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Effects of Diet on LCN2 Expression and Onset of Neuroinflammation in an Alzheimer's Disease Mice Model

Abdirahman Hayir

An Honors Thesis Submitted to the Neuroscience Program at Macalester College, Saint Paul, Minnesota, USA

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Abstract

This project covers the findings regarding the impact of diet on lipocalin 2 (LCN2) and the effects it has on neuroinflammation Alzheimer's Disease (AD). LCN2 is a protein that is critical to the functionality of mitochondria and inflammatory responses. Evidence has shown that mitochondrial dysfunction is a potential central event in driving AD pathogenesis and contributing to formation of pathological hallmarks such as chronic inflammation. Furthermore, studies have shown that LCN2 can be deficient under metabolic conditions such as high-fat-diet (HFD). This study investigates if HFD induces LCN2 deficiency and increased neuroinflammation in an AD mice model.

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There are many individuals that I want to thank for their support and help in completing this project. First and foremost, I would like to thank the Butterick & Nixon lab for granting me the opportunity to start out in their lab last summer. It has been a tremendous experience and I have learned and grown a lot under the mentorship of both Dr. Tammy Butterick and Dr. Joshua Nixon. Additionally, Kendra Fleming for helping me learn all the new techniques and procedures of the lab and teaching me the concepts of the study along with the help of Simon So. Additionally the endless support from my fellow interns from the summer, Cody Thao, Bethel Tadesse and Sheymaa Mohamed who have given me encouragement and positive words for all the times I felt like giving up. Apart from the Butterick & Nixon lab, I wish to thank the Chen lab for being our collaborators on this project and their support during this long journey. My professors from the Neuroscience and Biology department for their teachings and assistance have prepared and inspired me to take on this journey. I would also like to thank my honors committee members; Dr. Darcy Burgund, Dr. Eric Wiertelak, and Dr. Tammy Butterick for accepting to be on my committee. I want to especially thank Darcy for being a tremendous advisor during this journey and dedicating so many hours these past semesters to help me accomplish this project, you are an invaluable mentor who I will never forget. Lastly, I want to thank my family and friends who have stayed by my side through the long nights I spent working on this project to ensure that I am not overworking myself and neglecting my health. This thesis would not have been possible without all these contributions to help me get to where I am today.

Table of Contents

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder that is characterized by gradually progressive cognitive and functional decline as well as behavioral changes in individuals (Apostolova et al., 2016). The disorder is associated with common pathological hallmarks such as amyloid beta plaque formations and tau tangles. The common cognitive deficits expressed through this disorder include deficits in short-term memory, language, and executive functions (Silva et al., 2019). AD is the most common neurodegenerative disorder and the sixth most common cause of death in the United States. Even though the first clinical symptoms start to present themselves after the age of 65, evidence suggests that AD pathology starts depositing in the brain in midlife. AD prevalence has started to rapidly increase in large part due to the proportion of people 65 years and older. Between 1997 and 2050, the elderly population, defined as people 65 years of age and older is predicted to increase from 63 to 137 million in America alone with similar large-scale increase in other parts of the world (Apostolova et al., 2016). Furthermore, in a nationally representative US data set, the Aging, Demographics, and Memory Study (ADAMS), it was estimated that 14% of people over age 71 and older have dementia. AD dementia accounted for 70% of the dementia cases across this age population (Apostolova et al 2016). Although aging is the most prevalent risk factor for the onset of AD, there are other risk factors that can contribute to the prevalence of AD onset. Obesity is a risk factor that can increase the chance of individuals developing AD over time and one that is explored in this study, it has been shown that obesity in midlife can increase an individual's likelihood to develop Alzheimer's drastically (Povova et al., 2012).

Effect of Diet

Obesity is a worldwide health problem that affects many people due to excessive consumption of saturated fat and lack of exercise. It has been linked to cognitive deficits, impaired long-term potentiation, and synaptic plasticity along with a smaller brain volume (Flores-Cordero et al., 2022). These changes result in an increased probability of developing AD and other forms of dementia.

Neuroinflammation

Obesity is known to cause a state of low-grade chronic inflammation that can lead to dysregulation of homeostatic systems and lead to development of various disease, including neurodegenerative disorders such as AD (Flores-Cordero et al., 2022) This is important because chronic neuroinflammation is one of the pathological hallmarks of AD. The chronic neuroinflammation is detrimental due to not only causing damage to healthy tissue but prolonged propagation of inflammation can become overwhelming and lead to exhaustion of the immune system, resulting in poor host defense. Additionally, it has been shown that obesity can have a negative effect on the propagation of neuroinflammation (Nam et al., 2017). Diets composed of high fatty acids have been shown to be capable of passing through the blood brain barrier and interacting with cells in the brain (Mitchell et al., 2011). Furthermore, it has been observed that LCN2 expression in adipocytes is highly inducible in response to inflammation and metabolic stressors such as high fat diet (HFD) feeding (Zhang et al., 2014). This is proposed to be a protective mechanism against overactivation of stress responses which could potentially cause the damage and inflammation of tissues. It has been demonstrated that HFD increases neuroinflammation and cognitive decline in rodent models of obesity (Duffy et al., 2019). The Duffy et…al 2019 was a study conducted previously by our lab and was done using a mice

model with a progressive orexin neuron loss. Orexin A(OXA) is a hypothalamic peptide that is important in obesity resistance and contributes towards neuronal protection. To assess the neuroprotective aspect of orexin, immortalized hypothalamic cell lines were pretreated with OXA for 24 hours followed by an additional 2hrs of incubation with palmitic acid in the presence or absence of OXA. Palmitic is a saturated fatty acid and is common in western diets. It was observed that reactive oxygen species (ROS) production is significantly increased following 2hrs of palmitic acid exposure and that OXA treatment attenuated palmitic acid-induced ROS production (Duffy et al., 2016.) To test how diet would influence cognitive performance they utilized WT mice and Orexin-Ataxin-3(O/A3) mice (Duffy et al., 2019.) O/A3 mice lacked the neuroprotective peptide OXA. These two mice groups were kept on either a HFD treatment or on a normal-chow (NC) treatment for 4 weeks. The mice were assessed cognitively using a Two-Way Active Avoidance (TWAA) task. The mice completed a spatial memory task where they were allowed to explore a box and received a 0.3 mA scrambled foot shock that was delivered 5 sec later through the floor grid. The scrambled nature of the shock prevented the mice from finding a no-shock position on the floor. To avoid the shock, the mice learned that to avoid the shock they had to move to the other compartment. Upon analyzing the TWAA activity it was seen that O/A3 mice on a HFD treatment had significantly impaired long-term memory indicated by reduced shock avoidances and longer latency. It was also observed that HFD O/A3 mice had decreased locomotor activity compared to WT-O/A3 mice indicating that even with the same groups diet had a detrimental effect on performance levels. Furthermore, upon analyzing the effects of HFD on neuroinflammation, it was observed that there was an upregulation of ionized calcium binding adaptor molecule 1 (IBA1) markers in both HFD-WT and HFD-O/A3 mice. IBA1 is a marker for microglia which are the resident macrophages of the brain. They are

activated during inflammatory responses and are responsible for clearing up foreign pathogens, bacteria, and debris inside the CNS. Additionally, it was seen that proinflammatory markers such as TNF- were also upregulated in HFD mice compared to NC mice in both groups.

Cognitive Deficit

Furthermore, apart from impacting neuroinflammation, it has been shown that diet can affect functionality of other brain regions such as the hippocampus. AD is a disorder that results in progressive loss of cognition which includes deficiency in memory, decision making, language and orientational decline due to neuronal cell death. The brain regions primarily affected are areas associated with memory storage and cognitive processing such as the cortex and hippocampus. It has been shown that exposure to HFD strongly affects the hippocampus and alters the homeostatic system of the hypothalamus and leads to hypothalamic inflammation (Velloso et al., 2011) In Lizarbe et al., 2018, they investigated alteration of hippocampal dependent spatial memory when mice were put on different amount of fat diet (10 %, 45%, and 60%) The mice were assessed via a Y-maze spontaneous alterations test where they are allowed to explore a Y shaped maze and assessed based on their spatial memory of the maze. It was observed that there was impaired spatial memory in the 45% and 60% HFD groups compared to the control group. It has also additionally been observed that even short-term exposure to HFD can have impacts on hippocampal functions. It was demonstrated that mice experience impaired memory function and depressive-like behavior already in the first week of a HFD exposure (de Paula et al., 2021). These changes were followed by a reduction in synaptic density and changes in mitochondrial function in the hippocampus. There was additionally an induction of pro-inflammatory cytokines along with increased permeability of the BBB. Treatment with TNF-

alpha inhibitors mitigated the BBB-alteration suggesting that inflammatory signaling was critical for this change (de Paula et al., 2021).

Amyloid Cascade Hypothesis

Apart from obesity another risk factor for AD is a genetic predisposition to AD due to mutations to the amyloid precursor protein (APP) and Presenilin-1(PS1) protein, the catalytic subunits of the y-secretase complex that is involved in the cleavage of the APP (Silva et al., 2019). Improper cleavage of the APP is what results in the formation of one of the neuropathological hallmarks of AD and that is the amyloid beta plaque deposits observed in patients' brains. There are two main pathways for APP processing: a non-amyloidogenic α-secretase-mediated pathway and an amyloidogenic β and γ-secretase mediated pathway (Silva et al., 2019). Cleavage of APP by α-secretase results in a soluble molecule known as sAPPα, which has probable neuroprotective function and plays important roles in plasticity and survival of neurons. However, the Aβ peptide is produced through cleavage of β-secretase (mainly BACE1 enzyme). This gives rise to an APP soluble fragment and a carboxy-terminal complex(C99) linked to the cell membrane. C99 is then cleaved by γ-secretase, which is composed of different proteins including the PS1, to give rise to the Aβ peptide. Aβ peptides range in size from 40 to 43 amino acids, however it's the Aβ42 that is more prone to aggregation and is found in higher concentrations in amyloid plaques. Mutations of the PS1 or APP protein results in more improper cleavage of the C99 giving rise to more Aβ42 peptides compared to the other sizes. The current most popular hypothesis regarding AD pathogenesis is the amyloid-cascade hypothesis (Barage et al., 2015). The hypothesis states that it is the deposition of Aβ protein that is the causative agent of Alzheimer's pathology and that the neurofibrillary tangles, inflammation, and dementia follow because of this deposition (Ricciarelli et al., 2017). Most of

the evidence in support of the amyloid beta cascade theory stems from the occurrence of pathology in individuals carrying autosomal dominant mutations and AD seen in individuals with down syndrome (Ricciarelli et al., 2017). Furthermore, the theory has received support from generations of a variety of APP or APP/PS1 transgenic mice models that have shown anatomopathological and behavioral aspects of the disorder (Ricciarelli et al., 2017). However, there are some aspects of AD that unfortunately are not supported by this theory or don't align with it. This is due to the observed bioenergetic disruptions observed in AD even prior to the formation of amyloid beta plaque formations. These symptoms include Ca2+ homeostasis dysfunction, increased oxidative stress, and phospholipid imbalance (Swerdlow et al., 2015). Furthermore, apart from these symptoms it has been noted that extraction and purification of soluble oligomers in AD patients or mice is a complex process and it is uncertain whether if the isolated amyloid-beta oligomers are endogenously produced or artificially created during the process (Swerdlow et al., 2015). Additionally, it is noted that AD can occur in individuals with no genetic predisposition due to mutations, this form of AD is known as sporadic AD(SAD).

Mitochondrial Theory

With the Amyloid beta cascade theory not being able to account for the unexplainable bioenergetic symptoms observed prior to formation of Aβ deposits along with the individuals affected by SAD, studies have investigated other underlying events and mechanisms that could explain the AD pathogenesis better. A lot of studies have focused on understanding the role that mitochondria could play in being the central event behind onset of AD symptoms and events in patients. The mitochondria carry out multiple essential functions in different cellular processes (Yu et al., 2021). Neuronal activities require heavy energy expenditure and the nervous system in general consumes a great deal of energy and the mitochondria is the major energy source

providing ATP through oxidative phosphorylation to allow for normal neuronal homeostasis and function (Area-Gomez et al., 2018). Additionally, the mitochondria are vital in regulating cell survival and death under various stress conditions through interacting with other cellular organelles to regulate mitochondrial stress response under physiological and pathological conditions (Yu et al., 2021). It is therefore evident that disturbances of mitochondrial function are closely associated with mechanisms underlying neurodegenerative diseases such as AD (Area-Gomez et al., 2018). It was shown in a study from 1987 by N R Sims. et al that analyzed oxygen consumption in the brain from dementia subjects and control subjects. It was shown that under sub-maximal conditions oxygen consumption from dementia patients exceeded that of the control subjects indicating that their mitochondrial function was unstable. It was still unclear at this time whether these mitochondrial or bioenergetics lesions contributed to AD or whether they simply represented a byproduct of the disease.

Mitochondria Dysfunction

To understand this predicament better, studies focused on the mitochondria and amyloid beta (Aβ) plaque, a byproduct caused by improper cleavage of the amyloid-β protein precursor (APP). It is argued that these byproducts initiate the neurodegeneration of AD, so it became evident to understand the relationship between these plaques and mitochondria. It was shown that cultured cells that are maintained in the presence of Aβ show reduced electron transport chain enzyme activities. Additionally, Aβ also impairs respiratory chain function in isolated mitochondria. While these results indicate potentially important relationships between Aβ and mitochondria, they did not provide compelling evidence of how mitochondria lesions could induce AD. It was in a study by Cardoso et al, 2001 that this question was more clarified. This study was looking at Aβ-induced toxicity via mitochondria. To achieve this the study added Aβ

to the medium of human neuronal NT2 cells, which induce cell death. They additionally also added Aβ to the medium of NT2 ρ0 cells, which are NT2 cells depleted of their mitochondrial DNA (mtDNA). Since they lack mtDNA, ρ0 cells do not produce key respiratory chain subunits and are respiration-incompetent. The treatment with Aβ did not harm these cells since they lacked a mitochondrion, but the other cells were induced with cell death. Through this study it could be inferred that the mitochondria and respiratory chain may mediate Aβ-toxicity. With the mitochondria becoming more tied in with the pathogenesis of AD there was the possibility that the mitochondria and its destabilizing effects were the central event in the cascade of AD pathogenesis. The mitochondrial being the cause of this would explain the unusually bioenergetic symptoms that occurred prior to the formation of amyloid beta plaques. Additionally, the mitochondria theory maintains genetic aspects while also accounting for AD cases that were more sporadic. The theory states that genes set a baseline mitochondrial function. Inherited factors(genetics) along with environmental exposure determine the rate at which age-associated mitochondrial changes develop and manifest. It has been shown in studies that mitochondrial DNA mutations can aggravated again and brain impairment (Ross et al., 2013), Furthermore it was shown that increase in somatic mtDNA mutations in mice is associated with premature onset of aging related phenotypes such as dementia (Trifunovic et al., 2004). In essence the mitochondrial theory states that mitochondrial function affects AB accumulation based on interactions between genetics and environmental factors.

Reactive Oxygen Species

While it is evident that mitochondria play a role in AD pathogenesis, there are many different causes that can result in mitochondrial dysfunction. One of the primary causes of mitochondrial dysfunction is through oxidative stress caused by an overproduction of reactive

oxygen species (ROS) (Guo et al., 2013). ROS are a byproduct generated during mitochondrial oxidative metabolism such as when producing ATP. ROS is toxic which can start a series of harmful reactions to the cell (Ray et al., 2012). Although ROS are produced under normal conditions, they are counterbalanced by endogenous enzymatic cellular antioxidant systems. The damage caused by free radicals is due to an imbalance between their production and neutralization system. Both systems appear to be altered in AD and these changes play a role in the degeneration of the mitochondria over time (Ray et al., 2012). Apart from the nucleus, the mitochondria are the only organelle in the cell that contains their own DNA and machinery for synthesizing RNA and proteins (Guo et al., 2013). ROS is known for attacking nucleic molecules which compromise the DNA and RNA found inside the nucleus and mitochondria and causing damage. The persistence of mitochondrial DNA damage ultimately leads to mutations of the mitochondrial genome and gives rise to mitochondrial dysfunction that aggravates the disease (Guo et…al, 2013).

While ROS is one possibility for inducing mitochondrial dysfunction over time, another possibility is through mitochondrial-associated-ER-membranes also known as MAMs. Biological procedures that are essential for maintaining homeostasis in cells are effectively completed through MAMs. MAMs are highly fluid membranes that provide direct, fast, and reciprocal signaling molecules transport between the mitochondria and the ER (Yu et al., 2021). MAMs are integral regulators of calcium homeostasis, Phospholipid transportation and metabolic homeostasis of mitochondria. Additionally, MAM's play a role in the generating of intracellular Aβ peptides in the cell. Initial evidence for intracellular Aβ was when formation of intracellular Aβ was observed in differentiated neuronal cell lines (Wertikin et al., 1993). Furthermore, it was implicated that intracellular Aβ deposits accumulation induces toxicity in

AD and that extracellular Aβ deposits could be secondary to the intracellular Aβ accumulation where the mitochondria accumulation specifically plays a significant role (Fernandez-Vizarra et al., 2004). It has also been observed in both postmortem AD brains and transgenic AD mice models that mitochondrial Aβ deposition has negatively affected neuronal functions and lead to cellular dysfunction (Yu et al., 2021). The γ-secretase has been reported to be localized in MAM's which is also supported by the findings of Aβ40 and Aβ42 at the MAM platforms near the mitochondria and are expected to be transported into the mitochondria via the Outer Mitochondrial Membrane (OMM) (Schreiner et al., 2015). It has also been shown that Aβ can promote ROS production through binding to the mitochondrial alcohol dehydrogenase(ABAD) along with altering mitochondrial DNA expressions (Lustbader J.W. et al., 2004) Through these findings we are able to understand that primarily not only are MAM;s involved in various important metabolic pathways necessary for maintaining cellular function but that MAM dysfunction can result in a chain reaction of events that can lead to the failure of the mitochondrial via increased Aβ42 production and ROS production inside the mitochondria itself. While there are many different MAMs involved in maintaining the mitochondrial function, our study focuses on trying to identify a potential novel MAM known as Lipocalin 2 or LCN2.

Lipocalin 2 (LCN2)

Lipocalin 2 (LCN2) is a MAM that is important for maintaining the architecture and dynamics of the mitochondria. The functions of LCN2 have been more elucidated in the peripheral nervous system (PNS) compared to the central nervous system (CNS). It has previously been explored in how it impacts inflammation in the PNS and how diet can influence the expression of LCN2. LCN2 has been shown to have high specific binding for phosphatidic acid (PA) which is one of the critical phospholipid regulators of the mitochondrial dynamics

along with cardiolipin (CL) (Kameoka et al., 2017, Su et al., 2022). Mitochondrial dynamics refers to the continuous fusion and division that the mitochondria undergo (Kameoka et al., 2017). Since there are hundreds of mitochondria in cells they have to be able to communicate with each other effectively in order to regulate the overall mitochondrial population. Fusion and division are processes that control the size and number of mitochondria which are accomplished by importing proteins from the cytosol and lipids from the ER (Kameoka et al., 2017). Each mitochondrion must have at least one copy of mtDNA since oxidative phosphorylation depends on the components encoded by mtDNA. However, creating too many mitochondria by excess division would generate mitochondria that lack mtDNA and compromise the oxidative phosphorylation process. Inadvertently if too many mitochondria are overly connected via fusion, they will end up containing too many mtDNA molecules which can cause them to be aggregated and unable to function properly (Kameoka et al., 2017).

Mitochondrial Dynamic

PA and CL are two lipids that make up the inner and outer membrane of the mitochondria. CL is synthesized from PA via multiple enzymatic reactions and comprises about 10-15% of the mitochondrial membrane (Kameoka et al., 2017). CL is important for maintaining large protein complexes such as the electron transport chain complexes and the protein import machinery in the inner membrane. Studies have also revealed that both PA and CL control mitochondrial fusion and division coordinating a balance between two processes in contain the mitochondrial dynamic (Kameoka et al., 2017). CL plays an important role in fusion of the mitochondrial inner membrane through the biogenesis and assembly of a dynamin related GTPase, Opa1, that mediates the fusion process (Kameoka et al., 2017). Similarly, CL is also involved in the division process in the outer membrane through regulation of another dynamin

related GTPase known as Drp1 (Kameoka et al., 2017). With PA and CL being vital for the regulation of the fusion and division process of the mitochondria, there can be detrimental effects if these processes are negatively regulated or hindered. Imbalance of fusion and division can result in increased production of ROS, and this can have detrimental interactions with the mitochondrial proteins and DNA resulting in mitochondrial dysfunction. Therefore, disruption of LCN2, which is important for the transporting of PA into the mitochondrial membrane, can result in loss of the mitochondrial dynamic over a long period of time.

Inflammation

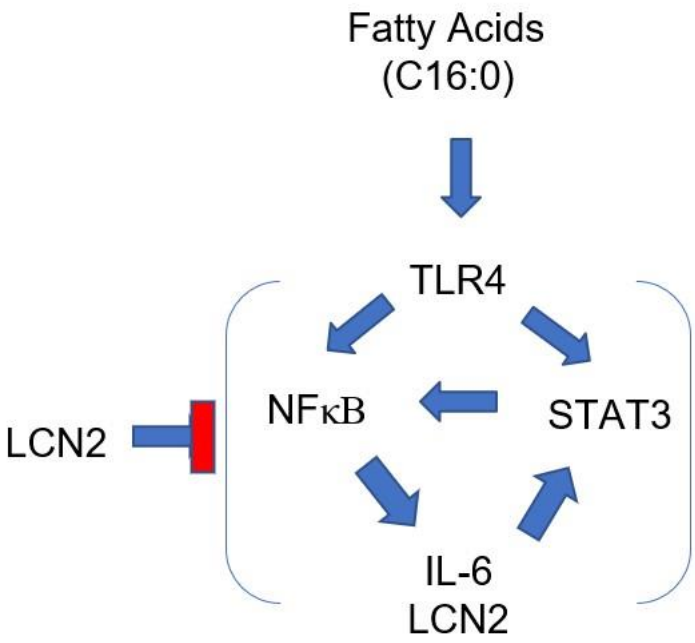
Furthermore, apart from being a regulator for the architecture and dynamic of the mitochondria, LCN2 has been characterized as an adipokine/cytokine and implicated in obesity and inflammation. The Chen lab investigated the role and potential mechanism of LCN2 in regulation of macrophage in obesity-associated inflammation (Zhang et al., 2014). Macrophages are the phagocytes of the PNS and are responsible for cleaning up debris and foreign materials such as bacteria. Macrophages can switch between a M1 and M2 mode set that result in promotion of different cytokines. M1 macrophages produce pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are involved in the propagation of inflammation and contribute to the host defense system (Leslie et al., 2015). Meanwhile, M2 macrophages produce anti-inflammatory cytokines such as IL-10, which are responsible for signaling the clearing of the foreign pathogens and bacteria in the host, ensuring that inflammatory cells such as neutrophils, T-cells and B-cells start to cease their activity (Leslie et al., 2015). This is important because, if inflammation is maintained for a long period of time, it can become chronic and lead to degeneration of host tissue, rather than just clearing foreign materials in the host (Leslie et al., 2015). This is why free fatty acids can induce activation of inflammatory pathways and induce a

M1 polarization, meaning that the pro-inflammatory state is more prevalent (Leslie et al., 2015). LCN2 has been shown to be highly upregulated when exposed to inflammatory conditions and plays a role in modulation of macrophage activating in the lung, liver, and brain. The Chen lab sought to investigate the mechanism by which LCN2 modulated inflammatory conditions in Guo et al., 2014 study. They examined the gene expression of M1 and M2 macrophage markers in adipose tissue from the liver of LCN2 knockout mice after a high fat diet feeding. The results indicated that the M1 marker Cd11C was increased whereas the M2 marker Arg1 was decreased. LCN2 deficiency also resulted in the upregulation of M1 markers and pro-inflammatory cytokines when looking at Bone Marrow Derived Macrophages (BMDMs) that were isolated WT LCN2 knockout mice. These BMDMs also expressed a down-regulation of anti-inflammatory cytokines and M2 markers. With these findings at hand, the Chen lab sought to understand the inflammatory pathways that LCN2 could potentially be regulating and how a change in LCN2 expression would affect these pathways. In order to achieve this they stimulated BMDMs from LCN2 knockout mice with LPS and observed the activation of inflammatory pathways and looked for pathways that increased with LCN2 deficiency. Stimulating with LPS induced increased activity of the NF-κB signaling pathway, which is a signaling pathway that plays a central role in mediating inflammatory signals and controlling production of proinflammatory mediators. Furthermore, there was also an increase in activity of the STAT3 pathway. The STAT3 pathway has been demonstrated to have a direct interaction with NF-κB in studies in cancer cells and contributes to the continuous activation of NF-κB and inflammation (Guo et…al,2014). The interactive relationship between LCN2 and the STAT3 and NF-κB pathways proposed by Guo et al., (2014) is depicted in Figure 1. The diagram depicts how, upon stimulation of the TLR4 receptors, the NF-κB pathway is activated and leads to the upregulation

of pro-inflammatory cytokines. These cytokines result in the activation of the STAT3 pathway, which further reinvigorates the NF-κB pathway and creates a forward feeding loop. Figure 1 also illustrates how there is an increase in LCN2 expression when the NF-κB pathway is initially activated. This is to ensure that there is a checking system to the forward feeding loop and ensures that once the foreign pathogen is cleared the cycle can be regulated and shutdown. However, since LCN2 is the anti-inflammatory regulatory, when it enters a deficient state there is no break to the system leading to a continuous forward feeding loop between the NF-κB and STAT3 pathway.

Figure 1

Relationship Between LCN2 and STAT3 and NF-κB Pathways



The Present Study

The perspective on the central driving event behind AD pathogenesis has progressed immensely through the years. Initially amyloid beta plaque deposits were thought to be the

causing AD pathogenesis and the associated symptoms. However, with the unexplainable bioenergetic symptoms observed, recent evidence has suggested that mitochondria and mitochondrial dysfunction could be occurring prior to formation of the common pathological hallmarks of AD. The mitochondria is central to a lot of the necessary biological functions of the cell and is crucial for maintaining functionality of the bioenergetic failures observed prior to the occurrence of AD pathology. With AD being such a complex disorder that is governed by the interplay of many different factors, such as genetics, environmental factors such as nutrition and diet, and intercellular players, our study aims to investigate the effects of a specific MAM. LCN2 as a novel MAM protein that is involved in the process of mitochondrial dysfunction through regulating and maintaining the mitochondrial architecture and functionality. Decreasing LCN2 levels would result in a loss of the mitochondrial dynamic over time leading to a loss of mitochondrial function. Additionally, like in the PNS, we expect LCN2 to be neuroprotective through acting as an anti-inflammatory regulator of neuroinflammation like as shown in the Guo et al., 2014 study by the Chen lab. LCN2 expression levels have been shown to be impacted via the influence of metabolic stressors and in this study, we are examining HFD as a source of metabolic stress and seeing if treatment with HFD will result in decreased LCN2 expression. We are hypothesizing that treatment with HFD will induce LCN2 deficiency and result in increased neuroinflammation in an AD mice model.

Method

Animals

In order to accomplish our study and investigate the role that LCN2 plays in the onset of neuroinflammation in Alzheimer's disease, we are working with a transgenic mice species known as APP/PS1. These mice have been genetically engineered to contain two mutant human

genes that will result in the onset of Alzheimer's disease as they age. The APP gene is encoding for the amyloid precursor protein (APP) that plays a central role in the pathophysiology of Alzheimer's disease in larger due to the sequential proteolytic cleavages that result in the amyloid-beta plaques, which are one of the two pathological hallmarks of the disease. Presenilin 1 (PS1) is a critical component of the gamma-secretase complex, an enzyme that is responsible for cleaving APP into amyloid beta. Mutation of the PS1 gene results in a similar phenomenon of increased AB_{42} to AB_{40} following the cleavage of APP. The amyloid beta plaques deposits found within Alzheimer's brains consist mainly of AB_{42}. With the mice having been genetically altered with these two mutated genes, the mice are predisposed to develop Alzheimer's as they age.

Diet Treatment

APP/PS1 (AD) and WT mice were put on two different treatment diets starting at 6 weeks old resulting in a total of four treatment groups. The diets were a high-fat-diet (HFD) and normal chow (NC) that the mice were kept on for a duration of 6 months prior to being sacrificed at 9 months. The HFD treatment was with a 60% high fat diet, specifically with saturated fatty acids and palmitic acid. The HFD treatment is meant to serve as a metabolic stressor that would decrease the secretion of LCN2 protein in the mice under that treatment group. Furthermore, HFD is also expected to exacerbate neuroinflammation due to the decrease in LCN2 expression, which would result in an increased level of microglial cells in the HFD treatment mice.

Behavioral Task

The behavioral task that the mice performed was the Y-maze spontaneous alternation test. The Y-maze is a behavioral test to measure the rodents' willingness to explore new environments in the maze. Rodents are typically seen to prefer to explore new environments and surroundings. During this test many parts of the brain are involved in this task such as the

hippocampus and prefrontal cortex. The test shows the activity of the rodents working memory and if they are capable of recalling exploring a certain environment and moving on to explore a new environment. The maze in this experiment is made up of three equally spaced arms that are 120 degrees apart and of similar dimensions designated as A, B, and C. The mice are allowed to explore the maze for 8 minutes and arm entries are grouped into consecutive sequences of 3 (i.e., ACBCAC=ACB, CBC, BCA, CAC). An alternation is scored for each set of three consecutive choices where no repeated entries occur. An alternation score (#alternations / # of possible alterations * 100) of 50% indicates a random selection. Young, healthy C57BL6 mice typically exhibit scores of 75-80% alternation and aging mice approach 50% alternation (Weiss et al., 1998). The Y maze data is collected by ANYMAZE software (V6.3). The order of arm entries is recorded and analyzed for spontaneous alternation (SA). This process does not require any rewards or punishments.

Brain Preparation

Following sacrificing at 9 months old, mice were perfused with saline to preserve the brain. For the perfusion procedure, the mouse is primarily deeply anesthetized with an IP injection of Ketamine/Xylazine. After the injection responsiveness from the mice was checked by pinching its tails or paws. The blood was collected via cardiac puncture and placed on ice until ready for serum collection. Place the mouse on a perfusion tray and use scissors to cut across the abdominal wall just below the diaphragm making sure to avoid cutting organs. Cut the diaphragm completely and expose the heart by cutting cranially up through the thoracic cavity. Using forceps pull the ribcage back and pin it down using needles to leave the heart exposed. Using forceps, gently hold the heart and insert a perfusion needle into the left ventricle. Start the pump slowly and cut the right atrium when it inflates. If fluids start to leak out of the nose, the

pump might be set to high. Since twitching is common make sure that the needle is not dislodged from the heart during the procedure. Allow 30-50 ml of saline to circulate through the mice while checking for paleness of the liver at regular intervals. Once the perfusion is complete turn off the pump to ensure that air bubbles are not introduced.

After the perfusion, the mice underwent brain extraction. The mouse were decapitated using a pair of large sharp scissors. The skin and skull of the mouse were cut open using micro scissors. After the removal of all the bone, the brain was removed gently using a micro spatula to scoop the brain from the skull. Brains are stored inside formalin for 48 hrs. in the fridge. Afterwards the brain is stored in 20% sucrose solution for 48 hours before being switched into cryoprotectant solution for long term storage.

Microtome

Following the harvesting of the brains from the mice, the brains were sliced into tissue sections via a microtome. To slice, brain tissues must have been immersed in 20% sucrose for at least 24 hours prior to slicing but not for more than 72 hours. To ensure proper slicing of tissue, the brain needs to maintain a cold temperature to ensure that sliced tissues are not mis formed or destroyed. To ensure a cold temperature, the stage of the microtome must be frozen utilizing dry ice to make a frozen layer of sucrose for the brain to be surrounded by. Dry ice and sucrose should be added continuously to ensure that the cold temperature is maintained, and the brain is stable. Once the brain is secured in the frozen sucrose, the brain is sliced, and tissues are collected into 12 well plates. The plates were labeled with the mice ID and genotype.

Immunohistochemistry (IHC) Protocols

Immunohistochemistry (IHC) staining was used to examine the effects of diet on LCN2 expression and whether this exacerbated inflammatory response in different mice groups. IHC is

a staining method that allows for the detection of an antigen within cell samples or tissues. This is generally accomplished through the use of a primary antibody that binds to the target antigen and a secondary antibody which is bound to the primary antibody. For our study we utilized two different methods of IHC, the first being a diaminobenzidine (DAB) staining method and the second being through the use of fluorescently labeled antibodies.

DAB Staining

Diaminobenzidine (DAB) IHC staining is a method where DAB is oxidized by hydrogen peroxide in the presence of antibodies conjugated with horseradish peroxidase (HRP). The oxidized DAB forms a brown precipitate at the location of the HRP which is insoluble in alcohol and can be visualized using light microscopy. Through the conjugation of HRP to antibodies, the DAB method can be utilized to stain these antibodies within the tissue and allow for their visualization. Thus, the DAB method is useful for visualizing the changes in the LCN2 antibody within study mice to see how the different treatments were affecting the presence of the antibody. The protocol process for the DAB staining spanned 3 days where the first day consisted of antigen retrieval process and incubation with primary antibody. During the second day the tissue was incubated with the secondary antibody followed by the DAB process to trigger the oxidation reaction of the HRP. Following the DAB reaction, the tissues were mounted on slides and allowed to air dry overnight. For the third day the slides were cleaned in different concentrations of ethanol and cover slipped in preparation to be imaged and analyzed.

Fluorescent Staining

Fluorescent IHC staining is a method that utilizes antibodies that have been tagged with a fluorochrome that emits light when excited. Immunofluorescence allows for great sensitivity and amplification of signals in comparison to regular IHC methods. Furthermore, it allows for the

visualization of multiple antigens simultaneously through the use of fluorochromes with different excitation levels. This results in the target protein fluorescing with different light colors allowing for identification of which protein is bonded to a certain fluorochrome based on the color of the light emitted. Immunofluorescence staining was utilized to visualize co-localization between LCN2 and two types of brain neurons, microglia, and astrocytes. Both neurons are part of the immune system of the CNS and are responsible for cleaning up debris and rapid response to brain tissue destruction. The protocol for fluorescent staining similarly to the DAB staining spanned over 3 days. Day one consisted of antigen retrieval and incubation with primary antibody. During day 2 tissues were incubated with secondary antibodies that had been tagged with different fluorochromes and then mounted onto slides to be air dried overnight. For day 3 slides were cleaned and prepared for imaging and analyzing. The process for fluorescent is like that of DAB but does not require utilization of DAB.

Image Processing and Analysis

In this study the preliminary images of the slides for both staining processes were done on an EVOS microscope. This allowed for confirmation of the staining in the tissues. For the DAB staining slides, the light microscope aspect of the EVOS was utilized and the images were observed for brown precipitate staining on the different brain regions. As for the fluorescent staining slides, the fluorescent channels appropriate for the fluorochromes used were necessary in order to visualize the fluorescent staining. The channels required are the GFP and TxRed channels to visualize the two different primary antibodies. Once the slides were verified with the EVOS microscope to clear staining and properly cleaned, they were sent to the University Imaging Center (UIC) at the University of Minnesota. The UIC were able to perform high

resolution images of the slides with the microscopes at their facility. Once these images were ready, they were analyzed for densitometry.

Results

Behavioral Performance

The Y-maze experiment was utilized to assess the short-term working memory of the mice from the different condition groups to observe the effects that changes in diet can exert on cognitive function. Spontaneous alternations were compared as a function of animal type (WT vs AD) and diet (NC vs HFD) to see effects on cognitive performance. Results are shown for both male and female mice.

Figure 2

Y-Maze behavioral assessment

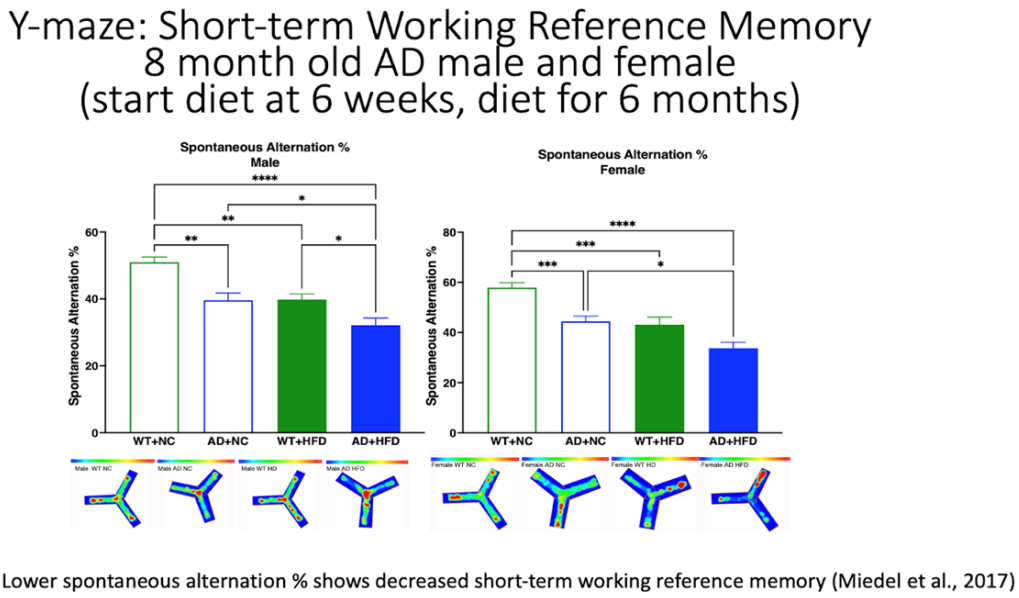


Figure 2 caption: P-values are indicated by asterisk linked between treatment groups. Asterisks indicate the following p-values: *= P≤0.05, **=p≤0.01, ***= p≤0.001.

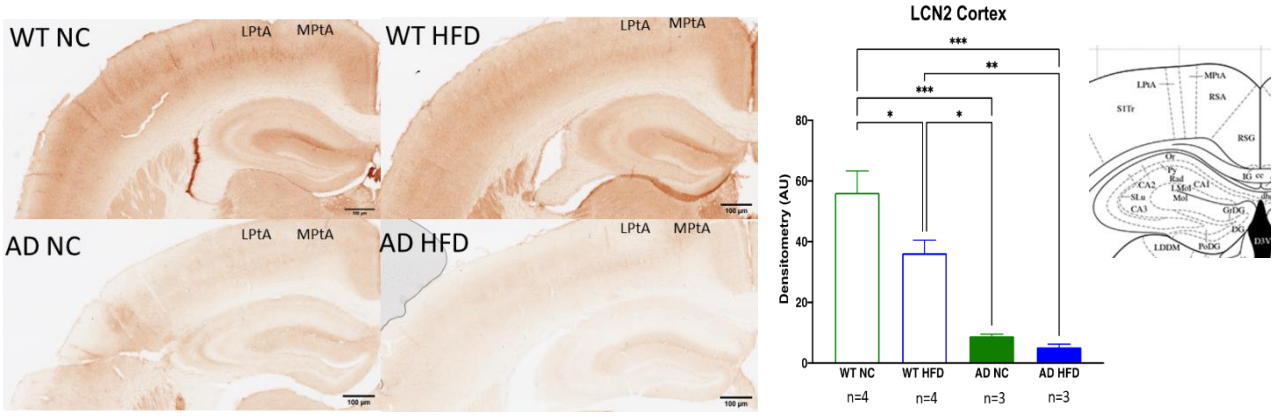
DAB Staining

LCN2 expression was stained in 3 different brain regions of mice brains, specifically the cortex, hippocampus, and hypothalamus. Figure 3 contains the staining images and densitometry for the cortex at the top followed by the hippocampus and ends with the hypothalamus. Treatment groups were analyzed for significant difference in LCN2 expression, which is shown through the brackets linking groups and comparing if there was a significant difference or not.

DAB staining for IBA1 was conducted in the same 3 brain regions as LCN2 staining. Figure 4 includes staining images that indicate the expression level of IBA1 in each brain region followed by a densitometry graph comparing the treatment groups and indicating if calculated p-value is significant or not through linking groups up in brackets

Figure 3

LCN2 Expression in Brain Regions



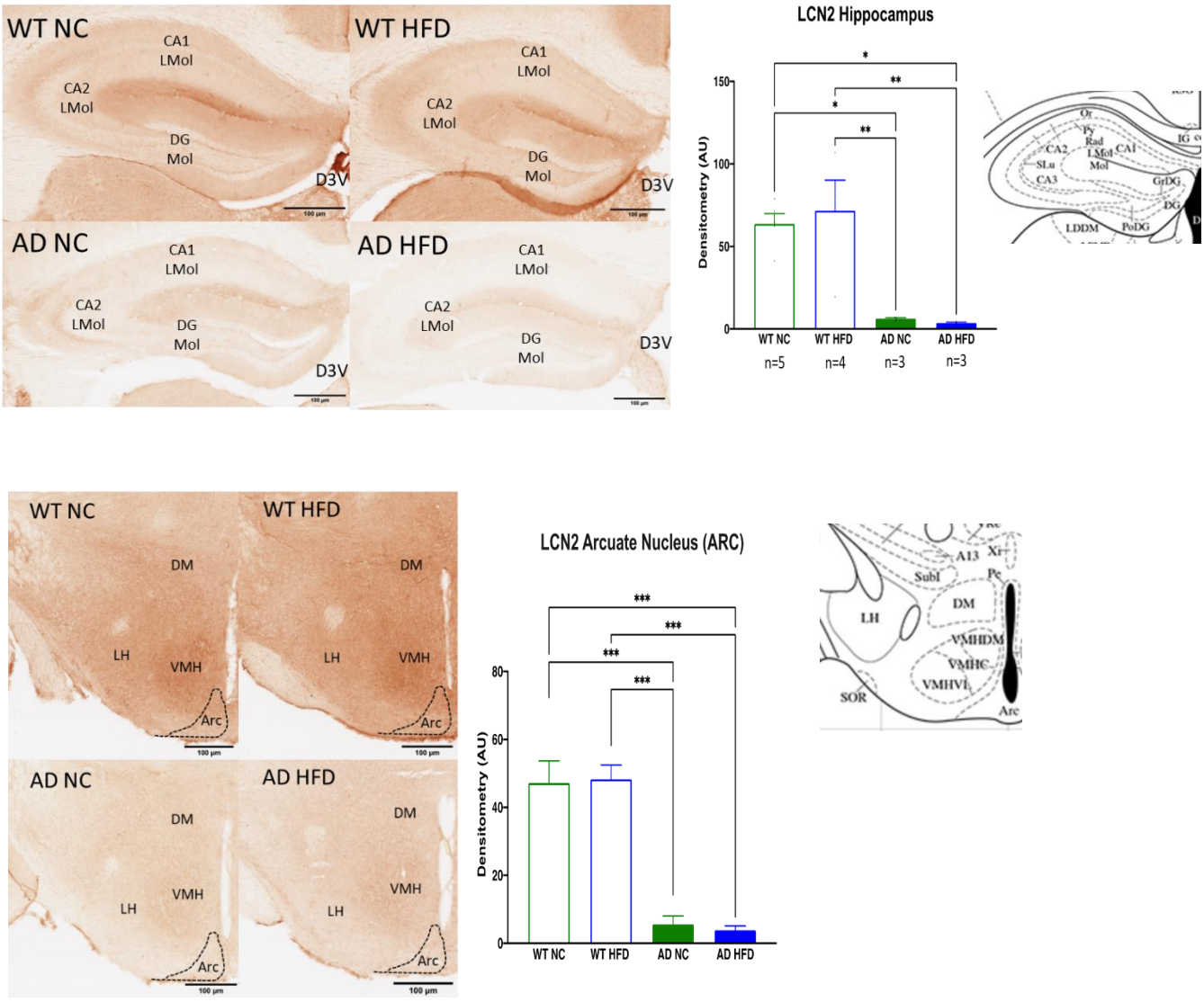


Figure 3 caption: Asterisks indicate the following p-values: *= P≤0.05, **=p≤0.01, ***= p≤0.00. N-values indicated in graphs, range from 3-5.

Figure 4:

IBA1 Expression in Brain regions

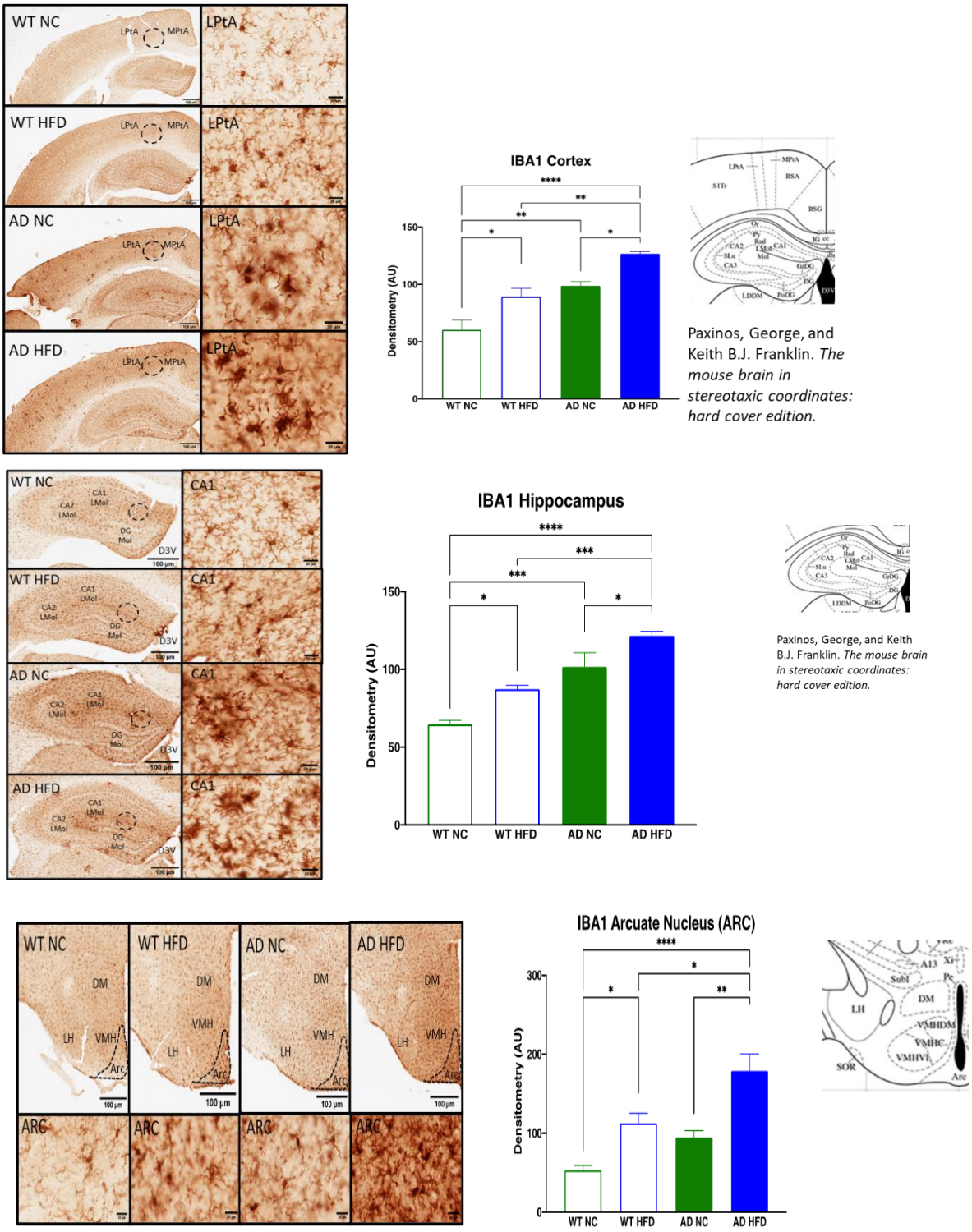


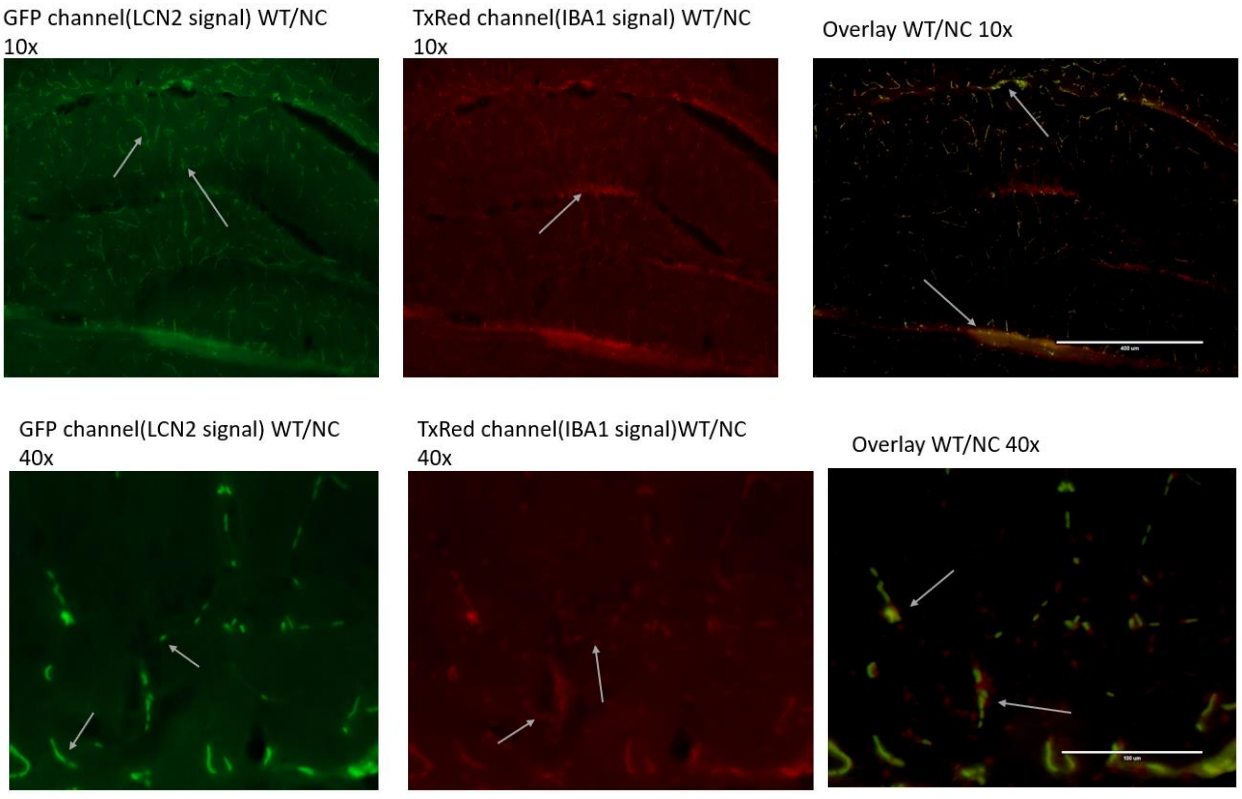
Figure 4 caption: Asterisks indicate the following p-values: *= P≤0.05, **=p≤0.01, ***= p≤0.001. N values of 5 for each treatment group.

Fluorescent Staining

Fluorescent Co-stain showing expression level of LCN2 and IBA1 in the hippocampus of WT/NC and WT/HFD mice are shown in figure 5. Green fluorescent markers indicate LCN2 expression while red fluorescent markers indicate IBA1 expression. Regions containing simultaneous expressions of LCN2 and IBA1 floures in yellow color. Figure 6 shows changes in fluorescent LCN2 expression in hippocampus of WT/NC and WT/HFD mice. The densitometry graph shows numerical representation and changes in marker density and indicate p-value. Figure 7 shows changes in fluorescent IBA1 expression in hippocampus of WT/NC and WT/HFD mice. The Densitometry graph shows changes in IBA1 expression based on treatment diet and represents this change numerically. Preliminary fluorescent staining images of AD/NC and AD/HFD treatment groups are shown in Figure 8.

Figure 5

WT Fluorescent Co-stain



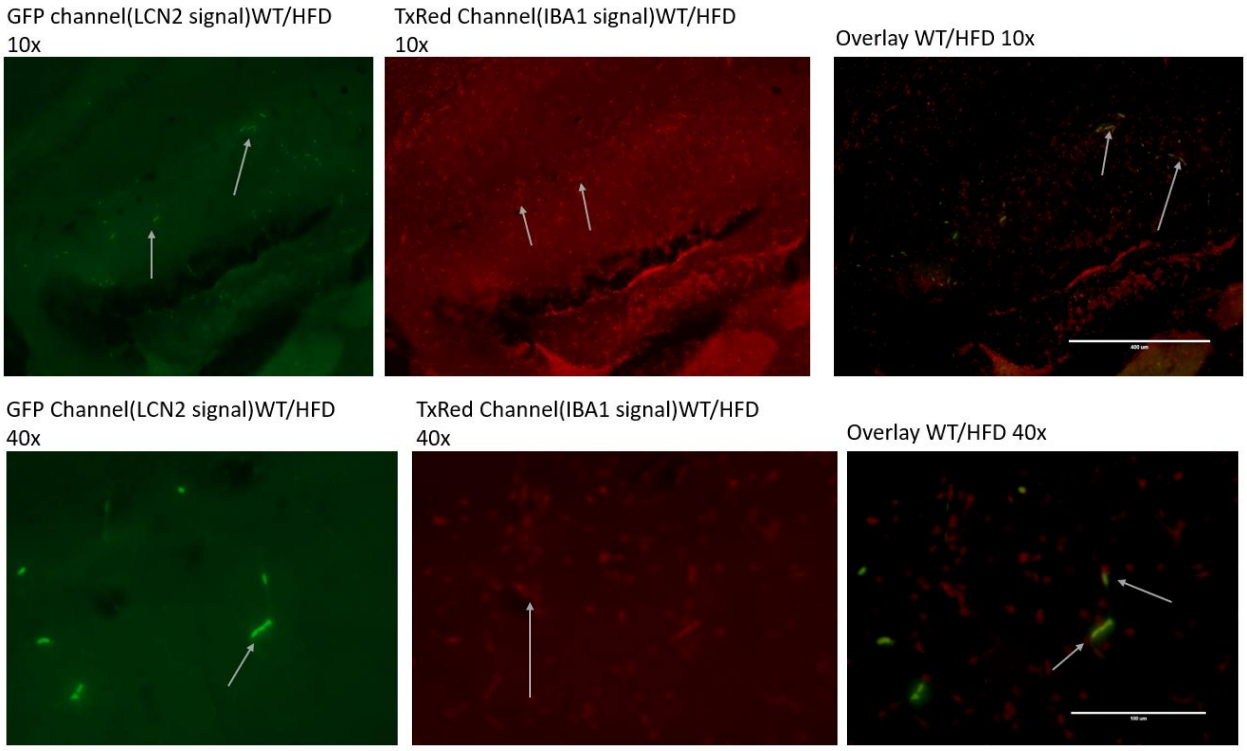


Figure 6

Fluorescent LCN2 in WT Hippocampus

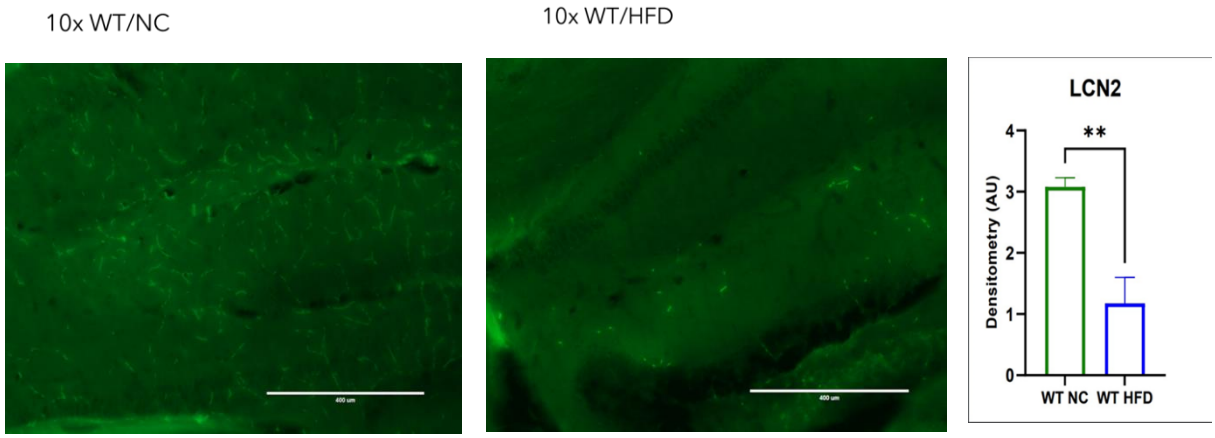


Figure 6 caption: Asterisks indicate the following p-values: *= P≤0.05, **=p≤0.01, ***= p≤0.001. N values of 3 for each treatment group

Figure 7

Fluorescent IBA1 in WT Hippocampus

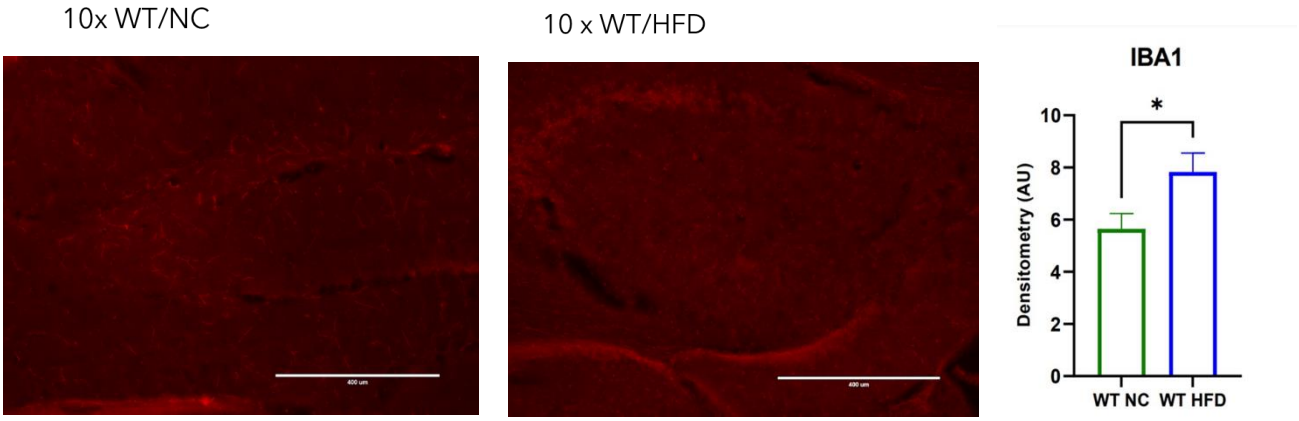
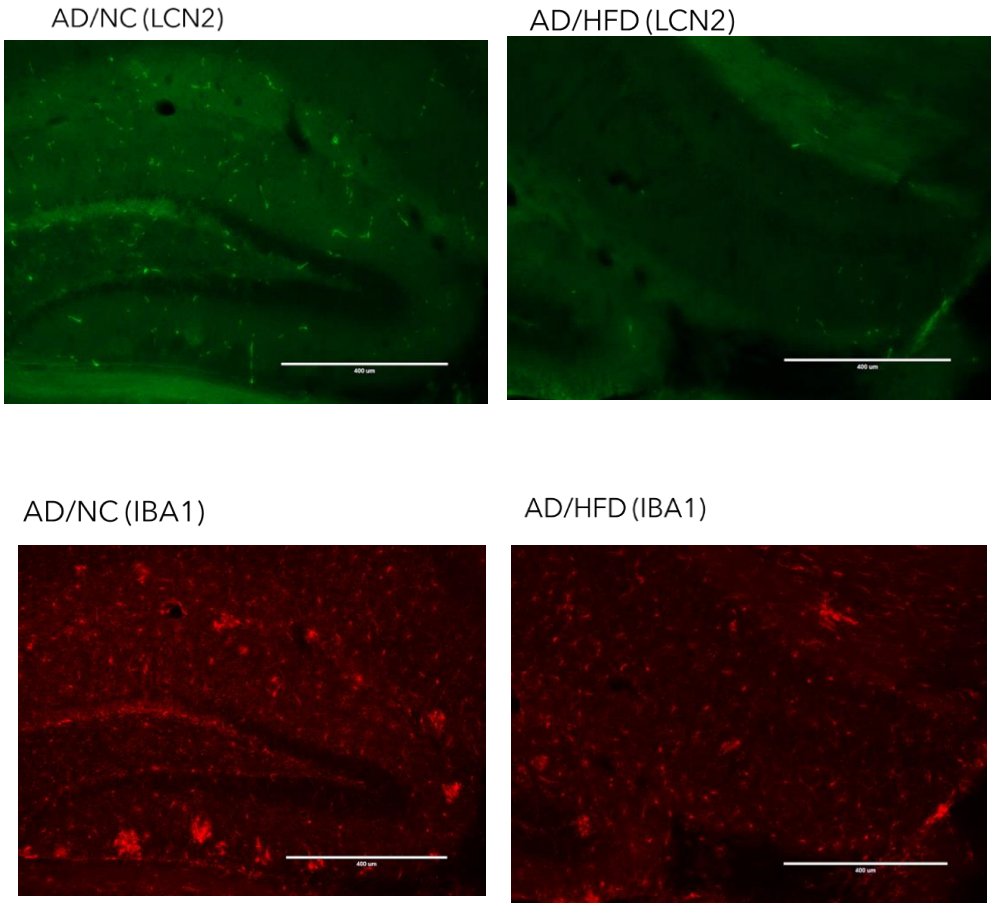


Figure 7 caption: Asterisks indicate the following p-values: *= P≤0.05, **=p≤0.01, ***= p≤0.001. N-values of 3 for each treatment group

Figure 8

Preliminary stains for AD mice



Discussion

Behavioral Performance

The goal of the present study was to investigate how diet would influence LCN2 expression and the effects that would have on inflammatory response within WT and AD mice in the brain regions of interest. By looking at the results from the behavioral assessment we see how changes in LCN2 expression influence cognitive performance in male and female groups based on their treatment diet. The WT mice did considerably better compared to the AD mice overall in both the male and female groups in exploring the maze. However, when looking within the treatment groups we can see the effects that diet has on cognitive performance. In the WT mice groups, there is an observed decrease in cognitive performance shown in a decrease in the WT/HFD group. In fact, the WT HFD group performance was like the AD/NC in terms of cognitive performance of exploring the maze when introduced to new areas of the maze. Despite the WT/HFD not being predisposed to developing AD symptoms, their performance was worsened considerably due to the HFD treatment only, indicating that even in healthy mice there can be cognitive decline because of diet.

LCN2 Expression

DAB staining for LCN2 markers in the AD and WT mice showed a significant difference in expression level between the two treatment groups. Overall, the WT mice expressed more LCN2 markers compared to the AD mice in all 3 brain regions of the tissues. This is primarily indicative of how the AD pathogenesis generally affects the LCN2 expression, especially when just looking at the NC treatment group of both the WT and AD mice. The AD NC animals have a significantly visual decrease in LCN2 graduation in the tissues which is also represented within the decrease in the densitometry value for that treatment group. Additionally, we can see how the

AD HFD treatment group has even less LCN2 expression compared to the AD NC treatment group. However, to see the impact of the HFD treatment, it is more evident in the difference between the WT treatment groups. Within the WT treatment groups, there is not a significant difference visually between the gradation of LCN2 in the tissue but through looking at the densitometry graphs we are able to see that there is indeed a difference, especially in the cortex region. The HFD treatment group in the WT mice are expressing significantly less LCN2 in the cortex region compared to the NC treatment group. The change in LCN2 between the AD mice is less noticeable due to the scale of the densitometry graph. However, the p-value test shows that there are significant differences in LCN2 expression, which are indicated through asterisk above brackets linking the treatment groups. There are similar trends observed in the hippocampus and hypothalamus in the AD mice, however the WT mice are showing a slightly different trend. The LCN2 expression in the hippocampus and hypothalamus of the WT/HFD mice don't show any significant decrease or increase in expression level compared to the WT/NC groups. One possible reason for this could be due to a difference in experiment group size between the two treatment groups indicated by the n value. A smaller size group might have resulted in less variable data collected from this treatment group. Especially since the preliminary LCN2 fluorescent staining indicated that there was a significant difference in LCN2 expression between the WT/NC and WT/HFD. Due to this, we would have to assess further experiments on the expression level of LCN2 in these brain regions and see whether diet treatment causes an impact on expression level or if there is some other explanation.

IBA1 Expression

The DAB staining for IBA1 expression in WT and AD mice showed similar trends of increase across all brain regions. Primarily the AD mice had more IBA1 expression compared to

the WT mice in general. This could be due to the AD pathogenesis and the associated increase in inflammatory response due to neuronal cell death. However, within the two cohort groups there is an increase in IBA1 expression due to changes in diet treatment. The WT/HFD and AD/HFD group had a significant increase in IBA1 expression compared to the NC group of each cohort group. This indicates that the exposure to saturated fatty acids over a long period of time does result in increased inflammatory response, even within a healthy mouse. Like the Cortex, the IBA1 expression in the hippocampus and hypothalamus follows a similar trend as observed. The AD mice primarily have a greater expression of IBA1 markers compared to the WT due to the AD pathogenesis triggering the inflammatory response in the mice CNS. However, we can see that even a change of diet within the cohorts once again results in a significant increase in IBA1 expression indicative of the effects that diet can have on the inflammatory response. In order to further assess the increased inflammatory response, future experiments could be focused on assessing activity of different cytokine of the microglial in these brain regions. The level of cytokines would indicate whether pro or anti-inflammatory cytokines are more upregulated.

Conclusion

As of currently based on the fluorescent co-stain images we can observe that there are regions of the brain areas where LCN2 and IBA1 are expressed in proximity of each other. This can be seen in the yellow stained areas in figure 4. Since LCN2 is expected to play a role in the inflammatory response system it is expected to interact with the immune cells of the CNS in some aspect. Through these staining we can see that LCN2 and IBA1 are in proximity of each other and might have interactions occurring during inflammatory response activity. It is important to note that while microglial are one of the primary immune cells of the CNS, astrocytes also play a role in the immune system. Therefore, during future experiments, it would

be crucial to compare co-localization staining between LCN2/IBA1 and LCN2/GFAP (marker for astrocyte) in order to understand which immune LCN2 acts more upon during an inflammatory response system. To further assess these interactions, utilization of DAPI staining, which stains the nucleus of cells, would allow for assessment of whether LCN2 is being expressed intracellular or extracellular of these immune cells. Furthermore, for future experiments we could do gene expression of different proteins and transcriptions factor based on LCN2 deficiency. Tracking which genes and proteins are downregulated when LCN2 expression is decreased would allow us to see which biological processes are impacted by this deficiency. Furthermore, since multiple proteins might contribute to one biological process, down regulation of these proteins could allow for investigation of new functions and roles of LCN2.

Significance

AD is a neurodegenerative disorder that currently affects a large portion of our population and is slated to increase as time goes on. However, there are still a lot of progressions and pathogenesis of the disorder that are still not fully elucidated making treatment option very ineffective. A lot of current AD treatments are focused on targeting the amyloid beta plaque deposits because they are seen as the causative factor of the neuronal loss and damage that patients experience (Castello et al., 2014). Infact no new treatment drug has been approved by FDA for treatment of AD since 2003 (Yiannopoulou et al.,2019). There are many possible reasons for why these disease-modifying treatments (DMTs) for AD have not been effective in treating the disorder. The most prominent reasons are due to late initiation of treatments during the course of AD development (Yiannopoulou et al.,2019). It has been shown that clinical symptoms of AD do not present themselves until after 20-25 years of their first amyloid plaques formation in the brain (Betthauser et al., 2022). For this reason, there can be significant buildup

of amyloid plaques even before patients start to develop clinical AD symptoms and make treatment very hard and ineffective due to the buildup of plaque formations. Due to these complications along with potential issues with the drug dosage and lack of understanding the complex pathophysiology of AD, there has been very little effectiveness in treating AD. However understanding how LCN2 and diet can work together to cause an early on-set of AD prognosis could be beneficial in shifting the treatment approach for AD patients. Since Diet is a significant risk factor for AD development and a metabolic stressor that can result in dysregulation of LCN2 over time, treatments focused on this would be to enable preventative measurements to lower the risk of AD onset. Patients would be able to put on a more regulated diet that consists of less saturated fatty acids to reduce increased low-grade chronic inflammation along with disruption of LCN2 expression. Furthermore, contingent upon further studies there might even be a possibility for more effective drug treatments that can be initiated early on apart from making changes to patients' diet and lifestyle.

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