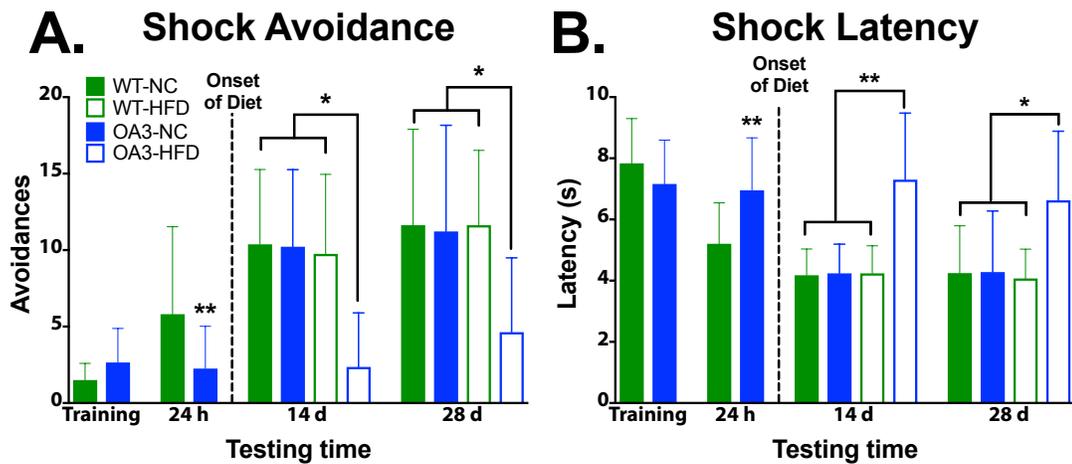
Figure 4.3. High fat diet impairs cognitive function in mice lacking orexin. There is no difference in learning ability between WT and O/A3 mice. When tested 24 h later, cognitive impairment in OA/3 is evident as measured via reduced avoidances and increased latency (A-B, $p<0.01$ vs. WT-NC). HFD significantly impairs long-term memory in O/A3 mice as indicated be reduced avoidances and increased latency compared (A-B; *$p<0.05$ vs. WT-NC, WT-HFD, O/A3-NC; **$p<0.01$ vs. WT-NC, WT-HFD, O/A3-NC).

Inflammatory genes are altered in response to HFD.

Our previous data indicate that orexin A attenuates PA-induced microglial inflammation (25). We demonstrate that hippocampal orexin 1 receptor is upregulated in mice fed HFD but orexin 2 receptor is unchanged (Fig. 4.4A-B, $*p<0.05$, $**p<0.01$ vs. WT-NC). Iba1, a marker of microglial activation, is increased in HFD-fed mice regardless of genotype, and in O/A3-NC mice (Fig 4.4C, $*p<0.05$, $**p<0.01$ vs. WT-NC). Pro-inflammatory marker TNF-α is increased in O/A3 mice (Fig 4.4D $*p<0.05$ vs. WT-NC, $\#p<0.05$. $\#\#p<0.01$ vs. WT-HFD). CX3CR1 is upregulated following HFD exposure (Fig 4.4E, $*p<0.05$, $**p<0.01$ vs. WT-NC). Irg1 expression is upregulated following HFD exposure (Fig 4.4F, $*p<0.05$, $**p<0.01$ vs. WT-NC).

Discussion

High fat diets and obesity increase neuroinflammation, neurodegeneration, and cognitive decline (4-7). While hippocampal neuroinflammation normally increases with age, risk of neuroinflammation and cognitive impairment is exacerbated by chronic consumption of diets high in PA (6, 7). Orexins are important mediators in cognitive function and metabolic function (104, 198, 204). Moreover, pro-inflammatory stimuli have been shown to reduce orexin signaling (107, 109, 205, 206). Here, we demonstrate that HFD increases body weight gain and fat mass despite genotype (Fig 4.1). In mice lacking orexin, increased body weight and fat mass is observed compared to WT mice maintained on NC (Fig 4.1). The loss of orexin also resulted in reduced locomotor activity and this effect is enhanced with exposure to HFD (Fig 4.2).

It is well accepted that orexins have a distinct role in the regulation of energy metabolism (73), and that loss of orexin signaling contributes to increased obesity in part via reduced energy expenditure and movement (199). Orexin has pleiotropic effects in the brain as the neurons project to regions involved in
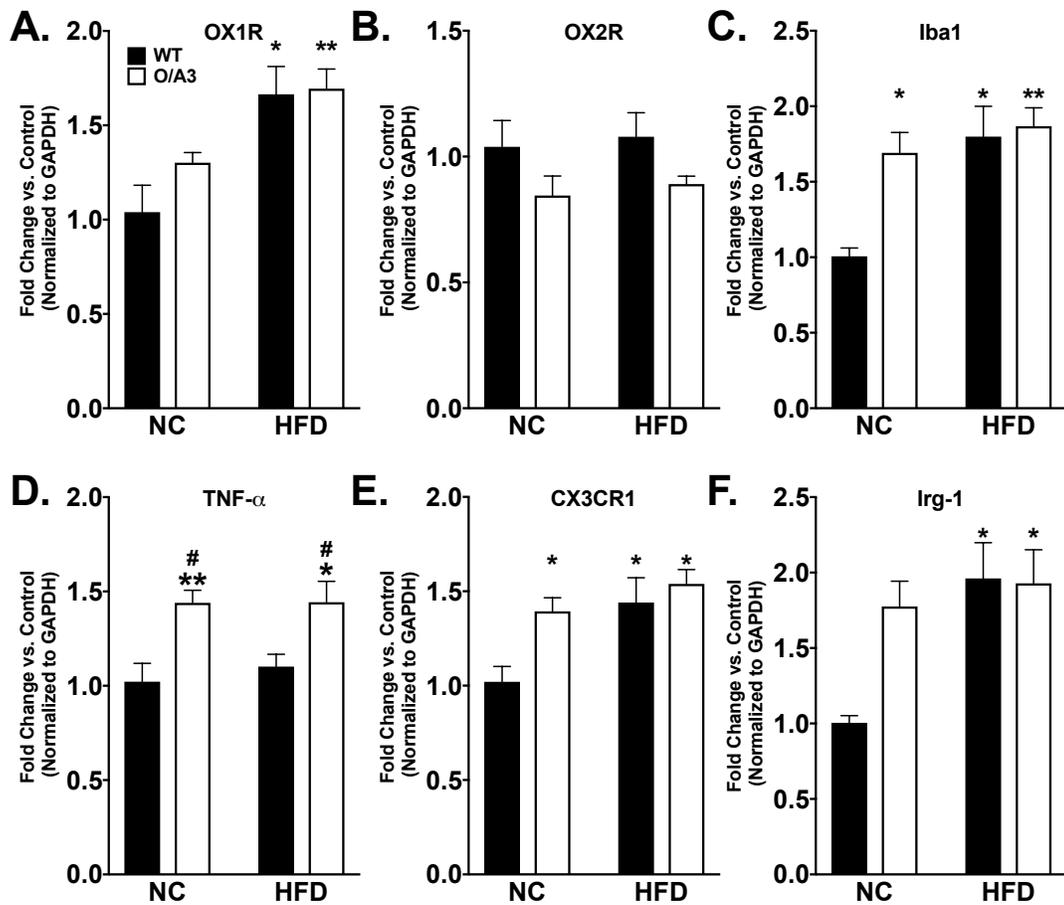
Figure 4.4. Inflammatory genes are altered in response to HFD. HFD but not orexin loss increases orexin 1 receptor (A). Orexin 2 receptor levels are unchanged by diet or genotype (B). HFD and loss of orexin induces microglial activation via increased Iba1 (C). CX3CR1 is upregulated following HFD exposure (D). The pro-inflammatory marker TNF-α is increased in O/A3 mice (E). Irg1 expression is upregulated following HFD exposure (F). *p<0.05 vs. WT-NC **p<0.01 vs WT-NC # p<0.05 WT-HFD.

cognition, executive function, and learning and memory (104, 207, 208). Orexin loss is the causal process in the neurodegenerative disease narcolepsy, and loss of orexin has been implicated in comorbidities such as sleep disturbances or metabolic changes noted in other neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's disease (209-212). While orexin loss has previously been considered largely to be incidental in these latter neurodegenerative diseases, data suggest that orexin loss may actually contribute to disease progression. We and others have demonstrated that OXA exhibits neuroprotective effects (22, 25, 105, 108). The loss of orexin could be a mechanism through which the brain becomes more susceptible to neuronal insults and increased degree of neuroinflammation. We demonstrate that mice lacking orexin neurons have increased cognitive deficits, and that exposure to HFD results in impaired long term memory in these mice (Fig 4.3). We show that both HFD exposure and loss of orexin increase markers of inflammation (Fig 4.4). Moreover, the upregulation of neuroinflammatory expression indicates that HFD accelerates hippocampal-dependent memory deficits and the onset of neuroinflammation in O/A3 mice. Our results are consistent with prior studies demonstrating upregulation of immune-related genes, including CX3CR1 and Irg1, in response to reduced orexin signaling and pro-inflammatory stimuli (126, 213). Additionally, the CX3CL1-CX3CR1 system, important in modulating hippocampus-specific memory tasks and synaptic plasticity (195, 214), is perturbed in HFD exposure, neuroinflammation, aging, and AD (195, 215-217). The CX3CL1-CX3CR1 system could represent a target for microglial-neuronal cross talk and microglial-mediated inflammation. Moreover, the neuroprotective immunomodulatory effects of orexin occur via CX3CL1-CX3CR1 signaling. Here we show mice exposed to HFD also have increased OX1R expression (Fig 4.4A). This is in agreement with our previous data demonstrating that OX1R expression is upregulated in microglia in response to PA, indicating that OXA effects primarily occur via OX1R signaling

(25, 198). Increased OX1R expression could be a mechanism in which microglia enhance orexin responsivity therefore enhancing the capability to appropriately respond to inflammatory insults.

Future studies will focus on the effects of orexin replacement therapy in O/A3 mice, using exogenous orexin to directly test if orexin improves cognition through a neuronal-glial mechanism. Orexin is necessary for consolidation of hippocampal dependent memory (104). Orexin treatment has been shown to improve cognitive performance on a variety of tasks (98-100, 204, 218-220), potentially via changes in long term potentiation (LTP). Other reports have demonstrated that orexin delivery to the CA1 region of the hippocampus improves cognition via increased LTP (98, 104, 221). Microglia have recently been appreciated to have a role in LTP and hippocampal cognition. Additionally, HFDs alter hippocampal LTP and microglial response (15). It is therefore reasonable to predict that orexin effects on cognition depend in part on modulation of microglia. To the best of our knowledge, we are the first to define a pathway linking orexin to phenotypic changes in hippocampal microglia. The data presented here indicate a targetable pathway to reverse diet-induced cognitive decline and treatment for neurodegenerative diseases involving orexin loss.

Chapter 5

Duffy, C. M., Xu, H., Nixon, J. P., Bernlohr, D. A. and Butterick, T. A., *Identification of a fatty acid binding protein4-UCP2 axis regulating microglial mediated neuroinflammation.* Mol Cell Neurosci, 2017. **80**: p. 52-57.

Chapter 5

Identification of a Fatty Acid Binding Protein4-UCP2 Axis

Regulating Microglial Mediated Neuroinflammation

Introduction

Saturated fatty acids (SFAs) such as palmitic acid (PA) contribute to the onset of metabolic inflammatory diseases, including obesity, in part through hypothalamic dysregulation and degeneration (8-11). In the hypothalamus, dietary PA activates microglia (immune cells of the brain) via a nuclear factor kappa B (NFκB)-mediated pathway to release pro-inflammatory cytokines and contribute to damage of neurons responsible for regulating body weight (26, 222).

Microglia are sensitive and highly dynamic in response to changes in the surrounding microenvironment. As microglia respond to the surrounding environment, they are activated to either a pro-inflammatory (M1) or anti-inflammatory, protective (M2) phenotype, depending on external stimuli. For example, PA activates microglia via a toll like receptor (TLR)-4 and induces the release of pro-inflammatory cytokines and factors such as tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) (9, 25). Conversely, microglia activated with the anti-inflammatory cytokine interleukin (IL)-4 polarize to an M2 protective state, characterized by the release of anti-inflammatory cytokines and factors such as arginase-1 (223). The polarization of microglial cells is a highly energetic process and dependent upon mitochondrial integrity and activation. Uncoupling protein 2 (UCP2) has been implicated in mediating energetic processes of microglial activation states (224). Microglia activated to an M1 phenotype have reduced UCP2 activity and expression, resulting in increased production of reactive oxygen species (ROS) (117). Conversely, UCP2 activity is

robustly increased following activation of an M2 protective phenotype (117), indicating a potential target to manipulate microglial activation states.

UCP2 activity is regulated by fatty acid binding proteins (FABP), lipid chaperones regulating metabolic and inflammatory pathways in response to fatty acids (28). Targeted deletion of the adipocyte FABP (FABP4, also known as aP2) is sufficient to prevent obesity induced insulin resistance, diabetes, atherosclerosis and asthma, (124, 225-227). Mice lacking FABP4 (also referred to as aP2 deficient mice) have been used to extensively characterize diabetes, atherosclerosis and asthma linking FABP4 signaling to important roles in metabolic homeostasis and immunometabolic diseases, as reviewed in (28). In peripheral murine macrophages, the loss of FABP4 protects against the development of atherosclerosis and dyslipidemia (228). The loss of FABP4 in macrophages via either molecular or pharmacologic means results in attenuated obesity-induced inflammation through a UCP2-redox based mechanism (125, 148). While the role of the FABP4-UCP2 axis in peripheral macrophages has been extensively characterized, this axis has not been explored in the brain immune cells such as microglia.

We hypothesized that inhibition of FBAP4 in microglia would attenuate PA-induced pro-inflammatory response through a UCP2 mediated mechanism (Fig 5.1). Herein we demonstrate that mice lacking FABP4 have increased expression of UCP2 and reduced expression of TNF-α, iNOS, and ionized calcium-binding adapter molecule 1 (Iba1, a marker of microglial activation) in the hypothalamus. Moreover, pharmacological inhibition of FABP increases UCP2 expression and reduces PA-induced pro-inflammatory response and ROS production. Further, this effect is negated in microglia lacking UCP2, indicating the FABP4-UCP2 axis is pivotal in obesity-induced neuroinflammation.
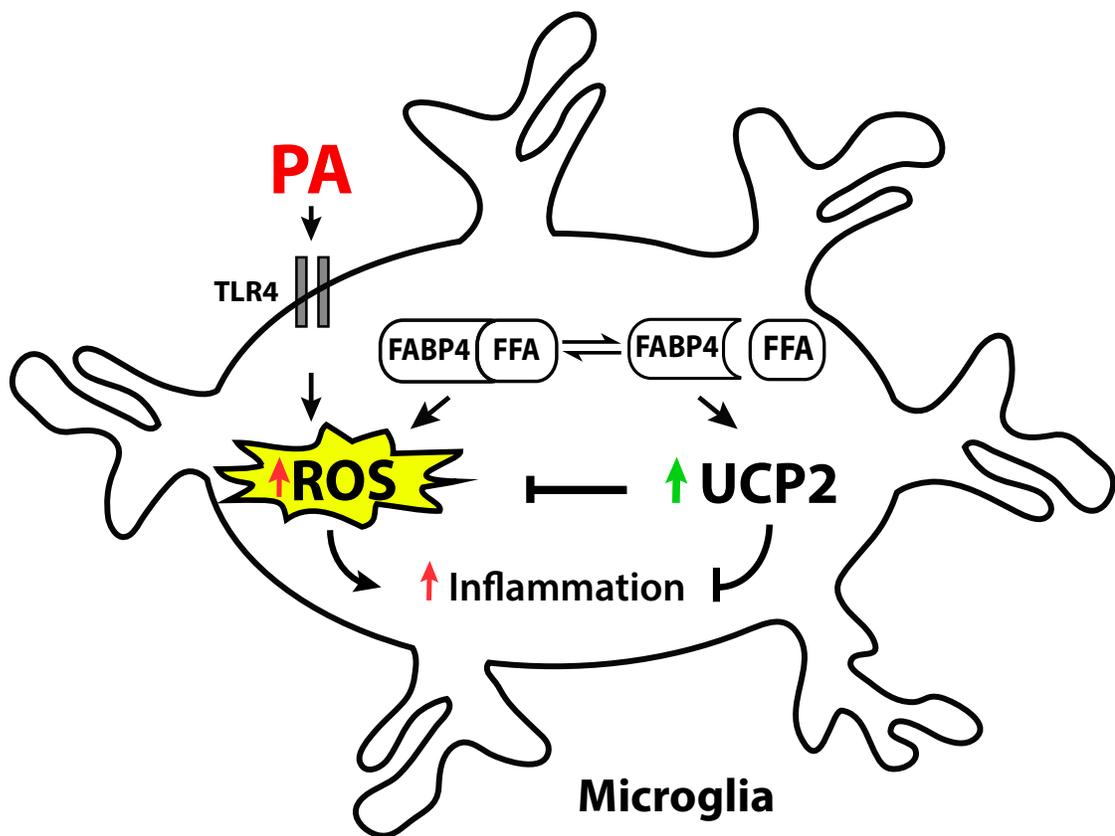
Figure 5.1. Hypothesized mechanism of FABP4-UCP2 axis in microglial mediated neuroinflammation

Materials and Methods

Cell culture and reagents:

Immortalized murine microglial cells (BV2) and UCP2 knockdown BV2 microglia (UCP2kd) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad CA USA) supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin, and neomycin and maintained at 37°C with 5% CO_2. Pan-FABP inhibitor (HTS01037; Cayman Chemical) was suspended in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis MO USA) and diluted to 30 µM in DMEM. Palmitic acid (PA; Sigma Aldrich) was conjugated to fatty acid free bovine serum (BSA; Sigma Aldrich) and diluted to 0.1 mM in DMEM.

shRNA knockdown of UCP2 in microglia:

BV2 microglia were transduced with a short hairpin RNA (shRNA) lentivirus as previously described (229). Green fluorescent protein (GFP) scrambled and UCP2 targeting sequences were obtained from Open Biosystems (Pittsburgh, PA USA). The following were used UCP2 (GenBank accession number NM_011671) targeting sequence (UCP2 knockdown; UCP2kd) 5′-CCGGTCTCCCAATGTTGC-CCGTAATCTCGAGATTACGGGCAACATTGGGAGATTTTTG-3′; scrambled sequence, 5′-AACGTACGCGGAATACTTCGA-3′. UCP2 expression knockdown is approximately 90% (Fig 5.2).

Real-time RT PCR:

Whole hypothalamic tissue was dissected from fifteen week old FABP4/aP2 knockout (also referred to as AKO) and wildtype (WT) mice maintained on 60% high fat diet (HFD) for 12 weeks (134, 230). Mice were obtained from our breeding colony and the experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. Total RNA was extracted from microglia and hypothalamic tissue with the aid of Trizol (Invitrogen) (22, 180).

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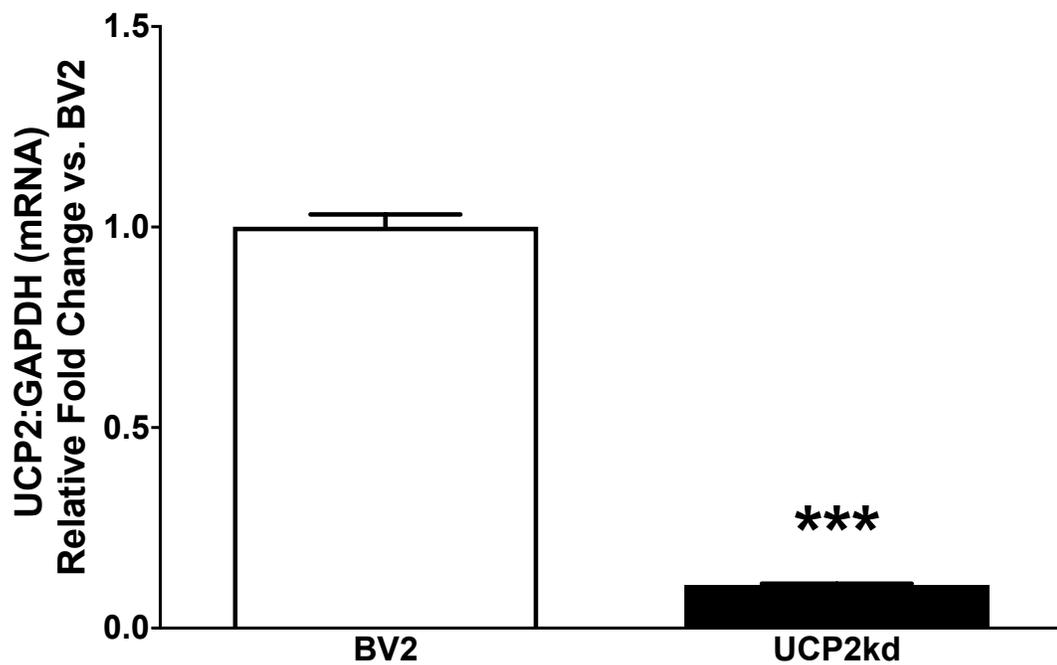
Figure 5.2. UCP2 expression is reduced following shRNA knockdown. UCP2 expression is reduced ~90% following shRNA knockdown (p<0.0001 vs. BV2 cells).

A final concentration of mRNA was determined spectrophotometerically (Nanodrop ND-8000; ThermoFisher Scientific, Waltham MA USA). Real-time thermal cycling were carried out in a Roche LightCycler (Roche Diagnostics Corporation, Indianapolis, IN USA) by one-step RT-PCR using the general method as previously described (25). Target gene expression (Table 5.1) was determined using SYBR Green detection normalized to GAPDH using the $^{\Delta-\Delta}$CT method (146).

Amplification products were separated via electrophoresis on 3% agarose gels stained with SYBR green. qRT-PCR products were purified using a commercially available kit (MinElute PCR Purification, Qiagen Valencia CA USA) and validated using Sanger di-deoxyterminator sequence method at the University of Minnesota Genomics Center.

Reactive oxygen species assay:

Intracellular ROS production was determined using Deep Red Fluorescence kit (Abcam, Cambridge GBR) as previously described (108). Briefly, cells were pretreated with HTS01037 or vehicle for 3 h and then challenged with or without PA for 1 h (time points based on (117)). Cells were then exposed to the ROS Deep Red Dye for 1 h in 5% CO_2 at 37°C. Intracellular superoxide and hydroxyl radicals react with the deep red dye, producing a fluorescent signal which was measured using a spectrophotometer at $650_{Ex}/675_{Em}$ (SpectraMax-M5; Molecular Devices, Sunnyvale CA USA). Data are presented as relative fluorescence units.

Statistical Analysis:

Statistical differences were determined using a one- or two-way ANOVA followed by Tukey's post-hoc test (GraphPad Prism 7).

Table 5.1. Real-time qPCR primer sequences

Target	Forward primer (5′-3′)	Reverse primer (5′-3′)
Arginase	TAACCTTGGCTTGCTTCGGAAC	TCTGTCTGCTTTGCTGTGATGC
Gapdh	GACATCAAGAAGGTGGTGAAGCAG	AAGGTGGAAGAATGGGAGTTGC
Iba1	GTCCTTGAAGCGAATGCTGG	CATTCTCAAGATGGCAGATC
iNos	CCTACCAAAGTGACCTGAAAGAGG	TTCTGGAACATTCTGTGCTGTCCC
Tnf-α	AACACAAGATGCTGGGACAGTGAC	TGGAAAGGTCTGAAGGTAGGAAGGC
Ucp2	TCGGAGATACCAGAGCACTGTCG	GCATTTCGGGCAACATTGG
Fabp4	ATGAAATCACCGCAGACGACA	CATAAACTCTTGTGGAAGTCACGCC
Fabp7	TGGCAAGATGGTCGTGACTC	CCAGTGCTTCATTAGCTGGC
Fabp5	TCCCACCATGGCCAGTCTTA	ACCGTGATGTTGTTGCCATC

Results

Microglial cells express FABP4 and FABP5, but not FABP7.

To verify that microglial cells express FABP, real-time PCR products of FABP4 (Fig 5.3A), FABP5 (Fig 5.3B), and FABP7 (Fig 5.3C) were separated via electrophoresis and visualized on an agarose gel. Similar to macrophages, BV2 microglial cells express both FABP4 and FABP5, whereas in total brain lysates, FABP4, FABP5, and FABP7 are expressed.

Hypothalamic pro-inflammatory response is suppressed in mice lacking FABP4.

In peripheral macrophages, lack of FABP4 contributes to upregulation of UCP2 (125). To determine if the absence of FABP4 leads to upregulated UCP2 in microglial cells, gene expression in hypothalamic tissue from FABP4 knockout (referred to as AKO) and WT mice was assessed. Similar to peripheral tissue, brain tissue from AKO mice have upregulated UCP2 expression (Fig 5.4A; $p<0.05$ vs. WT). In peripheral macrophages, loss of FABP4 and upregulation of UCP2 is correlated with decreased NFκB activity, a polarization switch from M1 to M2 and reduced expression of inflammatory markers. Likewise, UCP2 mediates microglial polarization (117). Similar to the results in peripheral macrophages, hypothalamic tissues from wild type and AKO mice exhibited significantly reduced expression of Iba1, TNF-α, and iNOS compared to WT mice (Fig 5.4B-D; $p<0.05$).

Inhibition of FABP leads to increased UCP2 expression in BV2 microglial cells.

Prior reports demonstrate UCP2 expression is increased in macrophages treated with the FABP inhibitor HTS01037 (125). We tested whether the same was true in BV2 microglia when treated with HTS01037. As predicted, UCP2 expression was significantly increased following HTS01037 treatment in the presence or absence
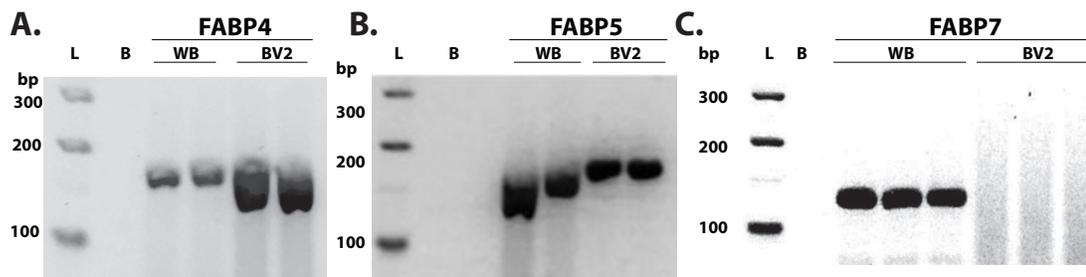
Figure 5.3. Microglial cells express FABP4 and FABP5 but not FABP7. A. Fatty acid binding protein is expressed in both whole brain (WB) and microglial cells. A visualization of real-time PCR products on agarose gel electrophoresis to evaluate gene expression. Gene product of 174 bp was expected for FABP4. B. FABP5 is expressed in both WB and BV2 microglial cells. Gene product of 168 bp was expected for FABP5. C. FABP7 is expressed in WB but not BV2 microglial cells. Gene product of 131 bp was expected for FABP7. L, ladder, B, water blank.

Hypothalamic tissue
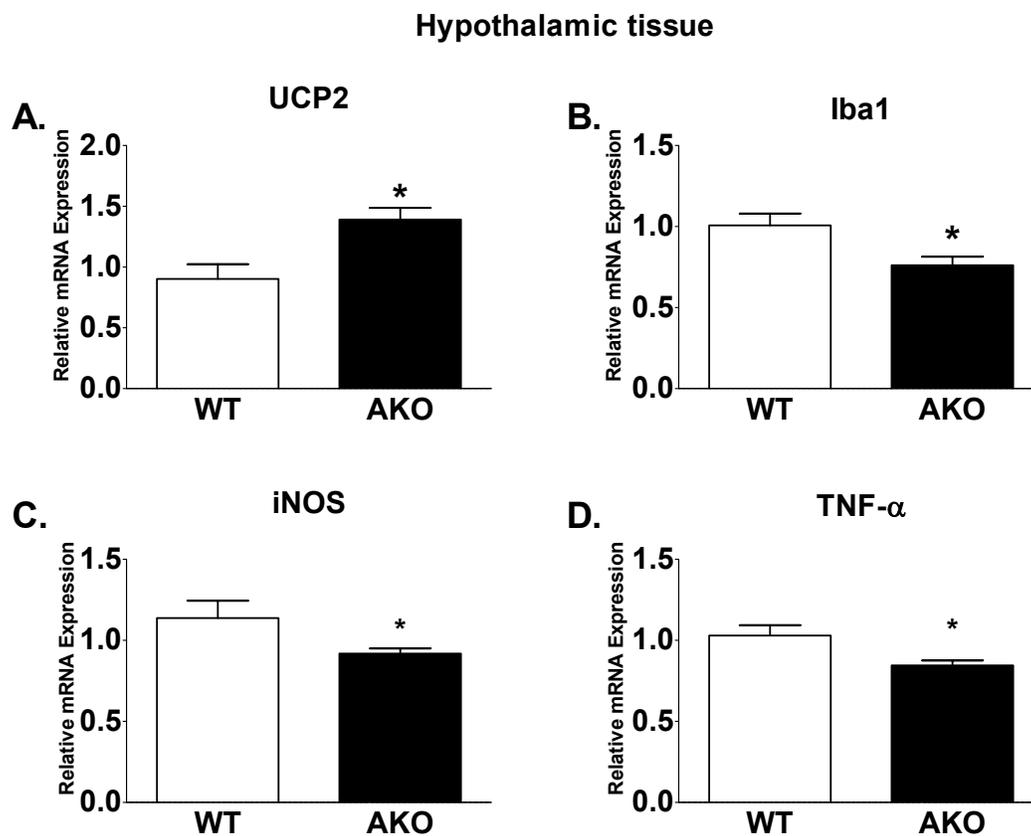
Figure 5.4. Hypothalamic gene expression is altered in FABP4/aP2 knockout animals. UCP2 expression is upregulated in FABP4/aP2 knockout mice (A; $p < 0.05$ vs. WT). Pro-inflammatory genes Iba1, iNOS and TNF-α trend to be suppressed in FABP4/aP2 knockout mice (B-D). Letters indicate significant differences between treatment groups.

of PA (Fig 5.5; p<0.0001 C vs. HTS, p<0.001 C vs. HTS+PA, p<0.001 PA vs. HTS, 0.05 PA vs. HTS+PA). Furthermore, the expression of UCP2 was dependent upon the presence or absence of FABP4, as PA alone did not alter basal UCP2 expression (Fig 5.5).

UCP2 is necessary for microglial inflammatory response.

To determine if UCP2 is necessary for mediating PA-induced inflammatory response, we assessed changes in gene expression of iNOS in BV2 versus UCP2kd microglia. Exposure to PA significantly increased iNOS expression in BV2 and UCP2kd microglia (Fig 5.6; p<0.001 PA vs. C). Inhibition of FABP attenuated PA-induced iNOS expression below basal levels in control cells, but iNOS was significantly increased in UCP2kd cells (Fig 5.6; p<0.001 PA vs. HTS, HTS+PA). Further, UCP2kd microglia treated with HTS01037 no longer exhibited attenuated PA-induced iNOS (Fig 5.6; p<0.05 C vs. HTS+PA, p<0.001 C vs. HTS, p<0.0001 C vs. PA, p<0.0001 PA vs. HTS, HTS+PA). To determine if UCP2 is necessary to mediate the anti-inflammatory microglial activation, we evaluated changes in arginase-1 expression in BV2 and UCP2kd microglia. Arginase-1 expression was significantly increased in BV2 microglia exposed to HTS01037 alone or in the presence of PA (Fig 5.7; p<0.0001 C vs HTS, p<0.0001 PA vs. HTS, p<0.05 PA vs. HTS+PA). Remarkably, arginase-1 expression was significantly reduced in UCP2kd microglia treated with PA alone, HTS01037 only, or HTS01037 plus PA (Fig 5.7; p<0.0001 C vs. HTS, PA, and HTS+PA).

UCP2 effect of ROS production in microglia.

Next, we analyzed changes in production of ROS in BV2 and UCP2kd microglia. As predicted, we found that PA increased ROS production in BV2 cells (Fig 5.8; p<0.0001 PA vs. C), and that PA-induced ROS production was attenuated in BV2 cells when pretreated with the FABP inhibitor HTS01037 (Fig 5.8; p<0.001 vs. HTS and HTS+PA). In UCP2kd microglia, HTS01037 had no effect on ROS production,
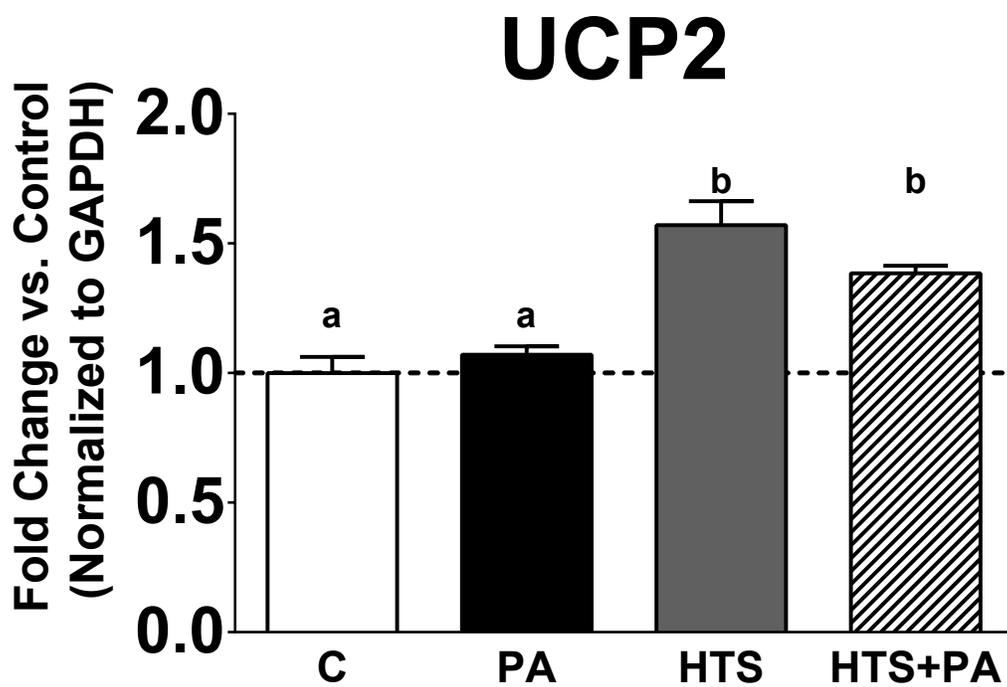
Figure 5.5. FABP abundance regulates UCP2 expression in microglia. UCP2 expression is significantly upregulated following HTS01037 treatment in the presence or absence of PA in BV2 microglia. ($p<0.0001$ C vs. HTS, $p<0.001$ C vs. HTS+PA, $p<0.001$ PA vs. HTS, 0.05 PA vs. HTS+PA). Letters indicate significant differences between treatment groups.
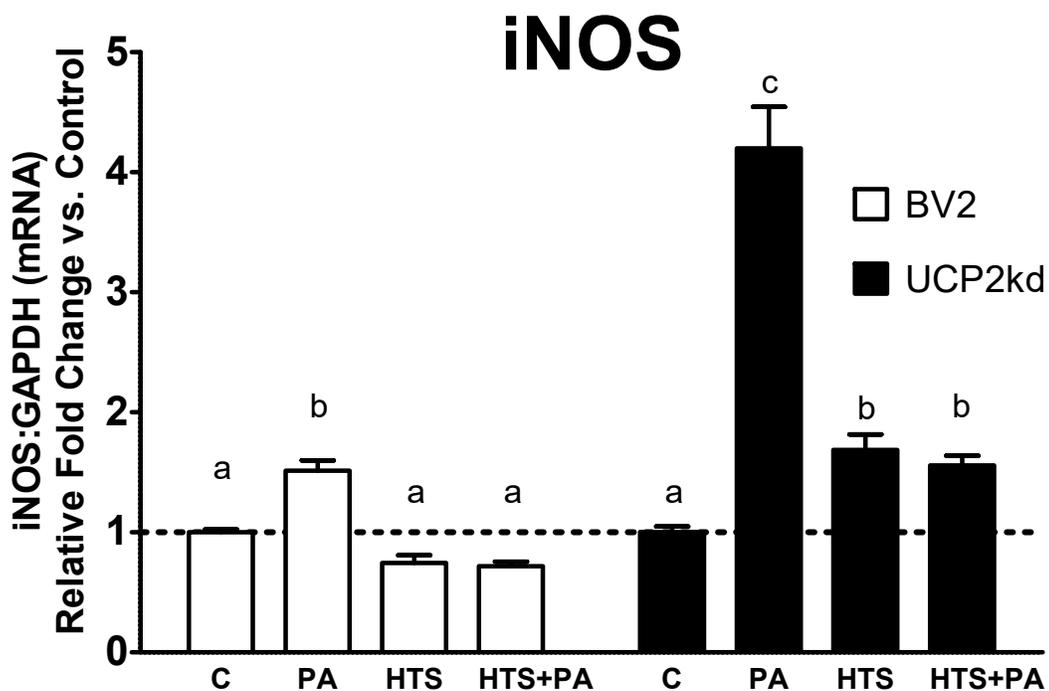
Figure 5.6. UCP2 is needed to oppose PA-induced upregulation of pro-inflammatory marker iNOS. Pretreatment with HTS01037 attenuates PA-induced iNOS expression in BV2 microglia (p<0.001 PA vs. C, HTS, HTS+PA). In UCP2kd microglia, PA increases iNOS expression, however, HTS01037 is no longer able to attenuate PA-induced iNOS upregulation (p<0.05 C vs. HTS+PA, p<0.001 C vs. HTS, p<0.0001 C vs. PA, p<0.0001 PA vs. HTS, HTS+PA). Letters indicate significant differences between treatment groups.
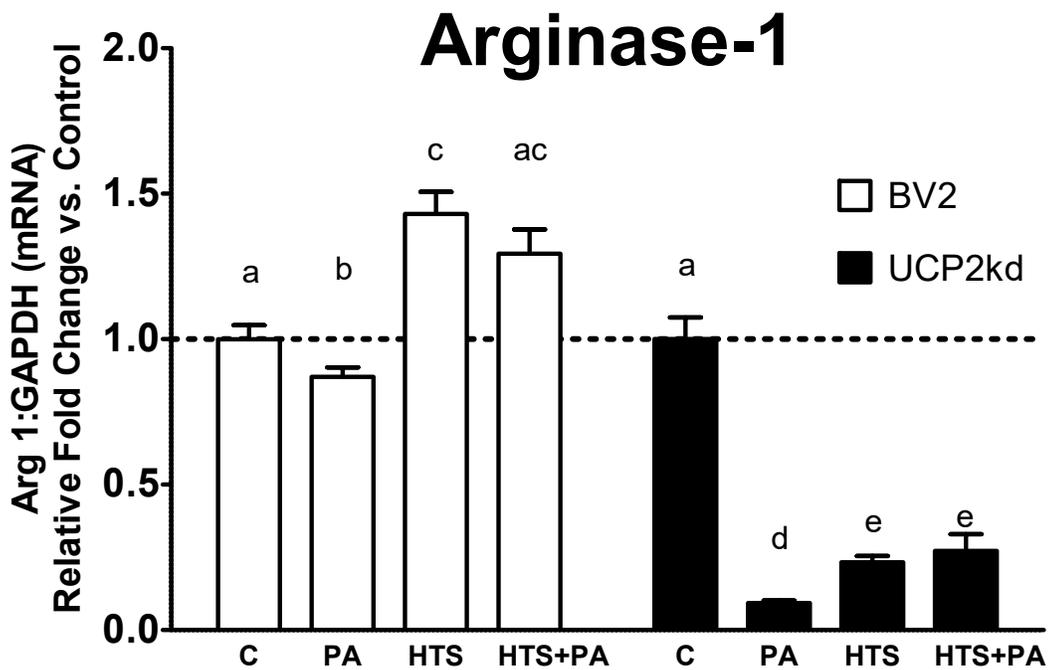
Figure 5.7. UCP2 mediates anti-inflammatory marker arginase-1. Pretreatment with HTS01037 in the presence or absence of PA robustly increases Arginase expression ($p < 0.0001$ C vs HTS, $p < 0.0001$ PA vs. HTS, $p < 0.05$ PA vs. HTS+PA). In UCP2kd cells arginase-1 is no longer upregulated following HTS01037 treatment ($p < 0.0001$ C vs. HTS, PA, and HTS+PA). Letters indicate significant differences between treatment groups.
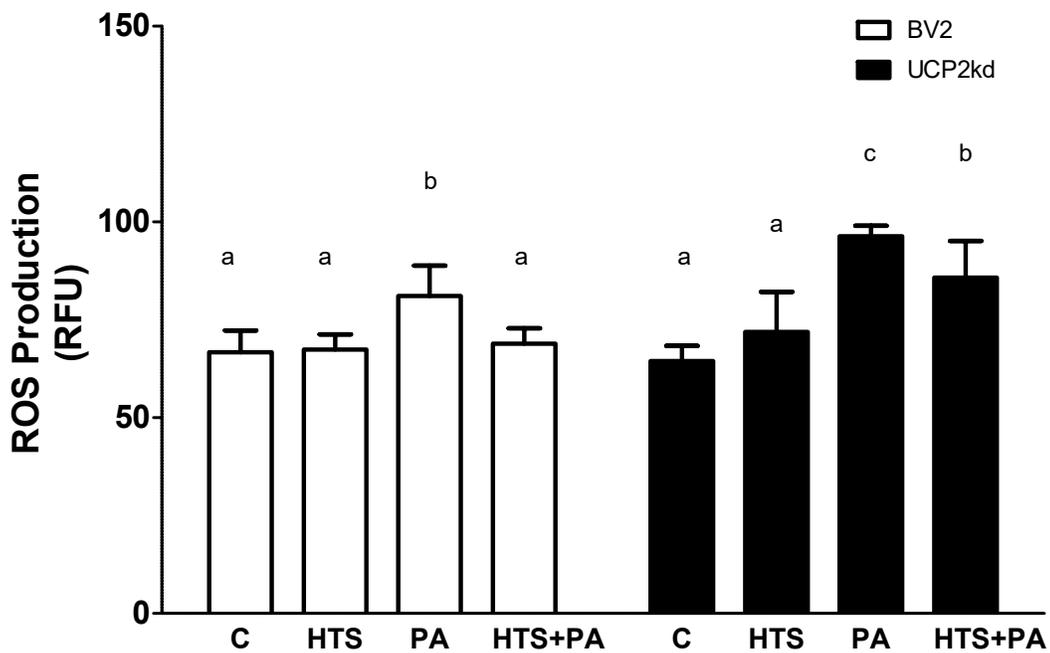
Figure 5.8. UCP2 effect on ROS production in microglia. BV2 cells exposed to PA significantly increases ROS production (p<0.0001 PA vs. C), however treatment with HTS01037 attenuates PA induced ROS production (p<0.001 vs. HTS and HTS+PA). In UCP2kd cells, PA increases ROS in the presence or absence of HTS01037 (p<0.0001 PA vs. C, p<0.001 HTS+PA vs. C). Letters indicate significant differences between treatment groups.

as PA significantly increased ROS production in all treatment conditions (Fig 5.8; p<0.0001 PA vs. C, p<0.001 HTS+PA vs. C).

Discussion

Obesity is often coupled with peripheral and central chronic low-grade inflammation (6, 196). Prolonged overnutrition and excess intake of SFA increase the amount of fatty acids in the brain and induce neuroinflammation (8-11, 39). The onset of chronic neuroinflammation has identified as a major contributor to HFD-induced hypothalamic dysregulation (9). In addition to influencing obesity risk, dietary fats, such as palmitic acid, have been linked to development of cognitive disorders such as Alzheimer's disease (AD) through increase in neuroinflammation (3, 31, 196).

Microglia promote neuronal health in part via maintaining a favorable microenvironment throughout the central nervous system (64, 184). Microglia, once thought to be passive support cells, are now appreciated to maintain neuronal-glial circuitry though removing damaged synapses and altering plasticity (50, 189). As observed in obesity, microglial activation state shifts towards a chronic pro-inflammatory phenotype (9, 185, 186). In peripheral macrophages, FABP dynamics alters macrophage polarization states (28, 125). Here, we demonstrate microglia express FABP4 and FABP5 but not FABP7 (Fig 5.3), similar to macrophages. Likewise, we show that mice lacking FABP4 have attenuated hypothalamic HFD-induced pro-inflammatory response (Fig 5.4). These findings are comparable to other published reports of diet induced neuroinflammation (9, 11). Others have demonstrated HFD induces M1 microglial activation, leading to impaired phagocytosis and contributing to worsening pathology of neurodegenerative diseases including AD (231). Although not tested in this study, it is likely that the microglia in the FABP4/aP2 knockout animals have increased phagocytic function and reduced neuronal stress. Future studies will examine the role of microglia and cognitive function in FABP4 knockout animals.

In peripheral macrophages, the loss or inhibition of FABP4 results in attenuated obesity-induced inflammatory response via a FABP4-UCP2 mediated axis (125, 148). Here we demonstrate the novel FABP4-UCP2 axis in microglia mediating PA-induced inflammation. We show UCP2 expression is increased following inhibition of FABP4 (Fig 5.5). Additionally, we demonstrate that inhibition of FABP4 upregulates the anti-inflammatory marker arginase and attenuates PA-induced increase in iNOS (Fig 5.6). Notably, we demonstrate that when UCP2 is knocked down, inhibition of FABP4 no longer suppresses PA-induced iNOS expression and significantly reduces arginase expression (Fig 5.7). These findings demonstrate that UCP2 is necessary for mediating microglial polarization. The mechanistic relationship between iNOS and arginase 1 exists functionally at the substrate level, as both enzymes utilize L-arginine (232, 233). However, arginase 1 can effectively outcompete iNOS to downregulate production of nitric oxide, thus suppressing iNOS activity (232). Interestingly, L-arginine metabolism is also known to regulate inflammatory function of macrophages however this is unknown in microglia. In macrophages, increased iNOS function is associated with the pro-inflammatory M1 phenotype and is thought to be a part of the metabolic adaptive component of immune cell polarization critical to immunity and inflammation. Although this has been defined in macrophages, it remains unexplored in microglia.

While evidence indicates UCP2 has a role in neuroprotection and in the inflammatory response, the underlying mechanisms remain unresolved. Our data support that promotion of the M2 microglial activation phenotype could represent one such mechanism, resulting in reduced ROS production and inhibition of the toxic M1 phenotype. We demonstrate here that treatment with HTS01037 attenuates PA-induced ROS production in microglia, and this effect is negated when UCP2, a known suppressor of ROS production, is knocked down (Fig 5.8). To the best of our knowledge, we are the first to describe a FABP4-UCP2 axis regulating ROS and inflammation in microglia. Mitochondrial ROS production is a

key driver in the pro-inflammatory response (234). When UCP2 deficient macrophages are stimulated with lipopolysaccharide (LPS), ROS production and pro-inflammatory cytokine release is upregulated (235, 236). In microglia, LPS induces the reduction of UCP2, causing mitochondrial depolarization and increased ROS production (117). Moreover, UCP2-silenced microglia fail to transition to an M2 activation state (117). Collectively these findings support that UCP2 is necessary for activation of the M2 protective microglial phenotype. These observations demonstrate UCP2 is an important regulator between the FABP-free fatty acids (FFA) equilibrium and inflammation, which to our knowledge has not been previously described.

FABP4 is expressed in multiple tissues including heart and kidney but the protein itself is only detectable in adipose tissue, peripheral macrophages and microglia (27, 28). Besides FABP4, other FABPs are also expressed in the brain. FABP7 is necessary for brain development, and FABP5 (mal1) is essential in transporting fatty acids across the blood brain barrier (27, 237). Although knocking out FABP5 peripherally results in attenuated diet-induced obesity, the effects are not as robust as in FABP4 knockout models (28). Despite the role of other FABPs in the brain, peripheral evidence indicates the lack of FABP4 alters the pool of free fatty acids, specifically, monounsaturated fatty acids (125). Monounsaturated fatty acids are ligands for receptors including peroxisome proliferator-activated receptor gamma (PPARγ), which opposes NFκB activity. Interestingly, PPARγ antagonists are neuroprotective in models of neurodegenerative diseases such as AD and PD, through a microglial-mediated mechanism (238, 239). Although not tested in the present study, upregulation of PPARγ could be a potential pathway though which the FABP-FFA equilibrium regulates the anti-inflammatory state of microglia. In summary, these data support a role for FABP4-UCP2 axis as a regulator of microglial activation states. Understanding the role of FABP and UCP2 in

modulating microglial activation state may provide therapeutic targets for prevention or treatment of neurodegenerative diseases including obesity.

Chapter 6

Chapter 6

Role of the fatty acid binding protein 4-uncoupling protein 2 axis

in diet induced cognitive decline

Introduction

Obesity is often characterized by chronic low-level inflammation, paralleling the prevalence of metabolic disorders such as type-2 diabetes mellitus (8, 11, 23). Chronic neuroinflammation may be a key factor in the linkage between obesity and cognitive decline (3, 9, 10). Neuroinflammation, accompanied with progressive neuronal loss, is heightened in cognitive decline and obesity (14, 15). Overconsumption of high fat diets (HFD), specifically those rich in the saturated fatty acid (SFA) palmitic acid (PA), exacerbate neuroinflammation, neurodegeneration, and cognitive impairment (8-16). Rodent studies indicate that this is more strongly influenced by dietary SFA content than by total calories consumed (8, 9). Dietary PA increases activation of a neurotoxic phenotype in microglia (brain immune cells), resulting in release of neurotoxic cytokines that may contribute to cognitive decline (11, 25, 26). For example, chronic activation of microglia to a pro-inflammatory state can lead to reduced hippocampal cognitive function and neurogenesis (40-42, 240). While neuroinflammation and cognitive impairment are correlated, the contribution and role of microglia in the context of obesity is undefined.

The link between inflammation and lipid metabolism indicates a key role for FABP4 (fatty acid binding protein 4; also known as adipocyte protein-2; aP2) in neuroinflammatory diseases such as obesity and Alzheimer's disease (AD). Importantly, molecular, genetic, or pharmacologic loss of FABP4 results in an anti-inflammatory phenotype preventing development of metabolic syndrome or neuroinflammatory response even in the presence of a HFD (24, 125). Without

FABP4, increases in UCP2, a mitochondrial membrane protein that attenuates the production of reactive oxygen species (ROS), prevents the switch to a pro-inflammatory state in both peripheral macrophages and microglia (24, 117, 125). We hypothesized that inhibition of FABP4 attenuates HFD-induced cognitive decline via reduced neuroinflammation. In vivo, we demonstrate that mice maintained on a HFD have reduced locomotor activity. Additionally, WT mice maintained on HFD have impaired long term and short term memory. Further, these effects are negated in mice lacking FABP4. We also demonstrate that pharmacological inhibiting of FABP4 in primary microglia attenuates PA-induced pro-inflammatory cytokine secretion. Moreover, microglial pharmacological inhibition of FABP4 is resistant to PA-induced mitochondrial dysfunction.

Methods:

Cell culture and reagents:

Primary microglia (Sciencell, San Diego, CA) were cultured in microglial growth medium (Sciencell) supplemented with 10% fetal bovine serum, 1% penicillin, streptomycin, and neomycin, and growth supplements (Sciencell) and maintained at 37°C with 5% CO_2. Pan-FABP inhibitor (HTS01037; Cayman Chemical) was suspended in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis MO USA) and diluted to 30 µM in DMEM. Palmitic acid (PA; Sigma Aldrich) was conjugated to fatty acid free bovine serum (BSA; Sigma Aldrich) and diluted to 0.1 mM in DMEM. Cells were seeded in 6 well plates treated with poly-L-lysine. Cells were pretreated with HTS01037 or vehicle control for 3 h and challenged with PA (or vehicle) for 12 h (24).

Mitochondrial respiration assay:

Mitochondrial function was assessed using the XF24 or XF^{e}96 extracellular flux analyzer (Seahorse, Agilent Technologies, Santa Clara, CA). Cell number and

inhibitor titration experiments were performed for optimal response based on manufacturer instructions. BV2 microglia were seeded at a density of 8×10^3 cells/well on XF96 microwell plates and incubated overnight at 37°C in 5% CO_2. Cells were pretreated with HTS01037 (30 µM or vehicle) for 3 h and challenged with PA (0.1 mM or vehicle) for 4 h. Following incubation, DMEM was replaced with XF Assay media (Sigma), supplemented with pyruvate (1 mM), L-glutamine (2 mM), and glucose (10 mM). During the assay, cells were treated with compounds in the following order as previously described: oligomycin (1 µM; Sigma), FCCP (carbonyl cyanide-4-phenylhydrazone; 0.5 µM; Sigma), and antimycin A (4 µM; Sigma) (108). The oxygen consumption rate (OCR) was automatically calculated and recorded by the Seahorse XFe software, respiration rates were calculated using Agilent Seahorse XF Cell Mito Stress Test Report Generator.

Cytokine Analysis:

Supernatant from stimulated primary microglia were analyzed for secreted cytokine protein using an automated magnetic 96-well plate format (MagPix; Millipore). Briefly, fluorescently labeled microbeads coupled with capture antibodies were bound to specific target analytes, and a fluorescent or streptavidin-labeled detection antibody. Change in fluorescence was used to yield quantitative analytical results (pg/ml) in cytokine abundance (54).

Animals:

Fifteen-week-old male FABP4/aP2 knockout (also referred to as AKO) and wildtype (WT) mice were maintained on 60% high fat diet (HFD) or normal chow (NC) for 12 weeks (230). Behavioral testing was performed following 12 weeks of HFD exposure. Mice were obtained from our breeding colony and the experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Locomotor activity:

To determine a general assessment of locomotion, animals were removed from the home cage and placed individually into a novel arena (22 x 42 cm) over a 2 hour period (between 1200 and 1600). Activity was recorded by digital video and analyzed using Any-Maze. Data is presented as total distance traveled over 2 hours.

Barnes Maze:

The Barnes maze was used to determine changes in spatial memory as previously described (241). Mice were placed on a white circular maze (91.44 cm diameter) consisting of 20 evenly spaced holes (7.5 cm apart, 5 cm diameter) located 2 cm from the perimeter. A black escape box, located under one of the holes (defined as target hole), remained in the same place throughout the training period. Distinct spatial cues surrounded the maze and were kept constant throughout the entire experiment. Animals were placed in a dark chamber in the center of the maze for 30 seconds prior to beginning the task. Once the chamber was lifted, mice were able to use spatial cues to orient themselves and locate the target hole. The session ended when the mouse completely entered the escape box or 3 min elapsed. If the mouse did not enter the hole itself, it was gently guided into the hole. At the end of each session, the lights were turned off, the mouse was allowed to stay in the escape box for 10 seconds before returning to their home cage. Mice were trained for a total of four training sessions per day with at least a 10 min inter-trail interval for a total of four consecutive days. Latency to target hole and average distance from target hole were measured over the 4 training days. On day 5, we tested memory of the escape location (the probe test). The escape hole was removed and the mouse was allowed to explore the maze for 90 sec. During the probe test, latency to the target hole, time spent in quadrant containing the target hole, and average distance from target hole were recorded (74-76).

T-Maze Spontaneous Alteration:

Spontaneous alteration using the T-maze was used to determine working and spatial reference memory as previously described (242, 243). Briefly, mice were placed in the start box after 30 s, the guillotine door was opened and mice were allowed free choice of either arm. Choice is made when the animal's body has completely entered the choice arm. Once choice has been made, animal was contained within the choice arm for 15 s. The mouse was then returned to the start box for 5 sec and allowed to again choose either arm for 10 additional trials. Choice arm and alterations were recorded. Percent alteration was calculated as the number of alterations divided by the maximum possible alterations (# of alterations/20*100%).

Statistical Analysis:

Statistical differences were determined using a one- or two-way ANOVA followed by Tukey's post-hoc test (GraphPad Prism 7).

Results:

High fat diet increases body weight and reduces locomotor activity.

We demonstrate that HFD increases body weight following 12 weeks HFD exposure despite genotype ($p<0.0001$; Fig 6.1). Prior studies indicate that HFD reduces locomotor activity (244, 245). To determine the effects of HFD on locomotor activity, mice were placed in an activity chamber for 2 h to measure general movement. We demonstrate that mice maintained on a HFD move significantly less compared to those maintained on NC, independent of genotype ($p<0.01$; Fig 6.2).

Loss of FABP4 protects against diet induced cognitive decline.

To determine the effects of FABP4 on cognition, WT and AKO mice maintained on HFD or NC underwent various cognitive tasks. Learning was apparent in all groups
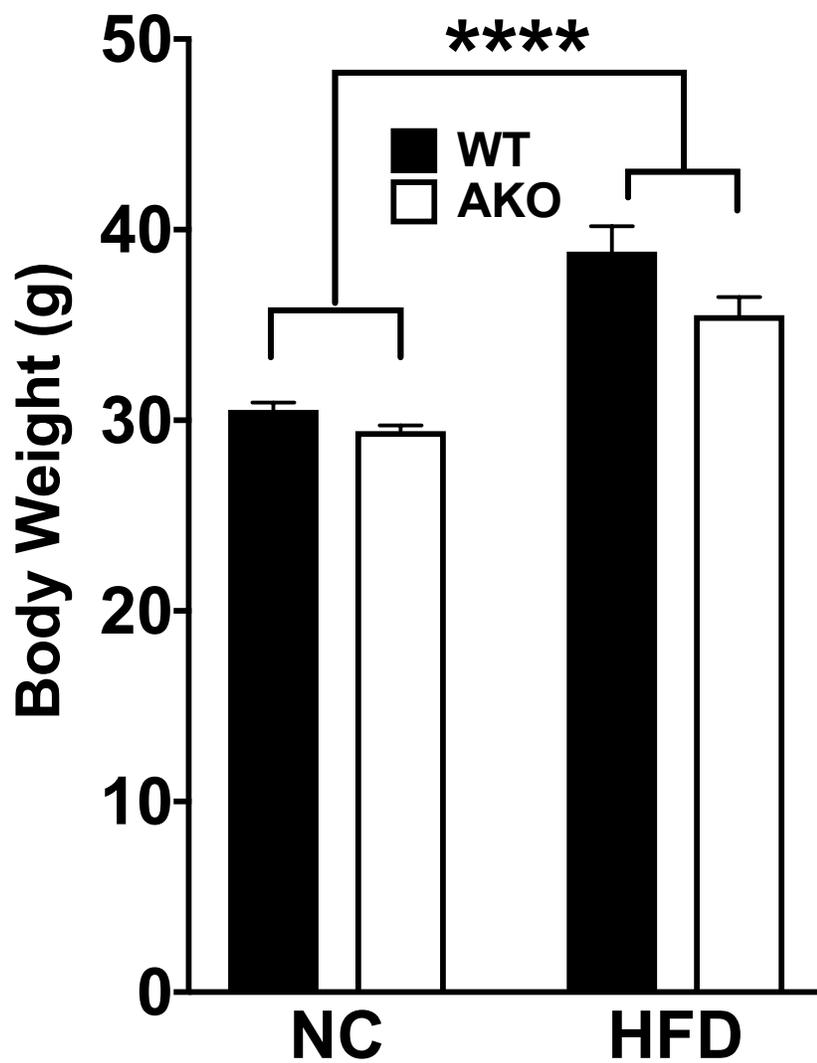
Figure 6.1. High fat diet increases body weight. Body weight is increased following 12 week HFD intake (p<0.0001).
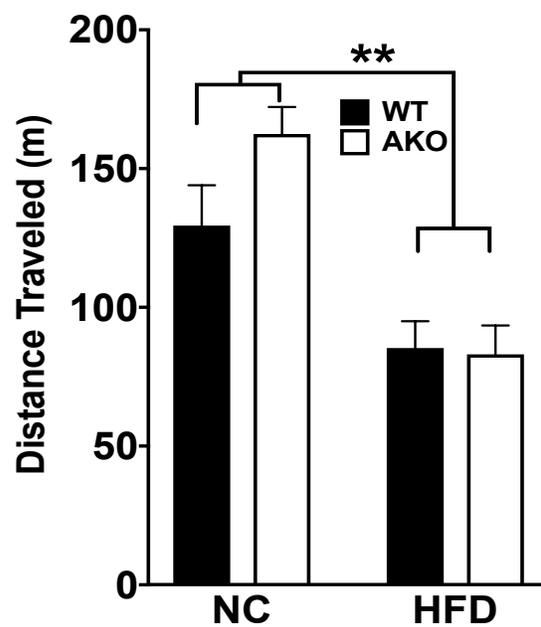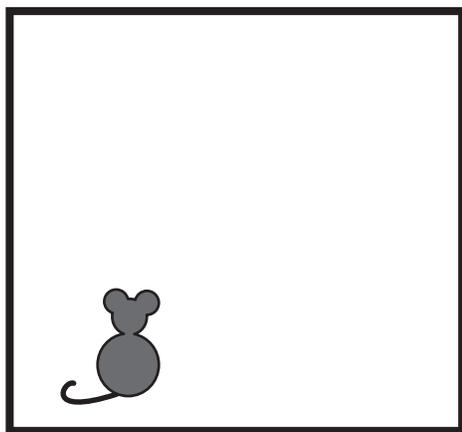
Figure 6.2. High fat diet reduces locomotor activity. Total distance traveled over 2 h is significantly reduced in mice fed a HFD (p<0.011).

in the Barnes maze task (p<0.0001; Fig 6.3B). AKO mice maintained on a HFD have attenuated HFD-induced memory impairment as measured by reduced latency to identify target hole during probe test in the Barnes maze task (Fig 6.3C). We demonstrate that WT mice maintained on a HFD have impaired memory as measured by increased latency to identify target hole during Barnes maze probe test (Fig 6.4C). We also demonstrated that WT-HFD mice have reduced alterations in T-maze, and this response is attenuated in AKO-HFD mice (Fig 6.4B).

Inhibition of FABP4 attenuates PA-induced pro-inflammatory response.

Our previous data demonstrated that inhibition of FABP4 attenuates PA-induced upregulation of iNOS in an immortalized microglial cell line (24). To determine a more detailed overview of the onset of inflammation in primary microglia, we utilized a more extensive cytokine array. Upon stimulation by either LPS or PA, several key mediators of inflammation were upregulated (TNF-α, IL-6, IL-10, IL-13, CXCL1 (KC), IL-1α, CCL5 (RANTES), CCL2 (MCP-2) and G-CSF), a finding in agreement with other studies (54, 246). More importantly, inhibition of FABP4 attenuated a wide range of key pro-inflammatory chemokines and cytokines (Fig 6.5 A-I).

Inhibition of FABP4 protects against PA-induced mitochondrial dysfunction.

Our previous work in macrophages indicate that lack of FABP4 protects against HFD-induced mitochondrial dysfunction (125). To determine if microglia had altered metabolic response, we analyzed real time mitochondrial response following exposure to HTS01037 and PA. Cellular respiration assays from BV2 microglia mirror AKO macrophage findings, (Fig. 6.6). Specifically, β-oxidative respiratory function was increased in microglia lacking FABP4 function when treated with PA.
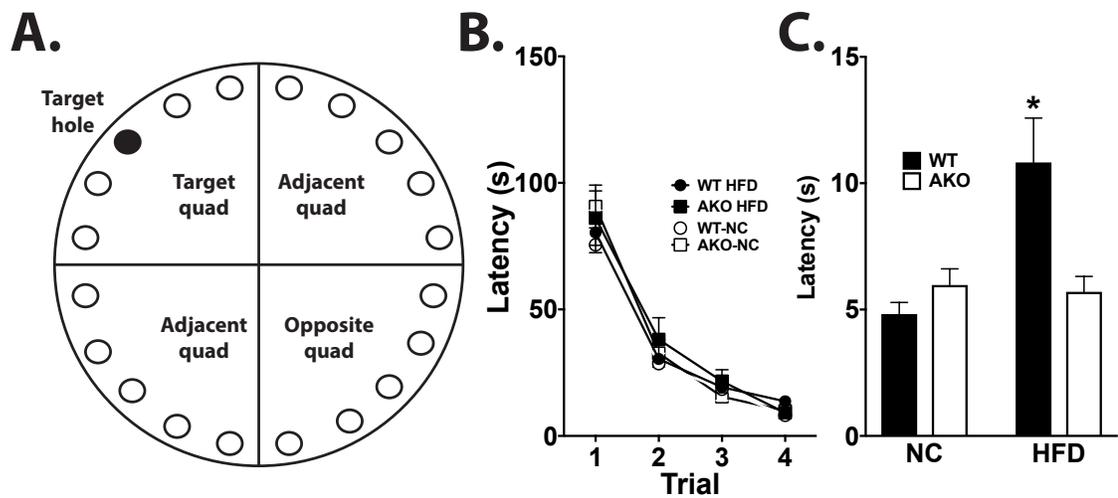
Figure 6.3. Loss of FABP4 is protective against HFD-induced cognitive decline. A. Representation of Barnes maze apparatus. Training occurred over 4 consecutive days where mice learned to identify the target hole containing an escape box. **B.** Performance was analyzed for latency to target hole over training days. Performance improved significantly in all groups over duration of training (p<0.0001). **C.** During the probe (day 5), the escape hole was removed, and latency to target hole was analyzed. WT mice fed HFD have increased latency to target hole during probe test (p<0.05 vs. WT-NC, AKO-NC, AKO-HFD).
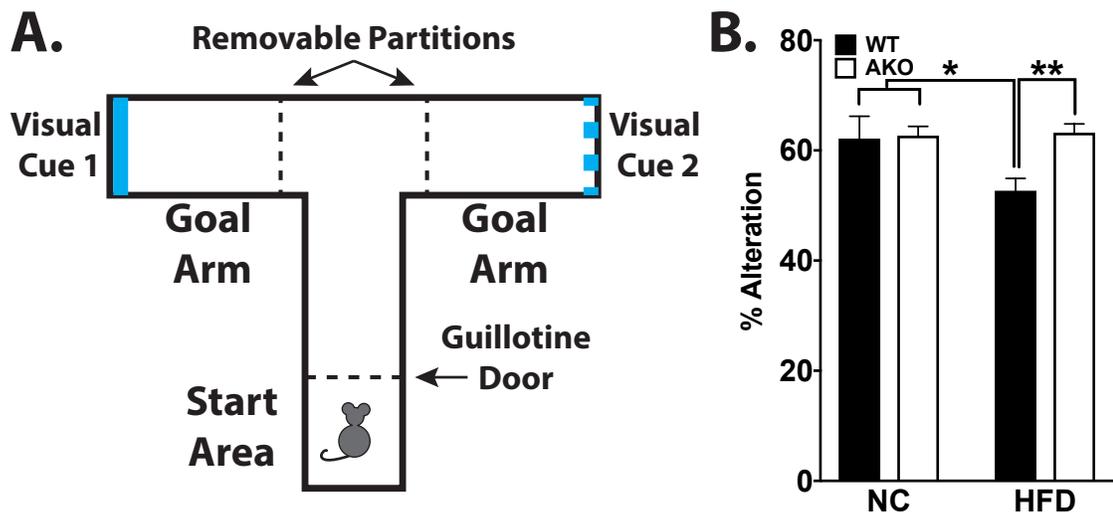
Figure 6.4. Loss of FABP4 rescues HFD-induced working reference memory decline. A. Representation of T-maze apparatus to measure spatial working reference memory. Mice were placed in start arm and allowed free choice to either goal arm. Once body fully in arm, removable partition was closed allowing mice to stay in goal arm for 15 s and then placed back in start area. Mice underwent an additional 10 trials per day for a total of 2 days. Percent alteration was calculated as the number of alterations divided by the maximum number of alterations. **B.** WT mice fed HFD have reduced alterations over all trails (*p<0.05 vs. WT-NC and AKO-NC, **p<0.01 vs. AKO-HFD).
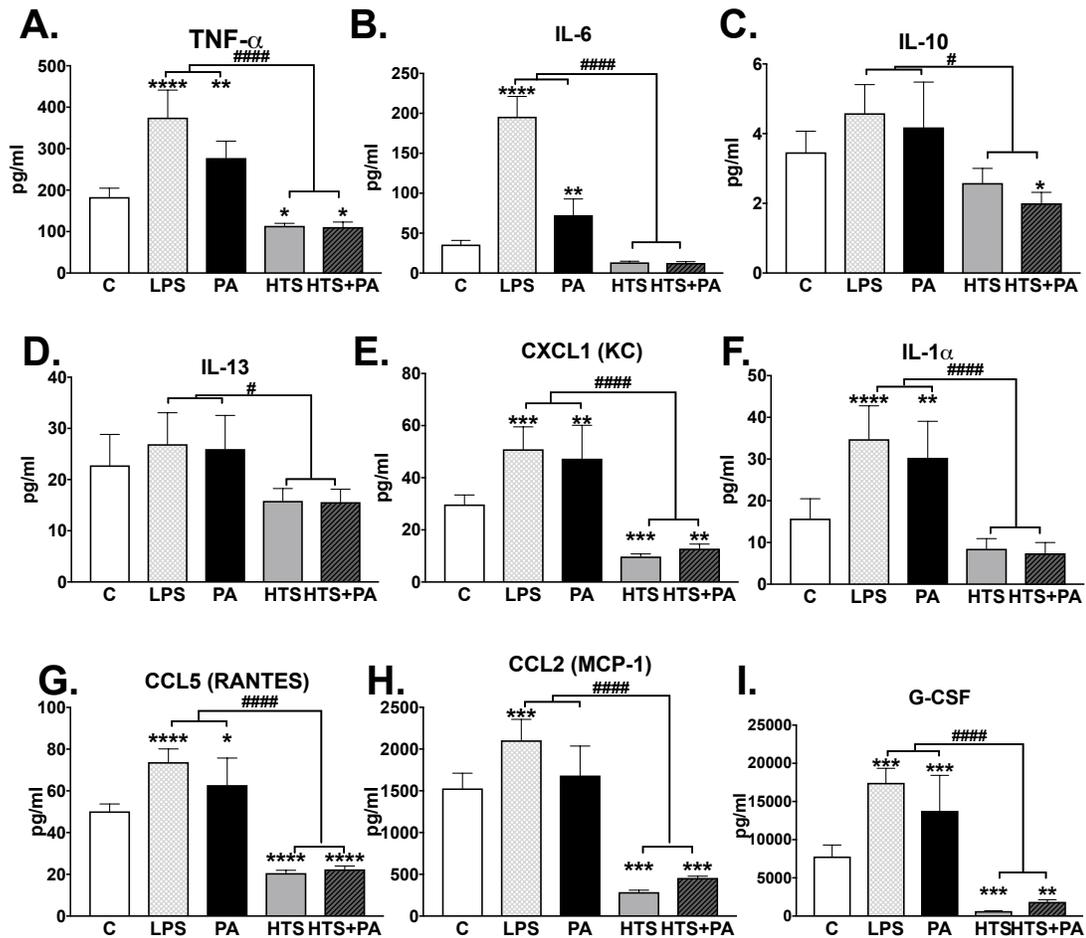
Figure 6.5. Protein expression for pro-inflammatory phenotype markers measured in primary murine microglia. Cells pretreated with vehicle or pan-FABP inhibitor (30 μM HTS) and 12 h PA (0.1 mM) challenge. LPS (100 ng/ml) used as a positive control. Expression shown as pg/ml protein, measured via multiplex ELISA-based Luminex Magpix assay. Data analyzed via one way ANOVA and Tukey's post-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. Control, #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs. HTS and HTS+PA.
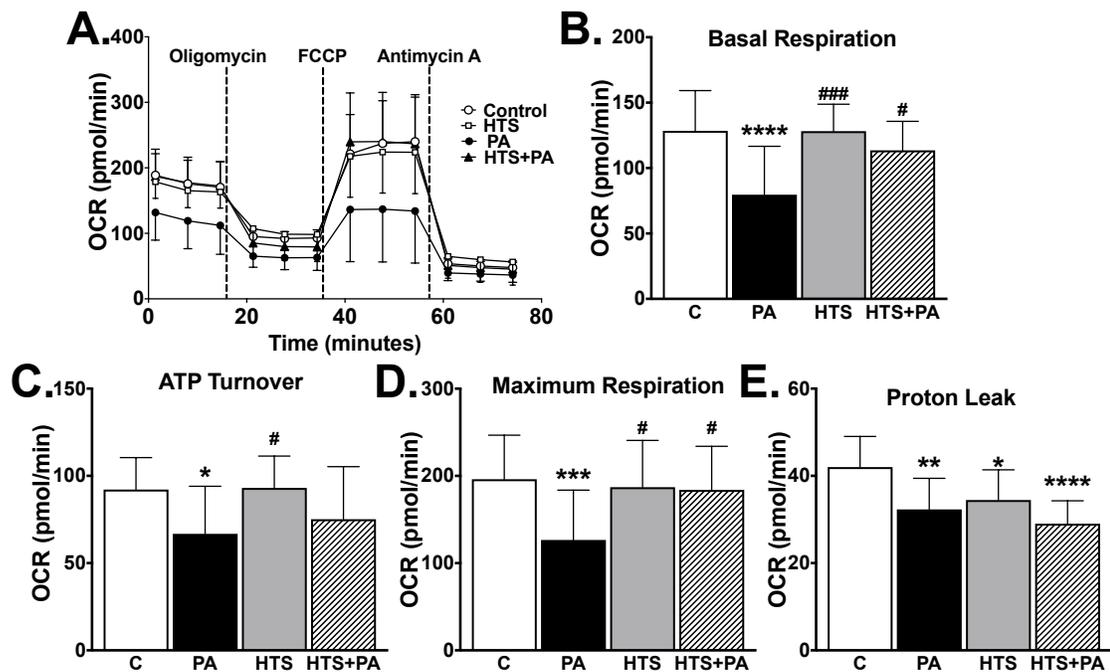
Figure 6.6. FABP4-UCP2 axis in microglial mitochondrial respiration. A. Oxygen consumption rate (OCR), basal respiration, ATP production, max respiration and non-mitochondrial respiration following 4 h PA exposure. Injections performed in the assay include oligomycin (ATP production), FCCP (maximum respiration) and antimycin A & rotenone (non-mitochondrial respiration), respectively. **B-E.** Microglia exposed to PA have significantly reduced basal respiration, ATP turnover, maximum respiration, and proton leak. HTS01037 (HTS) treatment rescues PA-induced changes in mitochondrial respiration. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. C, ###$p < 0.001$, #$p < 0.05$ vs. PA.

Discussion

Obesity and neuroinflammation are known risk factors for developing cognitive disorders (4-7). HFD induces activation of M1-like microglial phenotypes, leading to impaired immune response and contributing to either the onset of cognitive impairment and/or acceleration of neurodegenerative diseases such as AD (231). Herein, we provide evidence that HFD impairs memory, and the microglial FABP4-UCP2 axis is involved in attenuating diet induced memory impairments.

Microglia are dynamic cells that maintain and promote neuronal health throughout the CNS (64, 184). Neuronal-glial circuitry is maintained via microglia through removing damaged synapses and altering plasticity (50, 189). Depletion of microglia and subsequent neuroinflammation attenuates cognitive decline, indicating an important role for microglial in cognition (247). Previous studies have demonstrated that HFD alters microglial response contributing to cognitive decline (15, 248). Additionally, it has been demonstrated that microglial mediated synaptic pruning and hippocampal plasticity is impaired via HFD feeding (15). However, this effect is reversed when diet is returned to low fat (15), strongly indicating that dietary intake impairs microglial function. We and others have demonstrated that HFD, specifically PA induce microglial activation (9, 24, 249, 250). Our prior data demonstrate that the FABP4-UCP2 axis is central in mediating microglial inflammation (24). Here, we show that the microglial FABP4-UCP2 axis in part mediates protection against diet induced cognitive decline (Fig 6.3-4). Despite no differences in learning, long term spatial memory impairment was evident in WT mice maintained on a HFD as measured by increased latency to find escape hole (Fig 6.3). AKO mice maintained on HFD are protected against HFD-induced spatial memory impairment (Fig 6.3). Importantly, these differences are not attributed to motor deficits, as similar movement was observed in AKO and WT mice maintained on a HFD (Fig 6.2). We also demonstrate that short term working reference memory is impaired in WT-HFD mice and that AKO-HFD mice are

protected against this deficit (Fig 6.4). These findings are consistent with other reports (243). In adipose tissue FABP4 protein and gene expression is increased during HFD feeding (28, 125). Rodent and clinical data indicate that the FABP4 is a significant regulator of SFA-induced inflammation (24, 28, 125). Additionally, clinical findings support that increased FABP4 serum levels are associated with insulin resistance (IR) and secretion in patients with type 2 diabetes mellitus, suggesting that FABP4 plays an important role in peripheral glucose homeostasis (also linked to the onset of AD) (251, 252). Clinically, brain IR is considered an AD risk factor, as patients with AD present with increased IR in brain tissue (253). In HFD rodent models, neuronal IR paired with impaired spatial working memory has been reported (243). One mechanism in brain tissue could be lipotoxicity, which can impair glucose homeostasis and induce inflammation, due in part to activation of neurotoxic M1 microglial phenotypes, which can precipitate the onset of brain IR (21). The microglial FABP4-UCP2 axis represents an important mediator in this process, and under HFD conditions this axis is perturbed, ultimately resulting in cognitive decline. Future studies to determine the effects of the FABP4-UCP2 axis on brain IR are ongoing. While other groups have determined that FABP5 (E-FABP) KO mice demonstrate impaired cognitive function, it appears that this is due in part to decreased DHA (docosahexaenoic acid) metabolism within brain tissue (237, 254). A cell-type specific contribution has not been positively identified, but brain endothelial cells (at the blood brain barrier), not microglia, are thought to be the most salient contributing cell type (255).

Our prior work demonstrates that loss or inhibition of FABP4 results in reduced microglial pro-inflammatory expression of iNOS and TNF-α via a FABP4-UCP2 mediated axis (24). We show microglia treated with LPS or PA induce the upregulation of pro-inflammatory cytokines (TNF-α, IL-6, IL-10, IL-13, CXCL1 (KC), IL-1α, CCL5 (RANTES), CCL2 (MCP-2) and G-CSF; Fig 6.5), a finding in agreement with other studies (54, 246). More importantly, inhibition of FABP4

attenuated a wide range of key pro-inflammatory chemokines and cytokines (Fig 6.5 A-I). These findings indicate that UCP2 is necessary for mediating microglial inflammatory response. Seminal research has begun to define the links between dietary intake, metabolism, peripheral inflammation, and neuroinflammation (13, 24, 125-127). Diet-induced shifts in metabolism (metabolic remodeling) are directly coupled to immune effector functions and can directly impact the regulation of microglial/macrophage transition between pro- and anti-inflammatory phenotypes. For example, activation of macrophages to an M1-like (toxic) state via LPS stimulation of TLR4 is marked by a robust upregulation of aerobic glycolysis, impaired mitochondrial respiration, and disruption of the tricarboxylic acid cycle (TCA or citrate cycle) (126, 127). These shifts are directly coupled to accumulation of TCA intermediates such as itaconate and succinate, resulting in increased lactate and decreased intracellular oxygen consumption (126, 127). This pathway is driven by upregulation of the mitochondria-associated enzyme Irg1 (127). This remodeling of the TCA cycle in macrophages demonstrates the direct link between metabolic adaptation and immune function. Likewise, we show that inhibition of FABP4 protect microglia from PA-induced loss of β-oxidative mitochondrial respiration capacity (Fig 6.6). These results confirm other findings that pro-inflammatory stimuli shift from primarily oxidative metabolism to a glycolytic state (194, 256).

Multiple studies have demonstrated that lipid metabolism changes are associated with development of cognitive decline and increased risk of AD (5, 13, 15, 250, 257). In transgenic AD mice (APP23), HFD alters the brain transcriptome, lipid metabolism, and lipid composition, resulting in altered brain immune response (13). Additionally, HFDs alter phospholipid subspecies linked to neuroinflammation, mitochondrial dysfunction, oxidative stress, and cognitive deficits (13, 258). However, these studies largely focused on whole brain tissue samples, and therefore do not reveal individual contributions of microglia. Recent

data shows that single-cell RNA sequencing (scRNA-seq) is an effective tool for defining microglial immunoheterogeneity in models of AD (259, 260). To identify microglial phenotypes during HFD-induced cognitive decline, single-cell scRNA-seq to perform a comprehensive analysis for identification of genes regulating brain immune system and innate immune response pathways and regulatory factors within the FABP4-UCP2 axis are ongoing. Further work is necessary to understand the role of HFD, microglial lipid metabolism, and cognitive decline. Collectively, our data demonstrate that FABP4–UCP2 axis alters immune cell metabolism during HFD and obesity. The FABP4-UCP2 axis represents a novel target for the treatment of inflammation-induced neurodegeneration and cognitive decline.

Chapter 7

Summary

Despite extensive studies in models of obesity and aging understanding of neuroinflammation and cognitive decline is incomplete. The aim of my work was to define central regulators of microglial immune response in the context of obesity and cognitive decline. To pursue these studies, transdisciplinary approaches (nutritional biochemistry, pharmacology, and neuroscience) were applied to understand how dietary metabolites affect the central nervous system.

The epidemic of chronic diseases, including dementia and neurological related disorders, is linked to aging (261). Neurological diseases are one of the leading contributors to disease burden. Individuals over the age of 60 years, and results in disability rather than mortality (261). The societal cost of age-related diseases is rapidly increasing. The financial burden of dementia related care in the US was $157-215 billion annually (2010) and is projected to cost $511 billion by 2040 (262). Additionally, the economic cost of obesity is over $147 billion annually, and midlife obesity is a clinical risk factor for cognitive impairment and neurodegenerative diseases including AD (1-3). Therefore, the development for treatment of cognitive related disorders important. The work presented here lends to this public health crisis by leveraging transdisciplinary approaches (nutritional biochemistry, pharmacology, and neuroscience) to understand how obesity influences microglia and cognition.

Overnutrition induces metabolic syndrome (obesity, insulin resistance, and glucose intolerance) and inflammation (8, 23, 263). Chronic neuroinflammation represents a key link between obesity and cognitive decline (3, 9, 10). While neuroinflammation and cognitive impairment are correlated, few studies clearly define a mechanism that explores the relationship between diet-induced obesity the onset of neuroinflammation in microglia. The aim of my work was to define the

role of microglia in the context of obesity and cognition. The central hypothesis of my thesis was that HFD induce microglial activation resulting in altered immunometabolic response, neuroinflammation, and subsequent cognitive decline. Here, I presented data that support PA and HFD induce neuroinflammation and multiple durations of HFD exposure (chronic and short term) in multiple rodent models (O/A3 and WT) contributes to cognitive decline. Microglia are highly dynamic in that they mediate immune responses, maintain synaptic terminals, remove debris, and secrete factors for neuronal survival. Previous reports indicate that exposure to chronic overnutrition and HFD, synaptic pruning and maintained of synaptic terminals is impaired (15). In models of AD, microglial synaptic pruning is heightened and correlated to cognitive deficit (264). Therefore, maintaining microglial homeostasis especially in the hippocampus, where neuronal-glial crosstalk is highly dynamic and responsive to environmental stimuli such as HFD is important to preserving cognitive function (15).

I also demonstrate the loss of FABP4 attenuates diet induced neuroinflammation and cognitive decline. Importantly, molecular, genetic, or pharmacologic loss of FABP4 results in an anti-inflammatory phenotype and an M1-like to M2-like phenotypic shift in microglia, preventing development of metabolic syndrome or neuroinflammatory response even in the presence of a high saturated fat diet (24). While hippocampal neuroinflammation is part of normal aging, risk of cognitive impairment increases with chronic consumption of diets high in saturated fat and with metabolic perturbations such as insulin resistance (IR), especially during mid-life (3, 12, 14, 243, 265). HFD induces activation of M1-like microglial phenotypes, leading to impaired immune response and contributing to either the onset of cognitive impairment and/or acceleration of neurodegenerative diseases such as AD (231). Altered brain glucose is inherent to AD pathology and may present prior to symptoms of the disease (266). Additionally, increased concentration of brain glucose is associated with greater

severity of AD pathology (266). In human brains, AD pathology and increased brain glucose is also associated with increase markers of glycolysis (266).

Microglia activated to an M1-like state also have increased glycolytic markers (267). In macrophages lacking FABP4, markers of glycolysis are reduced (125). I demonstrate that the loss of FABP4 is protective against PA-induced disruption of mitochondrial biogenesis. The FABP4-UCP2 axis in microglia in the context of metabolic reprogramming, HFD and AD have been relatively unexplored, however it would be reasonable to predict that loss of FABP4 in microglia would reduce markers of glycolysis and could be a mechanism in which the loss of FABP4 is protective against HFD-induced cognitive decline. Previous work has demonstrated that HFD induces neuronal insulin resistance paired with impaired working memory in rodents (243). One mechanism in brain tissue could be lipotoxicity, which can impair glucose homeostasis and induce inflammation, due in part to activation of pro-inflammatory (M1-like) microglial phenotypes, which can precipitate the onset of brain IR (21). Interestingly, both microglia and macrophages exposed to pro-inflammatory stimuli have altered intracellular lipid content (increased SFA and reduced MUFA). This has been demonstrated to directly alter immune cell polarization. In macrophages lacking FABP4, MUFA concentration is increased and contributes to polarization to a protective phenotype in part via increased oxidative phosphorylation and reduced glycolysis (125). In the healthy brain, microglia are constantly surveying the surrounding microenvironment to promote structural formation and elimination of neuronal synapses (15). This process is especially important in the hippocampus, where neuronal-glial crosstalk is highly dynamic and responsive to environmental stimuli such as HFD (15). Future work should address the impact of the FABP4-UCP2 axis in regulating microglial polarization and metabolic reprogramming in response to HFD and cognitive decline.

Changes in lipid metabolism have been associated with cognitive decline and Alzheimer's disease (5, 13, 15, 250, 257). Collectively rodent and clinical data support obesity alters the brain transcriptome, lipid composition and metabolism, oxidative stress, and mitochondrial dysfunction, resulting in modified microglial response and cognitive deficits (13, 258, 268, 269). Therefore, metabolic adaptations in transcriptome and lipid composition would contribute to additional imprints on microglia that accelerate the progression of consequences of neurodegeneration. To delineate microglial heterogeneity the integration of "omics" studies will refine therapeutic approaches and enhance translation of bench top to bed side applications. The pathways studied in this thesis present the initial findings that could become important in clinical diagnosis. Further characterizing the contributions of metabolic response within microglia are a burgeoning field and will provide insight to treat neuroinflammatory diseases.

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Publication: Molecular and Cellular Neuroscience

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