

Intermittent fasting as a therapeutic strategy for alzheimer's disease: behavioral and molecular
evidence

by

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B.S., Minnesota State University, 2019

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AN ABSTRACT OF A DISSERTATION

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Department of Anatomy and Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Intermittent fasting (IF) has emerged as a promising non-pharmacological intervention to improve brain health and mitigate neurodegenerative disease progression. Alzheimer's disease (AD) is characterized by impaired metabolic functions that disrupt neuronal function and contribute to cognitive decline in aging populations. This dissertation examines IF as a therapeutic strategy for AD, focusing on behavioral parameters and molecular evidence. Chapter 1 provides a thorough review of the current literature from human and animal studies examining the impact of IF on brain health, brain aging, and neurodegenerative pathology with a particular focus on AD. Chapter 2 is an experiment into the effects of IF in young male and female rats, in which male IF feeding patterns resulted in lower body mass and lower mTOR expression in the cortex. The female IF feeding pattern, however, did not result in shifts in body mass or mTOR expression. This led to the next experiment (Chapter 3), which further explored the sex-specific adaptations observed in Chapter 2. Chapter 3 sought to determine IF-induced body composition shifts and metabolic adaptations. The animals maintained the same IF feeding patterns and mTOR cortical expression as in Chapter 2, and we now confirmed that IF did not induce differences in body composition in either males or females. Building on these findings, Chapter 4 sought to determine how IF-induced neuroprotective mechanisms influence the aged brain. Brain inflammation (via TNF alpha) was lower in aged IF females but not in males. Protein synthesis (via mTOR) remained unchanged following IF intervention. These results indicate that in aging brains, the benefits of acute IF may be driven by its anti-inflammatory effect rather than by modulation of the mTOR pathway. Chapter 5 evaluates the effects of IF in the transgenic AD model (TgF344-AD rats), focusing on cognitive functions and brain inflammation. IF attenuates neuroinflammation in AD brains. These findings highlight the potential of IF as a non-

pharmacological intervention to attenuate AD-related behavioral deficits and neuroinflammation. Collectively, these findings may provide insight into translating IF into clinical strategies aimed at preserving cognitive functions, reducing the prevalence of AD and improving the quality of life.

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Preface

Chapters 1,2 and 3 of this dissertation are currently in the peer-review process or have been published. Citations are listed below, and these articles are reproduced here with permission from the publisher.

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Chapter 1 - Introduction and literature review

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, memory loss, impaired communication, and widespread neuronal dysfunction and neuronal loss across multiple brain regions. According to the "*Alzheimer's disease facts and figures 2020*" report, AD currently affects approximately 6.5 million Americans aged 65 and older, a number projected to rise to 13.8 million by 2060. As the prevalence of AD continues to rise, there is an urgent need to identify effective and accessible interventions that can delay or prevent disease progression. Dietary interventions such as intermittent fasting have gained considerable attention for their metabolic and neuroprotective effects in AD.

Intermittent fasting (IF) is a dietary approach characterized by alternating periods of eating and fasting, with minimal or no calorie consumption for a period of time (1). IF was initially popularized for weight loss but has gained scientific attention for its ability to modulate molecular pathways involved in AD. These benefits are thought to be mediated through several mechanisms, first IF promotes metabolic switching from glucose to alternative energy substrates such as ketones and lactate. In AD, reduced expression of glucose transporters limits neuronal glucose availability and uptake; however, unlike glucose metabolism, ketone uptake remains preserved in AD brains. As a result, ketones such as β -hydroxybutyrate can serve as an alternative energy source to support neuronal function. Second, IF modulates nutrient-sensing pathways such as the mammalian target of rapamycin (mTOR) pathway, a key regulator of cellular energy balance. Dysregulation of the mTOR pathway, especially its hyperactivation, has been strongly correlated with increased amyloid β accumulation and progression of AD. Hyperactive mTOR promotes anabolic processes such as protein synthesis while suppressing

catabolic processes like autophagy. This imbalance contributes to the accumulation of toxic proteins, including amyloid- β and hyperphosphorylated tau, which are hallmark features of AD. Third, IF regulates both peripheral and central inflammation. In AD, impaired clearance of pathological proteins leads to sustained activation of glial cells, resulting in increased production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin -6 (IL-6). Additionally, adipose-derived cytokines, such as lipocalin -2, are upregulated in AD and have been shown to contribute to neuronal damage through crossing the blood-brain barrier. Increased adiposity is associated with increased systemic inflammation, which may further exacerbate disease progression. While IF is known to influence body composition and metabolic health, the underlying mechanisms by which peripheral adaptations contribute to central neuroprotection remain unclear.

Therefore, targeting these pathways through interventions such as IF may help restore the balance between catabolic and anabolic processes, reduce inflammation, and improve metabolic functions. In this dissertation, these pathways are examined across different conditions (wild type vs AD model) and age groups (young, middle-aged, and late-aged) to better understand IF-induced molecular adaptations in both sexes.

The overall objective of this dissertation is to investigate how IF affects cognitive functions and to identify the IF-induced mechanisms that regulate brain functions in both healthy and transgenic AD rat models. Specifically, this work examines (Chapters 2 and 3) sexual dimorphism in response to IF and its impact on the brain and skeletal muscles in young rats; the effects of IF on protein synthesis and inflammatory responses in peripheral tissues; and its impact on the brain (Chapters 4 and 5). Collectively, this research aims to elucidate how IF

influences brain function and to identify potential mechanisms underlying its neuroprotective effects.

Literature Review

Introduction

Intermittent Fasting (IF) is a dietary regimen that incorporates periods of fasting with or without overall calorie restriction. This regimen began as a trendy weight-loss strategy but has since gained significant research interest for its broad health benefits. IF has been shown to promote health and reduce the risk of various diseases, including cardiovascular disease (CVD), obesity, and type 2 diabetes (1–3). Beyond these metabolic benefits, clinical research suggests that IF may also support brain health by decreasing brain aging, reducing brain glucose and improving cognitive measures (4). There are several popular methods of IF, including 1) the 16/8 regimen, 16 hours of fasting followed by an 8-hour eating window (5), 2) the ADF (alternative day fasting), alternating between fasting days with zero or extremely low caloric intake and non-restricted feeding days (6) and 3) the 5:2 IF regimen, two days per week of reduced energy intake and normal intake on the remaining 5 days (7). While these methods differ, the key point is that they don't dictate where the calories come from (i.e., low-fat or low-carbohydrate); the most important part is the fasting period.

This chapter will examine contemporary evidence from human and animal studies examining the impact of IF on brain health, brain aging, and neurodegenerative pathology with a particular focus on AD. Specifically, it will begin with an understanding of brain metabolism during fasting, when the brain relies less on glucose and more on ketones and lactate, followed by a discussion of metabolic impairment in AD. The second section of this chapter will focus on

mechanisms of IF-induced neuroprotection, including neuroinflammation and autophagy. Lastly, the chapter will explore the role of peripheral-central signaling, particularly how adipose tissue and skeletal muscle communicate with the brain to enhance neuronal function and resilience against neurodegenerative pathology, with a specific emphasis on AD.

Metabolic mechanisms of IF-induced neuroprotection

This section will explore brain metabolism in health, in the fasted state, and the metabolic alterations present in AD.

The brain is a significant energy consumer; although the human brain accounts for only 2% of body weight, it uses approximately 20% of the body's total energy (10). This energy is primarily from the metabolism of blood glucose through glycolysis and the tricarboxylic acid (TCA) cycle. Glucose enters the brain via glucose transporters (GLUTs) located at the blood-brain barrier (BBB). Most glucose is transported into astrocytes via GLUT1, where it is metabolized into lactate before being transported to neurons via the lactate shuttle. Neurons can also directly take up glucose via GLUT3, although this is less common. In states of altered metabolism, such as neurodegeneration, these processes are disrupted, as discussed in subsequent sections.

Ketone Utilization as an Alternative Brain Fuel

Ketone bodies are produced in the liver through the oxidation of fatty acids. In the brain, they become an important alternative fuel source during periods of low glucose availability, such as fasting, exercise, or carbohydrate restriction, by entering mitochondrial oxidative phosphorylation to generate ATP. IF promotes the production of ketones such as β -

hydroxybutyrate (β OHB) and acetoacetate, which serve as alternative energy substrates when glucose metabolism is impaired (8). When glucose availability is low, fatty acids are broken down into acetyl-CoA, which accumulates and is converted to acetoacetate in the hepatic mitochondria, where it can be reduced to β OHB. Both of these ketone bodies circulate in the bloodstream and serve as alternative energy substrates for the brain.

Neurodegenerative diseases are characterized by reduced cerebral energy availability due to impaired glucose uptake (reduced expression of GLUT1 and GLUT3) and utilization (increased brain insulin resistance), as well as mitochondrial dysfunction (limiting ATP production), all of which contribute to cognitive and neuropathological impairments (9–11). Notably, preclinical studies have demonstrated that 20 weeks of ADF increases circulating β OHB levels and enhances brain ketone metabolism in 3xTg-AD mice, while also improving cognitive functions (8). ADF intervention also reduced AD pathology, specifically decreasing amyloid beta oligomers and tau hyperphosphorylation in the hippocampus (8). During prolonged fasting (31-41 days), ketones, such as β OHB, can provide approximately 60-70% of the total energy required for brain function (12,13). This metabolic shift is facilitated by increased expression of the ketone transporter, monocarboxylate transporter 1 (MCT1), at the blood-brain barrier, thereby enhancing the brain's capacity to utilize ketones (14).

Ketones as a Signaling Molecule

In addition to serving as an alternative energy substrate, ketone bodies cross the BBB and function as a potent signaling molecule in the brain. In vitro studies show that ketones such as 3-hydroxybutyrate induce brain-derived neurotrophic factor (BDNF) expression in cerebral cortical

neurons (15). BDNF is a key regulator of brain plasticity and neurogenesis (16) thereby linking ketone signaling to neuroprotection and cognitive resilience.

Lactate and the Astrocyte-Neuron Lactate Shuttle (ANLS)

Blood lactate serves as an important energy substrate for the human brain. In fact, up to 20% of cerebral energy is derived from lactate under eustress, such as during exercise or fasting (17). A study by Julie W Pan et al demonstrated that human brain lactate levels rise after fasting, reporting that non-fasted brain, specifically in the occipital lobe, lactate was 0.69 ± 0.17 mmol/L measured via high-field (4-T) MR spectroscopy, which increased to 1.47 ± 0.22 mmol/L after three days of fasting (18), indicating a critical role in maintaining brain energetics during fasting.

Within the brain, blood lactate serves as an energy substrate via the Astrocyte-Neuron Lactate Shuttle (ANLS). Astrocytes take up glucose from the bloodstream and metabolize it to pyruvate via glycolysis. Pyruvate is then converted into lactate by the lactate dehydrogenase enzyme (LDHA). Lactate is exported from astrocytes into the extracellular space via monocarboxylate transporters, MCT1 and MCT4, which are expressed in the astrocytes and adapted for lactate export within the brain. Neurons then take up lactate via MCT2, which has a high affinity for lactate. Once inside the neuron, lactate is converted back into pyruvate and oxidized in the mitochondria to produce energy (19). Disruption of MCTs impairs lactate transport, leading to neuronal energy deficits within the brain (20).

In addition to serving as an energy source, lactate promotes brain plasticity by modulating N-methyl-D-aspartate receptor (NMDAR) signaling. NMDARs are glutamate receptors and play key roles in synaptic plasticity and synaptogenesis. Lactate enhances neuronal signaling by elevating calcium influx and inward current following NMDA receptor activation

by glutamate (19). In that context, lactate acts as a signaling molecule that strengthens NMDARs. A study by Yang et al. shows that Lactate induces plasticity genes such as Arc, c-Fos, and Zif268 in neurons by potentiating NMDA signaling and downstream ERK1/2 signaling (21).

Metabolic Impairments in AD

Glucose transporters such as GLUT 1 (expressed primarily on endothelial cells at the brain barrier), GLUT3 (expressed primarily in neurons throughout the brain), and GLUT4 (expressed in hippocampal neurons throughout the brain) are essential for cerebral glucose uptake. Clinical studies show that deficiencies in GLUT1 and GLUT3 impair glucose metabolism and may contribute to neurodegenerative pathology. For example, a study by Liu et al. reported that in postmortem AD cortex, both GLUT1 and GLUT3 levels were decreased by 25-30% compared with controls, and these reductions correlated with increased tau hyperphosphorylation and neurofibrillary tangle density (22).

Preclinical studies in mice overexpressing amyloid β show that GLUT1 deficiency leads to early blood brain barrier (BBB) breakdown, cerebral microvascular degeneration (smaller capillaries in the brain, which can impair cerebral blood flow), and accelerated neuronal pathologies (23). In AD, GLUT1 reduction may result from endothelial cell damage, which can reduce GLUT1 at the BBB, or from amyloid beta toxicity (23). Glucose hypometabolism in AD occurs early and is driven largely by reduced expression of GLUT1/3 rather than by neuronal loss, thereby limiting neuronal glucose uptake and compromising energy production. In triple transgenic AD (3xTg-AD) mice, glucose hypometabolism preceded neuronal death and is associated with increased A β and tau phosphorylation, (24) suggesting that metabolic impairments exacerbate A β pathology and tau pathology, characterized by the accumulation of

A β into extracellular plaques and (11) intracellular aggregation of hyperphosphorylated tau into neurofibrillary tangles (25)

Beyond impaired glucose transport, disruption in brain insulin signaling also contributes to metabolic dysfunctions in AD. Unlike peripheral tissues, the majority of glucose uptake in the brain is insulin-independent and relies largely on GLUT1/GLUT3 glucose transporters; however, Insulin signaling in the brain remains critical for neuronal survival and energy homeostasis. A study by Schubert et al. shows that insulin receptors are required to inhibit apoptosis in neuronal cells (26). Administration of insulin directly into the brain inhibits food intake and body weight gain whereas deletion of neuronal insulin receptors leads to increased food intake (hyperphagic) and obesity (27,28). In addition, impaired neuronal insulin signaling has been linked to neurodegenerative pathology, as neuron-specific insulin receptor knockout mice exhibit increased tau hyperphosphorylation (26).

Clinical evidence shows that brain glucose uptake is impaired in patients with AD and mild cognitive impairment (MCI), whereas brain ketone uptake remains normal in both MCI and early AD (29). Under a normal mixed diet, plasma ketone levels are usually low (< 0.5 mM), accounting for less than 5% of brain energy metabolism (30). During a ketogenic diet, plasma ketone concentrations can increase 8-fold. After four days of ketogenic feeding, the cerebral metabolic rate of ketones can reach about 17% of the whole brain energy requirement in healthy adults (10). Suggesting that diet-induced ketones can serve as an alternative fuel to glucose for the human brain. In AD, glucose metabolism is impaired, possibly due to dysfunction of glucose transporters as previously described, and aging is associated with a marked reduction in cerebral glucose metabolism (31,32). This has been linked to cognitive decline, suggesting that ketones as an alternative fuel may be beneficial in age-related neurodegenerative diseases.

Non-metabolic mechanisms of IF-induced neuroprotection

This section will explore the proposed non-metabolic pathways whereby IF has been shown to promote brain health and prevent neurodegeneration.

IF reduces Neuroinflammation and Modulates Microglia

Microglia are the innate immune cells of the CNS and play a protective role by clearing debris via phagocytosis. This cellular process involves the engulfment and removal of damaged cells and cell debris (33). Specifically, microglia are also critical for the clearance of A β in the brain. The receptor Triggering Receptor Expressed on Myeloid cells 2 (TREM2) facilitates the clearance of aggregated proteins, bacteria, and apoptotic neurons, and low-density lipoproteins in the brain. Apolipoproteins such as ApoE/ApoJ activate the TREM2 and signal through its adaptor protein DAP12, which in turn activates downstream effectors, including phosphatidylinositol 3-kinase (PI3K) and Ca²⁺ signaling pathways (34). In AD, however, microglia become proliferative and morphologically altered, forming clusters around the A β plaques, but their containment capacity becomes reduced and overwhelmed as plaque deposition increases. When microglial containment fails, A β fibrils serve as a scaffold for further A β aggregation, generating plaques that spread into nearby neuronal tissue and exacerbate pathology. These reactive microglia also release inflammatory cytokines such as interleukin 1 (IL 1 β) and tumor necrosis factor alpha (TNF- α), which promote synaptic loss and exacerbate tau pathology (34). For example, overexpression of IL-1 β increases tau phosphorylation and reduces synaptic protein levels in neurons via activation of neuronal p38 mitogen-activated protein kinase (p38-MAPK) phosphorylation. p-38 MAPK is significantly increased in neurons treated

with recombinant IL-1 β within the microglia, and the inhibition of p38-MAPK significantly suppresses IL-1 β induced tau phosphorylation (35).

Disruption of intercellular lipid metabolism and transport capacity, leads to pathological accumulation of lipid droplets within microglia (36). Studies have shown that lipid-accumulating microglia, characterized by prominent lipid droplets, exhibit impaired phagocytosis and signaling through triggering receptors expressed on myeloid cells 2 (TREM2) (36). Importantly, IF significantly decreased the accumulation of lipid droplets within microglia, and these microglia with fewer lipid droplets may be involved in more phagocytic activity, thereby reducing A β deposition in the mouse brain with IF intervention (37). Similarly, Wu et al. demonstrated that six-month-old mice subjected to one month of fasting, during which food was provided for 8 h daily, followed by a 16-h fasting period exhibited improved cognitive function, along with microglial morphological changes characterized by enlarged cell bodies (soma), reduced process complexity, and fewer lipid droplet accumulations: adaptations that enhanced their phagocytic capacity and promoted engulfment and clearance of A β (37).

Brain inflammation occurs when immune cells respond to infection or other harmful stimuli, and it is primarily mediated by microglia and astrocytes. While acute inflammation can be protective, chronic neuroinflammation disrupts neuronal function and drives neurodegenerative diseases such as AD. Studies have found that stressed or apoptotic adipocytes recruit macrophages and secrete inflammatory mediators such as lipocalin 2 (LCN2) and galectin 3 (GAL3) (38). These mediators cross the BBB and bind neuronal receptors, activating astrocytic JAK/STAT3 and microglial TLR4 pathways and downstream inflammation. These adipose-derived inflammatory mediators, driving astrogliosis (which is proliferation and activation of reactive astrocytes characterized by enlarged soma morphology) (39) and microgliosis (which is

known as activated microglia and proliferative microglia within the CNS), lead to the release and secretion of cytokines that impair neuronal functions and synaptic plasticity-induced astrocytic LCN2 and microglial GAL3 protein expression in the brain hippocampus (38).

IF activates Autophagy and inhibits mTOR signaling

Autophagy is a catabolic process that degrades and recycles cytosolic components through the lysosomal-derived proteolytic pathway (40). This process involved several key stages: initiation (Unc-51-like autophagy activating kinase 1 (ULK-1 activation)), nucleation (beclin-1 activation), elongation (LC3II/ Atg5), fusion (with the lysosome), and final degradation via lysosomal proteolysis (41). When nutrients are unavailable, autophagy mediates cell breakdown and recycling unnecessary components such as proteins and organelles, whereas during the nutrient-sufficient stage, autophagy is suppressed and cells shift toward anabolic processes such as protein synthesis (42,43). Suppression of basal autophagy causes neurodegeneration by impairing autophagic flux, leading to toxic protein accumulation and neuronal loss (44). For instance, mice lacking autophagy-related protein 5 (Atg5), a protein required for autophagosome membrane elongation, developed progressive motor and behavioral defects after three weeks of age. They developed neurodegenerative changes, such as axon swelling, in the brain, specifically in Purkinje cells, the cerebral cortex, the hippocampus, and the cerebellum, by three months of age. Aging is also associated with a progressive decline in autophagy, and inducing cellular autophagy can reverse aging phenotypes. For example, a systemic overexpression of Atg5 in mice has been shown to extend lifespan by 17% (45).

Studies further revealed that mammalian target of rapamycin complex (mTOR), an upstream regulator of autophagy, is critical in regulating autophagy and provides protection

against aging and neurodegenerative diseases (46). mTOR plays a central role in regulating energy metabolism, and activation of the mTOR pathway inversely correlates with autophagy (42). mTOR functions in two distinct complexes, mTORC1 and mTORC2. mTORC1 (mammalian target of rapamycin complex 1) integrates nutrient, energy, and growth factor signals to promote anabolic processes while suppressing catabolic processes, whereas mTORC2 (mammalian target of rapamycin complex 2) regulates cell survival and cytoskeletal organization (46). mTORC1 is active in nutrient-rich environments (fed stage), during which autophagy remains at a basal level. In contrast, nutrient deprivation decreases mTORC1 activity and upregulates autophagy (47).

In AD, hyperactivation of mTOR signaling has consistently been observed (47,48) in the brain. Babygirija et al. demonstrated that in 3xTg-AD mice (which express human gene mutations in APP, pSEN1, and MAPT), CR combined with fasting reduced phosphorylation of mTOR downstream targets, including eukaryotic translation initiation Factor 4E Binding protein 1 (P-4EBP1)(49). Reduced phosphorylation of 4E-BP1 inhibits protein synthesis because unphosphorylated 4E-BP1 binds tightly to eukaryotic initiation factor 4E, a cap binding protein, thereby preventing initiation of translation.

Cellular energy balance is frequently monitored by adenosine monophosphate-activated protein kinase (AMPK), an upstream regulator of mTOR. AMPK closes pathways involved in energy consumption, such as mTOR activation. AMPK activates a protein known as the Tuberous Sclerosis Complex (TSC), and loss of TSC1/ TSC2 inactivates the TSC protein complex and leads to hyperactivation of mTOR (50,51). Hyperactivation of mTOR (losing the inhibitory effect of mTOR) leads to the inactivation of proteins responsible for autophagy, such as ULK1 (initiates autophagy), Light Chain 1 (LC3I, elongation of the autophagosome), and

Light Chain II (LC3II, maturation of the autophagosome) (52). Typically, activation of AMPK, a sensor of low cellular energy, inhibits mTORC1, thereby reducing anabolic processes and promoting catabolic processes (52). However, AD brains exhibit concurrent activation of both AMPK and mTOR in neurons with tau pathology and oxidative damage (48). This coactivation suggests a disruption in the usual inhibitory link between AMPK and mTOR, thus allowing mTOR to remain hyperactive despite AMPK signaling and potentially contributing to impaired catabolic processes. Furthermore, our own work in Fischer-344 rats introduced to ADF demonstrated that cortical mTOR reduction occurred independently of TSC (53), suggesting an alternative mechanism of regulation. For example, AMPK can directly inhibit mTORC1 by phosphorylating the mTOR binding partner raptor on Ser 722/ 792 (54).

Brain crosstalk mechanisms of IF-induced neuroprotection

This section will explore the proposed pathways through which IF promotes brain health, with a focus on skeletal and adipose tissues as mediators of these neuroprotective effects.

Brain-Adipose Crosstalk: IF improves leptin sensitivity and adipokine balance

Leptin is a hormone/adipokine secreted by adipose tissue that plays a crucial role in regulating hunger, body weight, and metabolism (55). A study by Annweiler et al. suggests that a non-linear U-shaped relationship exists between circulating leptin levels and cognitive performance in older individuals, indicating that both lower and higher leptin concentrations are associated with impaired cognition (56). Importantly, leptin crosses the BBB and influences hippocampal synaptic transmission by regulating NMDA (N-methyl-D-aspartate) receptors, a class of glutamate receptors that mediate excitatory neurotransmission in several brain regions.

Leptin enhances NMDA receptor activation and promotes hippocampal long-term potentiation (LTP), a long-lasting enhancement of synaptic communication (57). Indeed, animals with impaired leptin receptor signaling show disrupted hippocampal plasticity characterized by weakened LTP (58).

Leptin also has a dual role in inflammation; in obese individuals, leptin acts as a pro-inflammatory mediator. A study by Poosri et al. showed that individuals with obesity had significantly higher leptin levels, and these elevated leptin levels correlated positively with TNF-alpha and IL-6, both pro-inflammatory mediators (59). Leptin has been shown to activate immune cells to secrete pro-inflammatory mediators via activation of Janus kinase 2/activator of transcription 3 (JAK2/STAT3) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways (60). In addition, Poosri et al showed that high total fat intake increased leptin levels, contributing to diet-driven hyperleptinemia (59). Beyond its pro-inflammatory role, Leptin can also exert anti-inflammatory effects, such as stimulating innate immunity by activating natural killer cells, which play a key role in identifying and eliminating virus-infected cells. Lo et al. showed that leptin signaling is critical for NK cell development, as leptin-deficient mice exhibit reduced bone marrow NK cell numbers due to increased apoptosis (61). In addition, recombinant leptin can rescue NK cell survival in vitro. Supporting this, Tian et al reported that impaired leptin signaling and NK cell activation, further implicating leptin as a key regulator of NK cell function (62). HFD-fed mice also showed that 10 days of HFD feeding causes NK cell dysfunction and increased susceptibility to viral infection (63), suggesting that diet may affect NK cell regulation and that leptin likely plays a crucial role in immune regulation.

Several studies have shown that IF improves leptin sensitivity in preclinical models (64). A 16 h fast and an 8-hour feeding regimen improved leptin sensitivity in obese mice, along with metabolic benefits, such as improved glucose metabolism, thermogenesis, and reduced fat mass; these metabolic benefits were absent in leptin-deficient mice (64). Conversely, mice fed a high-fat diet and subjected to IF (24 hours of feeding and 24 hours of fasting for four weeks) exhibited decreased leptin levels and reduced leptin gene expression (in both hypothalamus and epididymal fat), while improving adiponectin levels, another adipose-derived adipokine that is strongly linked to anti-inflammation (65,66).

Brain-Skeletal Muscle Crosstalk: IF promotes skeletal muscle metabolic adaptations and myokine signaling

Lifestyle interventions such as exercise promote the release of myokines, including brain-derived neurotrophic factor (BDNF), into the systemic circulation, where they act on the brain to improve brain health and synaptic plasticity. Notably, a study by Yang et al. provides evidence that muscle-specific BDNF plays an important role in energy metabolism during fasting, as BDNF increases mitochondrial respiration and lipid oxidation in muscles (67). In addition, 24-hour fasting elevated *Bdnf* expression in gastrocnemius, EDL, and tibialis anterior in female mice; however, no changes in BDNF expression were observed in fasted males, suggesting that muscle-derived BDNF expression in response to fasting may be sex-specific (67). It has been shown that BDNF knockout mice undergo nutrient deficiency and impaired energy metabolism, while promoting muscle weakness as evidenced by reduced O₂ consumption. These animals also experienced lower energy expenditure, impaired autophagy and a higher respiratory exchange ratio (RER)(67).

The paradigm that reducing calorie intake improves mitochondrial health is widely accepted; for example, under dietary restriction, AMPK upregulates peroxisome proliferator-activated receptor gamma coactivator (PGC-1 α) in skeletal muscle (68). PGC-1 α is a master regulator of mitochondrial biogenesis and functions. Because mitochondrial impairment is a hallmark of AD, this pathway may be particularly relevant for preserving metabolic health and muscle mass with IF. Previous studies have shown skeletal muscle loss during fasting, yet AMPK appears to be essential for metabolic adaptations (69). In AMPK knockout mice, prolonged fasting led to hyperglycemia and hyperketosis, along with impaired autophagy markers, including ULK1. This study indicates AMPK is required to maintain blood glucose during prolonged fasting, which may be critical for glucose signaling in the brain (69). PGC-1 α has also been shown to prevent activation of the catabolic system and protect against disuse muscle atrophy (70). Together, these findings suggest that fasting-induced AMPK activation in skeletal muscle exerts protective effects primarily through the AMPK-PGC-1 α cascade (70).

Interestingly, Liu et al reported that a 10-week IF regimen significantly improved motor coordination and muscular strength compared with aged controls (71). Immunohistochemistry revealed increased expression of myelin-related proteins within axons, suggesting enhanced myelin integrity and repair. These findings indicate that IF not only preserves muscle functions but also supports neuromuscular health, ultimately mitigating age-related motor and cognitive decline (71).

Evidence of fasting's beneficial effects in AD from preclinical models

Transgenic preclinical models (APP/PS1, 3xTg) provide strong evidence that fasting mitigates AD pathology. In rodent models, fasting has been shown to reduce amyloid

accumulation (31,49,72). diminish tau pathology (8,49), and lower brain inflammation (37,49) A recent study in 3xTG-AD mice demonstrated that while both calorie restriction (CR) without fasting (AL feeding regimen but 30% calorie restricted diet) and with fasting (reduced calorie intake by 30% and these animals were once per day) reduce amyloid plaque burden in females, only CR with fasting improved cognitive function, reduced neuroinflammation, and reduced both amyloid plaque burden and tau pathology (49).

Similarly, IF has been shown to enhance phagocytic activity and reduce lipid droplet accumulation in microglia, thereby ameliorating amyloid deposition in APP/PS1-IF mice.³⁷ These findings suggest fasting contributes to not only through systemic metabolic improvements but also by directly modulating AD pathology (49). Mechanistically, fasting suppresses mTORC1 signaling, (49) promotes autophagy (40,72), and upregulates BDNF (49,73). In one study, male 3XTg-AD mice subjected to Alternative Day Fasting (ADF) for 20 weeks showed significantly increased systemic BOHB, enhanced brain ketone metabolism, restored ATP production, and reduced ROS levels, thereby counteracting mitochondrial bioenergetic deficits commonly observed in AD (8). Together, these adaptations contribute to neuroprotection against AD.

Behavioral studies provide further support. Six-month-old App/PS1 mice exposed to IF for one month showed significant improvements in cognitive performance (37). Likewise, 12-month-old 3xTg mice maintained on CR with a fasting period outperformed AL-fed controls in the Novel Object recognition (NOR) task, a measure of recognition memory (49). Yu et al. reported that IF also improved anxiety-like behavior and hippocampal plasticity, while reducing amyloid oligomers (8). Notably, these benefits occurred without adverse effects on muscle function or exercise tolerance (8). Furthermore, when old male 3xTg-AD rats underwent a

prolonged early-life fast and were tested at 17 months, they exhibited improved spatial memory in the memory test; however, IF failed to reduce both hippocampal tau and A β levels at this advanced age (74). This suggests that the timing of IF may be critical, and optimal duration and onset remain unresolved even in preclinical models.

Additionally, studies show that three months of fasting promotes neurogenesis (75) and neuronal differentiation and maturation (76), likely mediated by enhanced BDNF signaling. Specifically in the hippocampal dentate gyrus in 3xTg-AD mice, fasting upregulates glycogen synthase kinase 3 (GSK-3) signaling, which is essential for brain development. Interestingly, fasting also reduced insulin signaling while improving adenosine monophosphate-activated protein kinase (AMPK) signaling (77). Majid et al proposed that one aspect of AD metabolic dysfunction involves hyperactivation of the AMPK-mTOR metabolic axis, leading to abnormal neuronal energy metabolism and AD pathology (48). They concluded that both AMPK and mTOR are highly upregulated in AD brains, suggesting that the inhibitory link between AMPK and mTOR is disrupted (48). Whether fasting-induced AMPK activation is a direct regulatory mechanism or a secondary effect of AMPK-mTOR dysregulation in AD remains unknown. Nevertheless, most studies consistently report that IF suppresses mTOR signaling. In peripheral tissues, AMPK activation is essential for metabolic adaptation during fasting (69). These findings underscore the importance of examining the mTOR-AMPK metabolic axis as a regulator of both systemic and brain energy during fasting.

Evidence of fasting beneficial effects in AD and mild cognitive impairment from clinical populations

IF has been shown to improve metabolic health in patients with metabolic diseases (2,78). Human evidence supports improvements in metabolic functions. For example, obese, overweight, and prediabetic individuals subjected to ADF significantly reduced body weight and body mass index compared to controls, whereas 16/8 and 20/4 time-restricted feeding regimens did not produce significant changes (79). A study by Anna Obermayer found that IF is a safe dietary approach for patients with type 2 diabetes receiving insulin therapy. They have improved glycemic control and reduced daily insulin dose (78). Similarly, patients with metabolic syndrome who followed a two-day modified IF protocol for eight weeks significantly reduced fat mass, oxidative stress, and inflammatory cytokine levels, and improved vasodilation (80). Individuals introduced to IF also showed favorable alterations in gut microbiota, all of which are linked to reduced cardiometabolic risk (2). In healthy adults, an 18-hour daily fast for one month reduced body weight, BMI, and fat-free mass in both sexes, while lowering resting energy expenditure (REE). Notably, the reduction in REE was greater in females than in males (8.1% vs 4.6%) (81). This underscores the importance of measuring sex as a variable in IF studies.

While rodent models strongly support IF as a strategy to reduce AD pathology and improve behavioral outcomes, the human evidence is limited but is steadily growing. Several clinical trials suggest metabolic and behavioral alterations with fasting. For example, obese older adults undergoing 5:2 IF for eight weeks showed reduced neuronal insulin resistance and improved memory functions (4). However, despite weight loss and memory improvement, the cerebrospinal biomarkers of AD pathology (amyloid beta, tau, and neurogranin) were not significantly altered (4). A study by Bartholomew reported that adults engaged in a low-

frequency IF regimen (24 hours twice a week for four weeks, then once weekly for 22 weeks) reduced metabolic syndrome score, but showed no improvements in cognitive markers (82). Faris et al. investigated the effects of Ramadan fasting on circulating inflammatory and immune markers in 50 healthy adults. Blood samples were collected before Ramadan and in the third week of fasting, showing significant reductions in proinflammatory cytokines, including IL-1 β (78%), IL-6 (57%), and TNF- α (71%), as well as decreases in body mass, BMI, and body fat percentage (83). These findings suggest that three weeks of intermittent fasting attenuates systemic inflammation, providing a potential mechanism by which fasting may lower risk for chronic neuroinflammation.

Conclusions and future directions

IF may enhance brain health by modulating nutrient-sensing pathways, such as mTOR, which is a central regulator of energy metabolism. In AD, hyperactivation of the mTOR pathway has been shown to disrupt the balance between the anabolic and catabolic processes, suppressing autophagy and promoting the accumulation of pathological proteins. Instead of protecting neurons, sustained mTOR activation drives multiple disease pathologies. By downregulating mTOR pathways through intermittent fasting (IF) during the early stages of brain pathology, IF may restore this balance and improve brain health. This suggests that targeting the mTOR pathway may be a promising strategy to improve brain health in AD with IF intervention.

In addition, accumulation of amyloid beta and tau is associated with microgliosis and astrogliosis, which promotes the release of pro-inflammatory cytokines and contributes to neuroinflammation. Preclinical studies also demonstrate that IF decreases glial activation and proinflammatory markers, thereby reducing neuroinflammatory signaling in the brain.

Beyond the brain, peripheral tissues such as adipose tissue and skeletal muscle play a critical role in mediating the brain's benefits from IF. IF has been shown to reduce inflammatory adipokines in both adipose and brain tissues. Furthermore, IF has been shown to preserve skeletal muscle mass and enhance myokine (BDNF) release. This coordinated crosstalk may contribute to systemic improvements in metabolism and supporting brain health in AD.

Despite strong preclinical evidence, important gaps remain in translating these mechanisms to AD progression and therapeutic strategies. Even if intermittent fasting is not widely used as a clinical therapeutic intervention, the mechanistic insight gained from these studies has significant translational value. Understanding how IF modulates nutrient-sensing pathways, inflammation, and metabolic functions can inform the development of targeted therapeutic approaches. These include pharmaceutical agents, such as rapamycin and other mTOR inhibitors, designed to restore brain health and diminish disease progression. By targeting these underlying pathways via IF has the potential to slow disease progression and improve overall brain health in individuals with AD.

Future work should examine IF intervention across different brain stages (young, middle-aged vs. aged), explore sex-specific responses, and investigate mechanistic pathways in both the brain and peripheral tissues. Elucidating how IF interacts with age, sex, and genetic risk will strengthen the mechanistic rationale for clinical trials and identify personalized interventions that prevent AD progression.

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Chapter 2 - Sexual dimorphism in response to intermittent fasting and its impact on the brain

Abstract

Background: Intermittent fasting (IF) has been shown to improve cognitive functions in rodent models. In addition, mammalian target of rapamycin (mTOR) expression was lower in IF males. We hypothesized that IF would attenuate mTOR signaling while improving memory and strength in healthy rats. Methods: 10-week-old male and female Fischer-344 rats ($n = 48$) were randomly assigned to four groups: ad libitum (AL)-male, IF-male, AL-female, IF-female. The IF group had full access to food on an every other-day basis for 10 weeks. After 10 weeks, memory and strength were assessed. Following testing, brain and skeletal muscles were isolated, weighed, and mTOR pathway proteins were quantified via Jess Simple Western. Prism GraphPad was used to determine group differences (one-way ANOVA) and correlations (Pearson) with alpha 0.05.

Results: After 10 weeks of IF, only males experienced IF-induced declines in body mass ($p < 0.0001$) and average daily food intake ($p = 0.014$). Further, IF males displayed better memory performance compared to AL counterparts ($p = 0.0186$). Brain mass was lower in both males ($p = 0.0038$) and females ($p = 0.0205$) compared to their respective AL counterparts, while hippocampal mass was maintained. Weight loss in males correlated with lower expression of mTOR in the brain cortex ($r = 0.546$, $p = 0.006$), and mTOR was significantly lower in IF males cortex (vs. AL males, $p = 0.0003$) and skeletal muscle (EDL: vs. AL males, $p = 0.0377$).

Conclusions: In male IF rats, average food intake was lowered, and mTOR expression was

lowered in the brain cortex. In contrast, these effects were not observed in females, highlighting a sex specific response to IF.

Background

Among dietary restriction strategies, calorie restriction with adequate nutrition has long been the gold standard of health span and lifespan extension across species (1). Nearly a century ago, the seminal paper by McCay and colleagues found that animals fed a food-restrictive diet lived significantly longer than their ad libitum (AL) fed littermates (2). In rodents and nonhuman primates, restrictive diets such as a 10–40% calorie restriction (CR) have been shown to reduce the incidence of diabetes, cancer, cardiovascular disease, and neurodegeneration (3,4,5). Similarly, human trials like CALERIE and CRONies have reported benefits such as reduced body weight, and lower incidence of cardiovascular disease and cancer (6,7). However, CR is also associated with several adverse effects, including hypotension, osteoporosis, impaired wound healing, reproductive irregularities, and increased reports of psychological conditions (8). Considering these adverse effects, more work is needed to understand the link between CR and longevity.

Recently, Acosta-Rodriguez et al. (2017) suggested the need to reevaluate CR research, highlighting that animals on CR often engage in self-imposed fasting periods (9). They found that animals given a restrictive calorie diet drastically shortened their food intake duration and, consequently, previous CR studies should be reevaluated to assess for fasting periods (9). Thus, whether health benefits in previous CR studies were mediated by caloric intake or fasting periods remains unclear.

Intermittent fasting (IF), which involves time-restrictive eating with or without calorie restriction emerged as a form of dietary restriction with recent popularity. IF is known to improve healthy aging (increased autophagy, enhanced mitochondrial function) (10,11,12), obesity (13), insulin resistance (13,14), dyslipidemia (15), and inflammation (11) in both human and animal models such as rodents. IF has also been shown to reduce several disease pathologies in animal models, i.e., diabetes, cardiovascular disease, cancer, and neurological disorders (16). Indeed, IF has been found to improve memory and cognition in models of epilepsy, Alzheimer's disease, multiple sclerosis, Parkinson's disease, ischemic stroke, autism spectrum disorder, and anxiety (17). However, the underlying molecular mechanism of the effects of IF on memory and cognition in healthy, neurotypical brains remains unclear. While research suggests that IF improves brain function in the presence of disease, its effects on healthy brain function are not well understood (18).

One potential mechanism of IF's benefits is the inhibition of the mammalian target of the rapamycin (mTOR) signaling pathway (19,20,21). mTOR is a protein kinase that controls cell growth and proliferation, protein synthesis and autophagy throughout the body, including the brain. In the brain, mTOR is required for normal neurological function but in neurodegenerative diseases such as Alzheimer's disease (AD) the mTOR pathway is hyperactivated and results in decreased autophagy, impaired glucose metabolism, and dysfunctional mitochondria. mTOR's inhibitor, rapamycin, has shown promise in AD animal models by decreasing plaques and tangles and improving cognition (22). IF has been shown to regulate mTOR signaling while activating catabolic pathways such as autophagy. During fasting, mTOR phosphorylation is suppressed due to a lack of nutrients and insulin signaling (23). The absence of mTOR activates cellular processes such as autophagy, which aids in removing cell debris (24). Once the food is

reintroduced, mTOR signaling is activated and plays an important role in the growth and repair of tissues (25).

While historically CR has been the most recognized life-promoting dietary intervention, the fasting periods present in CR studies should also be further evaluated. IF has recently become a popular diet trend for its positive impacts on aging, metabolism, and brain function.

Additionally, there is evidence that IF may have sex-specific effects, with differences observed between males and females. However, the lack of control in nutrition trials (i.e., self-reporting, self-administering) has limited our understanding of these differences. We hypothesized that IF intervention in early brain stages improves memory performance and inhibits the mTOR pathway. This experiment will investigate the effects of IF-induced mTOR inhibition in both male and female healthy young animals, focusing on their brain and skeletal muscle tissues.

Materials and Methods

Ethical approval

All methods carried out in this experiment were performed in accordance with the guidelines for the Care and Use of Laboratory Animals under the approval of the Institutional Animal Care and Use Committee of Kansas State University (protocol code 4466 and date of approval Oct 9, 2020). This study has been reported following the ARRIVE guidelines (26).

Experimental design

Forty-three, 10-week-old male and female Fischer-344 rats (SAS Fischer rat, Charles River, Wilmington, MA) were randomly assigned into two interventions (male/female ad-libitum (AL) or intermittent fasting (IF)) and housed 3–4 animals per cage, on a 12:12 light: dark cycle in the

Kansas State University (K-State) Comparative Medicine Group facility accredited by AAALAC International (000667), OLAW (A3609-01) and USDA (48-R-0001) assured Food was provided each day at noon and removed at noon the following day. Group numbers were as follows: female AL n = 7, female IF n = 12, male AL n = 12, male IF n = 12. Based on preliminary data (memory, and protein expression) an n of 10 is required to detect significant differences in the primary outcomes (power of 0.8 at $p < 0.05$ level) (46). The ad-libitum group had 24/7 access to food (LabDiet 5001, protein 29%, fat 14%, carbohydrate 57%) and water while the IF group had 24/7 access to water and full access to food every other day (i.e., alternate day fasting) for ten weeks. After 10 weeks of IF (20 weeks of age), spatial memory and muscular strength were assessed. Following behavioral tests, animals were euthanized under isoflurane and decapitated, with tissue masses documented. The brain (hippocampus and prefrontal cortex) and skeletal muscle (soleus and extensor digitorum longus (EDL)) tissues were sectioned and snap-frozen in liquid nitrogen for subsequent homogenization and protein analysis via western blot.

Morris water maze

Spatial learning and short-term memory were assessed using the Morris Water Maze (MWM) (27). Briefly, this MWM protocol consisted of a single day of learning trials, and one test trial conducted one hour later. During learning and test trials, the pool (180 cm in diameter) was filled with water to a level 1 inch above the platform, and nonfat dry milk was added to make the water opaque. Under a red light and at a water temperature of $25 \pm ^\circ\text{C}$, the animals were placed in the pool and allowed to find the hidden platform for 60 s, using visual clues. Four learning trials were conducted consecutively, and the time to reach the platform was recorded. Each animal underwent four consecutive learning trials on the same day, with each trial starting from a

different quadrant. During the 60-minute trials, animals were allowed to swim freely and locate the hidden platform. The animals were directed to the platform if they were unable to find it within 60 s on their own. Upon completion of the last learning trial, the animal was dried and returned to their cage. One hour following the final learning trial, a test trial was performed. In the test trial, the animal began in the quadrant opposite the platform to assess short-term memory. The latency time during the test trial was recorded and used for analysis, as video tracking was not available during this experiment.

Grip strength

Due to IF's impact on skeletal muscles, we measured changes in skeletal muscle strength with a four paw grip strength protocol (28). To do this, animals were removed from their cage by grasping the base of the tail with the thumb and forefinger. Then, the animals were gently lowered over the top of the grid so that all four paws were positioned to grasp the grid. Animals were allowed to attach to the grid properly before being pulled away. The torso was kept horizontal as the rat was pulled back steadily until the grip was released. When the animals released the bar, the force was recorded as the strength of the grip, and the maximal value over the 3 trial assessments was analyzed. We repeated the procedure three times with a 2-min rest period between each trial.

Tissue harvest

The animals were placed under anesthesia with 3–4% isoflurane in an induction box. Absence of response to tail and toe pinch reflexes ensured they were fully anesthetized. Animals were decapitated with a large rodent guillotine. The frontal cortex, the whole hippocampus and

skeletal muscle tissues were removed to be used later to investigate molecular mechanisms induced by IF. For size-based comparison, the hippocampus, EDL, and soleus were isolated and weighed. These harvested tissues were snap-frozen in liquid nitrogen and stored at -80 °C until homogenized.

Homogenization procedure

Samples were homogenized using RIPA (Radio-Immunoprecipitation Assay) buffer. Frontal cortex, hippocampus, and skeletal muscles were cut and weighed to 20 mg and transferred to a 2 mL glass Dounce homogenizer. 25 μ RIPA buffer per 1 mg of tissue was added, and samples were homogenized (4–6 steps with the Dounce pestle for brain tissue and 8–10 steps for skeletal muscle). Thick, rounded glass rods and heavy-walled test tubes were used for manual homogenization. The homogenized samples were transferred into an intermediate microcentrifuge tube and incubated on a rocker (4 °C) for 2 h. Homogenized samples were centrifuged at 17000 x g (RCF) for 20 min at 4 °C. Then supernatant was subsequently transferred into a microcentrifuge tube, and protein concentration was determined using the Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA), following the manufacturer's instructions. Samples were stored at -80 °C for future Western blotting analysis.

Western blotting procedure

Following homogenization, western blotting was performed using Jess Protein Simple automated western blotting (Bio-Techne, Minneapolis, MN). The following primary antibodies were used to detect target proteins: mTOR (Santa Cruz, sc-517464, mouse, 1:100) and tuberous sclerosis complex 1 (TSC-1 Abcam, ab200728, mouse, 1:10). For detection of primary

antibodies, a 20X anti-rabbit HRP conjugated (protein simple, 106872, 20x) secondary antibody was used according to the manufacturer's instructions. In the Jess automated system, samples were mixed with sample buffer and fluorescent Master Mix and then denatured at 95 °C for 5 min. The pre-filled separation module provided by Bio-Techne was used, with a separation time of 25 min. Primary antibodies were incubated for 30 min, followed by a 30-minute incubation with secondary antibody at room temperature. Standard reagents, including the antibody diluent, wash buffer, and chemiluminescent substrates, were supplied by Bio-Techne.

Statistical analysis

Group behavioral differences and tissue masses were analyzed via one-way ANOVA with Prism GraphPad Prism software (version 9.5.0 (730)). Protein expression data were collected via Compass software, generating area under the curve values (AUC). The group differences were analyzed with a one-way ANOVA. Statistical significance was also determined using two-tailed t-test. Over time, body mass and food intake data were analyzed via mixed-effect analysis. Pearson correlation was performed with GraphPad Prism software. All statistical significance was set as $p < 0.05$. All the data were presented as mean $\pm$ SE.

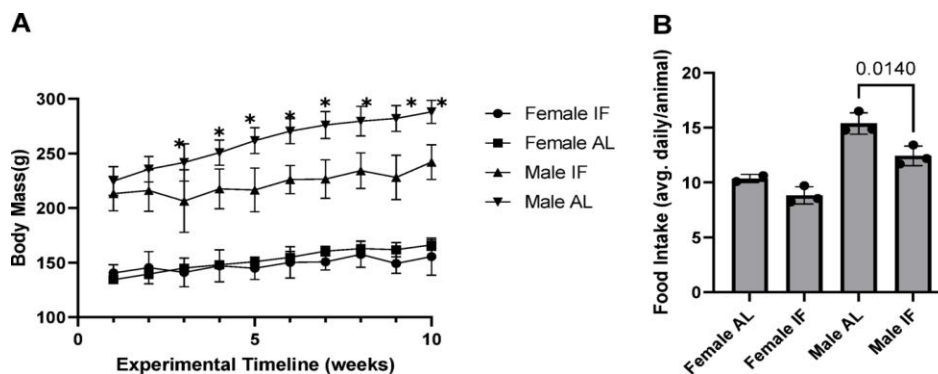
Results

Body weight and food intake changes during intermittent fasting in male and female rats

Figure 2.1A shows the body weight changes over the 10-week study for all experimental groups. Male IF animals weighed significantly less over the course of the experiment compared to male AL ($p < 0.0001$). Interestingly, the female IF group did not have significantly reduced body mass compared to the female AL. Figure 2. 1B shows the average animal food intake during the 10th

intervention week. The data points indicate the cage-per-animal intake. Due to group housing, individual animal intake was not available, and group housing was preferred to allow social interactions. Data analysis of food consumption showed a significant reduction in the male IF group compared to the male AL group ($p = 0.014$). Females did not show IF-induced declines in food intake compared to their AL counterparts. As expected, body mass change was significantly correlated with food intake in both males ($r = 0.92$, $p < 0.0001$) and females ($r = 0.875$, $p < 0.0001$)

Figure 2-1 Body weight and food intake changes during intermittent fasting

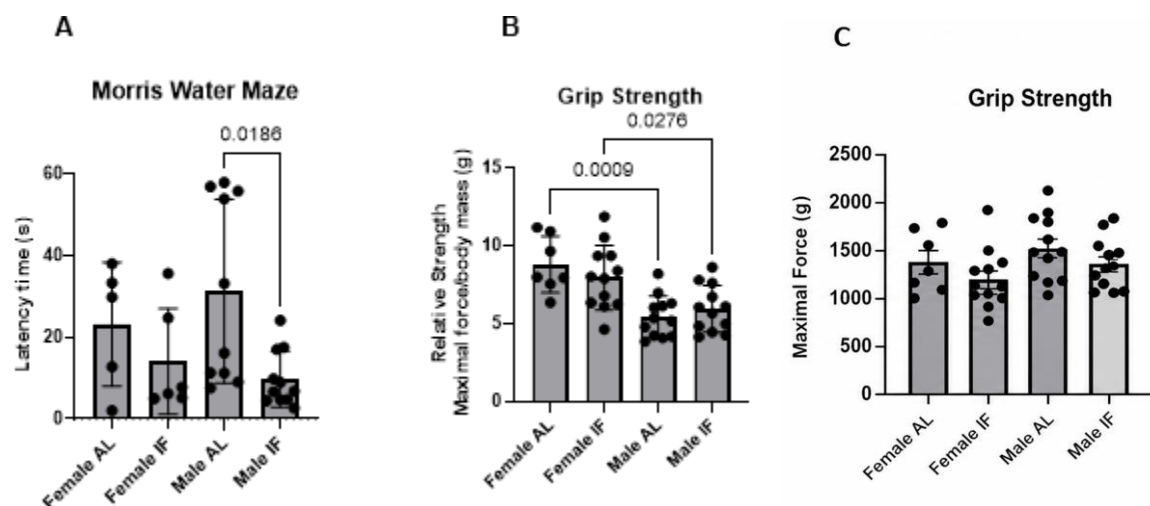


(Figure 2.1A) The alterations of body weight shift in females and males following 10 weeks of IF treatment. Intermittent fasting slowed age related growth rate in male rats ($p < 0.05$). Females did not display IF-induced declines in weekly body weights. (Figure 2.1 B) Food intake during the final intervention week as measured by cage and divided by the number of animals in the cage. Data points represent each cage's average intake. Male IF animals decreased their food intake ($p = 0.014$) while females did not.

Effects of Intermittent Fasting on memory performance in Fisher-344 rats

Figure 2.2 shows the effects of IF on rats' memory and grip strength. Male IF groups significantly decreased the latency time in the Morris Water Maze (MWM) (vs.AL males, $p = 0.0186$) (Fig. 2.2A). A shorter latency time indicates better memory and learning, as the male IF animals were more quickly able to remember the platform location after the training. However, no significant effect on memory was observed in the females (Fig. 2.2A). Max grip strength (Fig. 2.2B) was significantly higher in females compared to their male counterparts. Grip strength did not change after fasting.

Figure 2-2 Memory and motor functions in young healthy rats

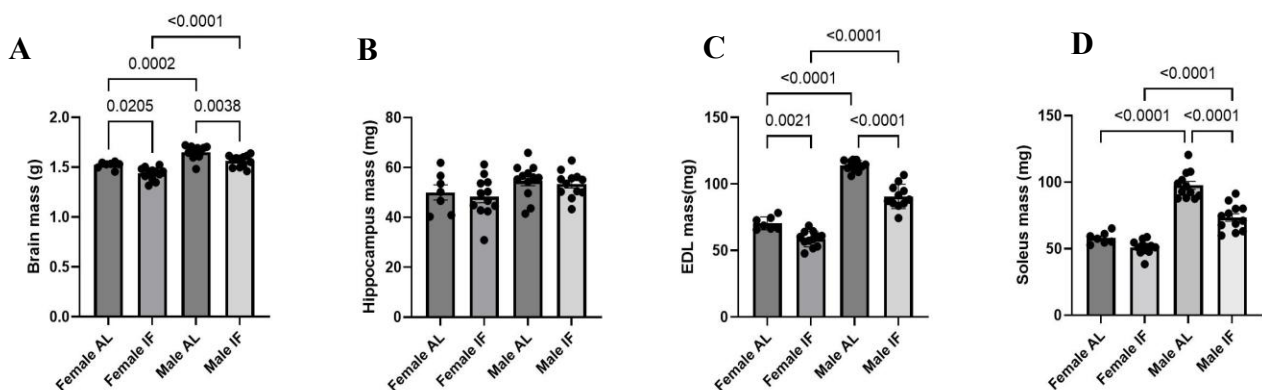


Memory and motor functions in young Fischer-344 rats. Latency time was lower in males compared to AL counterparts. ($p = 0.0186$) (Figure 2.2A). No significant difference was documented with memory in females. Normalized to bodyweight, grip strength was significantly higher in females compared to their male counterparts (Figure 2.2B). (Female AL vs. Male AL; $p = 0.0009$, Female IF vs. Male IF: $p = 0.0276$). Maximum force was not significantly altered among groups (Figure 2.2C).

Brain and skeletal muscle masses following Intermittent Fasting

Brain mass was lower in both IF males and females compared to their AL counterparts (Fig. 2.3A) (vs. Female AL, $p = 0.0205$, vs. Male AL, $p = 0.0038$). Brain mass was significantly higher in male AL animals compared to female AL counterparts ($p = 0.0002$). Similarly, brain mass was higher in IF males compared to IF female counterparts ($p < 0.0001$). Absolute brain masses were lower in both females and males following IF intervention; however, brain mass expressed relative to body weight was increased only in males, with no significant change observed in females, highlighting sex-specific differences. Hippocampus mass was not significantly changed (Fig. 2.3B) in Fischer-344 rats by the IF. Additionally, skeletal muscle (Soleus and EDL, vs. male AL, $p < 0.0001$) masses were lower in the IF males (Fig. 2.3C and 2.3D). After 10 weeks of IF, absolute EDL masses were lower in the female IF group compared to female AL counterparts ($p = 0.0021$) while absolute soleus mass remained unchanged in females (Fig. 2.3C and D). Relative skeletal muscle masses did not differ significantly among groups.

Figure 2-3 Brain and skeletal muscle masses following IF

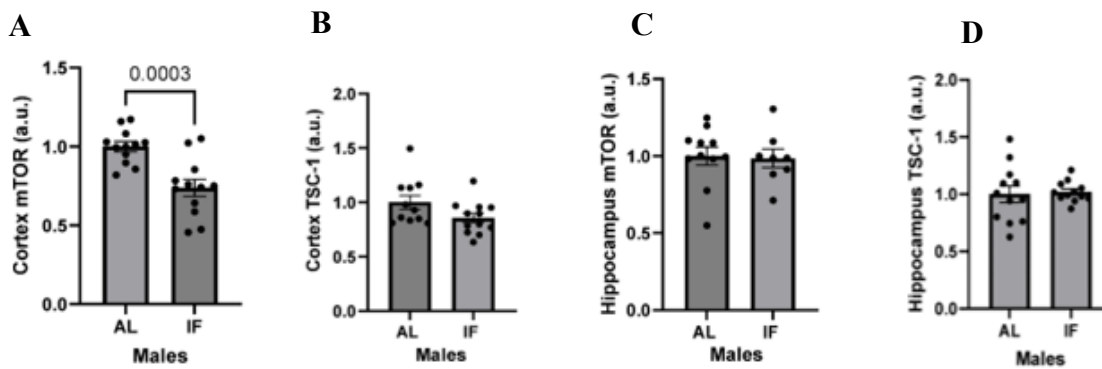


The alterations in the brain (Figure 2.3 A), hippocampus (Figure 2.3 B), and skeletal muscle (Figure 2.3 C, D) masses after 10 weeks of IF compared to their AL counterparts. The absolute brain masses were significantly lower in female ($p = 0.0205$) and male ($p = 0.0038$) IF groups compared to their AL counterparts. A lower absolute EDL mass was observed in response to fasting in both females ($p = 0.0021$) and males ($p < 0.0001$) compared to AL counterparts. Alternatively, soleus mass was lower in males as a result of IF ($p < 0.0001$).

mTOR expression in the brain following intermittent fasting

Figure 2.4 displays mTOR and its upstream regulator, TSC-1 (tuberous sclerosis complex 1), expression in the frontal cortex and hippocampus. As shown in Fig. 2.4A and 10 weeks of IF in males show lower expression of mTOR in the brain cortex ($p = 0.0003$); however, TSC-1 was not altered (Fig. 2.4B). In the hippocampus, mTOR expression and TSC-1 expression remained unchanged following IF (Fig. 2.4C and 2.4D).

Figure 2-4 mTOR expression in the brain following IF



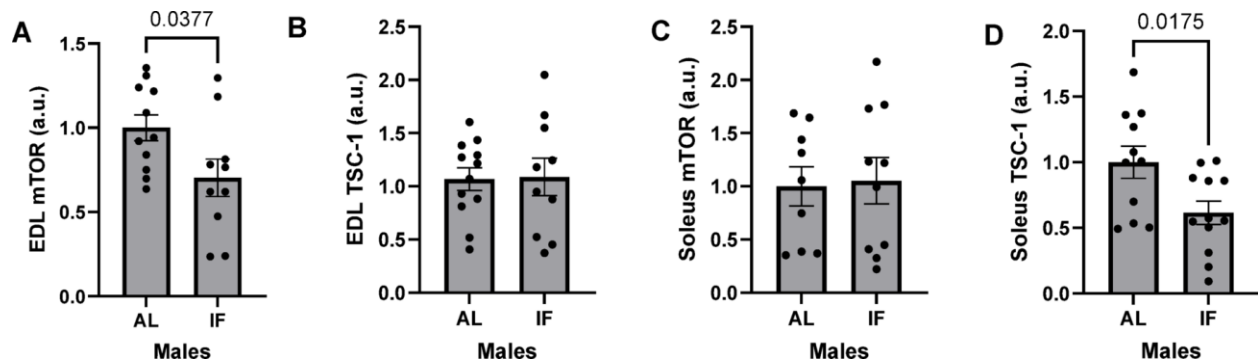
Cortical and hippocampal protein expression of mTOR, TSC-1 expression following intermittent fasting (IF) and ad libitum (AL). (Figure 2.4A) Cortex mTOR expression was significantly lower in male IF cortex tissue compared to AL males ($p = 0.0003$). (Figure 2.4B)

Cortex TSC-1 showed no significant differences. (Figure 2.4C) Male hippocampus did not have significantly altered mTOR or TSC-1 expression (Figure 2.4D).

mTOR expression in the skeletal muscles following intermittent fasting

We investigated whether 10 weeks of IF affects mTOR and TSC-1 in young Fischer-344 rats (Fig. 2.5). To understand the impact of IF on type 1 and type 2 muscles, we measured mTOR expression in both AL and IF groups. Statistical analysis revealed that mTOR expression was significantly lower in the fast-twitch EDL muscles (Fig. 2.5A), but not in the soleus muscle (Fig. 2.5C), in response to fasting in males. In the male soleus, TSC-1 expression was lower in the IF group (Fig. 2.5D).

Figure 2-5 mTOR expression in the skeletal muscle following IF



The skeletal muscle protein expression of mammalian target of rapamycin (mTOR) and Tuberous Sclerosis Complex (TSC-1). (Figure 2.5A) mTOR expression was significantly lower in EDL ($p = 0.0377$) but not in the soleus muscle (C) in response to fasting. (Figure 2.5B) TSC-1 expression was not changed in EDL in response to fasting. (Figure 2.5D) TSC-1 expression was significantly lower in male IF soleus tissues in response to fasting ($p = 0.0175$).

Pearson correlation against weight change in males and females

The correlations between weight change and cortex mTOR expression in males and females are displayed below (Table 2.1 and Figure 2.6). Interestingly, there was no correlation between weight change and hippocampus mass in both males and females. As suggested by the mTOR expression, males showed a significant correlation between cortex mTOR and weight change. Figure 2. 6 displays a heatmap of the full correlation matrix.

Table 2-1 Pearson correlation table against weight change in males and females

	MALES		FEMALES	
	Weight change		Weight change	
	Pearson r	<i>p-value</i>	Pearson r	<i>p-value</i>
Memory	-0.417*	0.043	-0.215	0.408
Brain mass	0.651*	0.001	0.731*	<0.001
Hip mass	0.241	0.257	0.212	0.385
Soleus mass	0.827*	<0.001	0.669*	0.002
EDL mass	0.913*	<0.001	0.756*	<0.001
mTOR Cortex	0.546*	0.006	0.304	0.235
mTOR Hip	0.233	0.273	N/A	N/A
mTOR Sol	-0.072	0.763	0.120	0.648
mTOR EDL	0.267	0.242	-0.122	0.629
TSC Cortex	0.090	0.675	0.001	0.998
TSC Hip	-0.033	0.879	0.054	0.826
TSC Sol	0.377	0.076	0.046	0.855
TSC EDL	-0.014	0.967	0.155	0.631

Weight change was calculated as post-intervention body weight minus pre intervention body weight. Memory values were inversely entered into the correlation data set so that a high value indicated high memory performance and a low value indicated low memory performance. Tissue

masses were unaltered and mTOR and TSC-1 protein expressions are the area under the curve values generated with the Compass for Simple Western software. * notes significant p-values.

Figure 2-6 Pearson correlation against weight change in males and females following IF

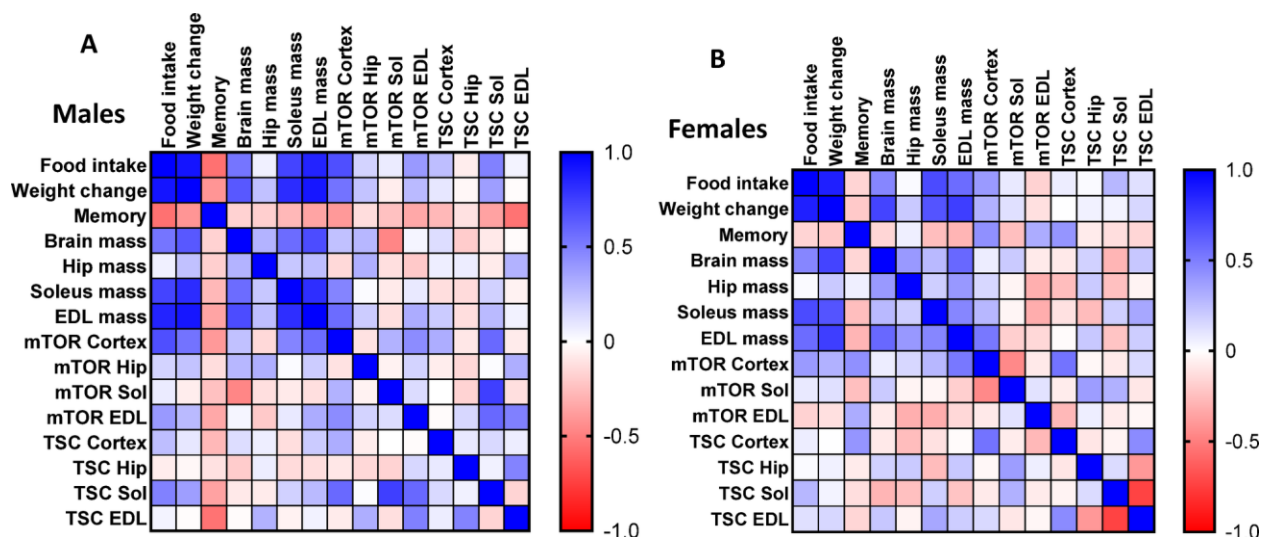


Figure 2.6: Heatmaps of Pearson correlations A males and B females. Blue indicates a positive correlation with the shade, noting the strength and red indicates a negative correlation.

Discussion

The current study investigated the effects of 10 weeks of IF on memory, muscle strength and mTOR and TSC-1 expression in young, healthy rats. We assumed that both males and females in the IF groups would incur declines in growth rate via reduced food intake. Interestingly, the females did not experience a change in growth rate after IF. IF improved memory in male rats but did not impact memory in females. IF males showed significantly lower mTOR expression in the brain cortex and skeletal muscles, whereas females did not show any differences. Additionally, there were significant changes in body weights and food intake in male IF rats compared to their AL-fed counterparts. Female IF animals did not show alterations in

behavior, mTOR expression, body weight shifts, or food consumption; however, absolute brain mass was lower in IF females compared to AL males.

In the present experiment, male and female rats were housed and fed identically in either the AL or IF intervention groups. We observed that male IF animals ate significantly less on average compared to their AL counterparts. Specifically, we found that IF males ate 27% less. This is in agreement with Singh et al. who demonstrated that male IF animals consumed 25–30% less compared to AL animals when following an alternate day fasting regimen (29).

We observed that female IF animals did not eat significantly less on average than their AL counterparts. Our observations contrast with Munhoz et al who found that young female Wistar rats subjected to a 12-week IF (24-h fed/fast cycle) significantly reduced their average food intake, with the IF group consuming 35% less chow compared to their control AL groups (30). We suggest that the observed differences might be attributed to strain differences (Wistar vs. Fischer 344) and feeding behavior in different rat strains. Alternatively, it may be link to binge eating. AL-fed females have a propensity for binge eating (31), and the effects of IF on binge eating behavior in females is unknown. In the Klump et al. (2013) study, they found that female Sprague-Dawley rats subject to different binge eating paradigms showed 2–6 times higher propensity for binge eating compared to males (31). Thus, binge eating differences between the Wistar and F344 females may account for the F344s not showing reduced growth rate here. However, we have no data to support or refute the role in binge eating in the female F344 as playing a role in this discrepancy.

In the context of our study, the findings suggest a sex-specific response to IF. Our female Fischer-344 rats may have compensated during the AL feeding period, leading to increased food intake. This compensatory intake could be driven by to ghrelin signaling and predisposition

toward binge eating. This could explain the lack of reduction in overall food intake and the absence of weight loss in our IF females.

Not surprisingly, considering the discrepancy in food intake, this experiment reported a sex difference in body weight changes as well. IF intervention in males resulted in significant body weight loss while no changes were seen in females. This is likely due to the reduction in food intake that was measured in males and not females. The literature shows that with fasting every other day, males typically lose weight, and this weight loss is generally attributed to the reduction in fat mass (34). A pre-clinical study revealed that females introduced to IF (with food access limited to 3 h per day) experience an increase in fat mass (30). The lack of weight change in females may reflect compensatory increased food intake during AL feeding.

Lazic et al. (35) reported that a 4-month IF regimen in 2-month-old female transgenic 5XFAD rats did not cause significant changes in body weight, which is consistent with our finding in Fisher-344 female rats. Surprisingly, every other day feeding induced inflammation in the brain cortex of 6-month-old females. Additionally, they did not observe improvements in either short-term or long-term performance in the Novel Object Recognition test, which aligns with our findings in the MWM (35).

Another IF study indicated that twelve-week-old C57BL/6J male mice introduced to IF, with access limited to only 3 h per day, significantly reduced their body mass and fat mass while maintaining the same level of lean muscle mass as the AL groups (34). On the other hand, male Fischer-344 x Brown Norway rats (6 and 25 months) subjected to a 5-week IF regimen significantly reduced relative lean muscle mass without altering fat mass (36). While the current study did not measure whole-body lean muscle mass percentage directly, both soleus and extensor digitorum longus (EDL) were significantly reduced as a result of IF in male rats while

females only experienced a lowered in EDL mass. During aging and sarcopenia, there is disproportionate atrophy of glycolytic fibers compared to oxidative fibers (37), while periods of skeletal muscle disuse or inactivity shift fibers from oxidative to glycolytic (38). Given that the soleus is highly oxidative (type I fibers) and the EDL is highly glycolytic (type II fibers), you would expect the soleus to be more highly conserved which was the case in females. However, males experienced equal declines in type 1 and type 2 skeletal muscle.

Although the brain size was reduced, the hippocampus mass was preserved in our study, possibly due to increased neurogenesis and reduced inflammation via the brain-derived neurotrophic factor (BDNF) pathway (39). Previous studies have reported that BDNF plays an important role in brain volume and is linked to cognition (39). IF has been shown to increase hippocampal neurogenesis through the BDNF pathway (40).

Previous studies have suggested that adaptation to IF regulates protein synthesis/breakdown while reducing body fat (34). In the current study, IF altered mTOR expression in the male brain cortex tissues and the EDL skeletal muscle. Tuberous Sclerosis Complex 1 (TSC-1)(11,41), acts as an upstream regulator of mTOR and is widely distributed in multiple organs including the brain, lungs, kidney, heart, and skeletal muscles (41,42). This loss of mTOR inhibition results in an increase in protein synthesis, cell proliferation, and inhibition of autophagy (42). In the current study, TSC-1 significantly decreased in the male soleus, which could explain why we did not observe a lower mTOR expression in the soleus like was seen in the EDL muscle. Interestingly, TSC-1 expression was preserved in the EDL. Previous studies have shown that mTOR activity is highly active in a nutrient-rich environment and decreases with nutrient deprivation (43). Alireza et al. revealed that 24–48 h of IF diminished neuronal mTOR activity in the cerebellum, which also increases the abundance of autophagosome size

(24). This is an important process for the clearance of toxic proteins, such as amyloids. The mTOR inhibitor rapamycin has been shown to reduce amyloid-beta levels and deposition in 3xTg-AD mice (22). Likely, mTOR was not inhibited in the females because they did not experience reduced food consumption. In females, this lack of catabolic effect could be minimal due to estradiol, as it plays a major regulatory role in metabolism. Estrogen plays a major role in glucose metabolism and favors lipid utilization when lipids are more available than carbohydrates (33). This may be critical when carbohydrate availability is limited during fasting. In addition to its effect on carbohydrate and lipid metabolism, estrogen also impacts appetite regulations, energy expenditure, and overall energy homeostasis (44). One study revealed that IF in young animals exacerbates insulin resistance and bone mineral density loss at fasting in AD-induced estrogen deficit (ovariectomized) rats (45). mTORC1 is also a key integrator of estrogen signaling. Holz et al. found that estrogen stimulation promotes the interaction between estrogen receptor alpha and one of the regulatory proteins of mTOR (Raptor). Additionally, they provide evidence that mTOR directly phosphorylates estrogen receptor alpha at the serine residue (46). This further illustrates the role of mTOR signaling in the pathogenesis associated with aging in females as estrogen levels decline with age. Conversely, mTOR inhibition in skeletal muscle in males can be linked to a reduction in food intake. Reduction in food intake also correlates with weight loss in males. In the current study, we did not have enough evidence to determine whether the weight reduction in males was due to fat mass or lean mass. Fasting has been shown to induce molecular adaptations in type 1 and type 2 muscles (32,47). Ieko et al. found that fasting significantly reduces the weight of the gastrocnemius, a type 2 muscle, while also reducing mTOR expression in the gastrocnemius of fasted animals (48). Similarly, in our study,

EDL and soleus masses were decreased in male IF rats, along with a reduction in mTOR expression in EDL

In the fed state, insulin receptor activation triggers a signaling cascade that activates protein kinase B (AKT). AKT inhibits the TSC complex, ultimately activating mTORC1. This promotes anabolic processes such as protein synthesis. In contrast, during fasting, the absence of insulin signaling allows Adenosine Monophosphate Protein Kinase (AMPK) to activate the TSC complex. This inhibits mTOR, leading to catabolic processes such as autophagy.

Concurrent hyperactivation of adenosine monophosphate protein kinase and mTOR has been observed in diseases such as AD (49), which contrasts with the general rule of mTOR inhibition. In AD, impaired insulin signaling and insulin resistance in the brain impact mTOR regulations. Impaired AMPK activates TSC-1 complex which acts as an upstream regulator of mTOR. The most widely accepted mechanism is that loss of TSC1/ TSC2 inactivates the TSC protein complex and leads to hyperactivation of mTOR (42). mTOR complex 1 (mTORC1) can be activated by upregulating the RAS homolog enriched protein in the brain (Rheb), by binding to the Rheb GTP. TSC complex inhibits Rheb activity and further activation of the TSC complex prevents mTOR hyperactivation. In addition, mTOR inhibitors, such as rapamycin, have been shown to reduce neurodegenerative phenotypes in transgenic rodent models (50). Wang et al. reported that oral administration of rapamycin at a dose of 2 mg/kg/day for seven weeks significantly improved spatial learning and memory in APP/PSI transgenic mice, as assessed by the MWM test. Furthermore, proteomic analysis using HPLC (High performance liquid chromatography) analysis revealed that rapamycin treatment rescued 57 out of 100 altered proteins (within the hippocampus) and 167 out of 260 proteins (within the temporal lobe). This finding highlights the central role of mTOR signaling in brain tissues. Importantly, mTOR/TSC

signaling extends beyond the brain; it can also impact peripheral tissues such as skeletal muscle. In the current study, IF males experienced a lower mTOR expression in muscles and TSC-1 expression was lower in the IF male's soleus muscle. This could be linked to the reduction in muscle mass in the soleus muscles. Previous studies have supported the idea that TSC-knockout 12-month-old mice develop a late-onset phenotype with severe muscle alteration such as atrophy of type 2b and type 1 (soleus) and hypertrophy of type 2a /2x (Tibialis anterior) fibers (41,42). In the current study, a reduction in TSC-1 expression did not impact mTOR expression.

Limitations

Several limitations should be considered. Animals were group housed, preventing individual food intake measurements; intake was estimated at the cage levels. The absence of a pair-fed AL control group limits our ability to fully dissociate caloric intake from fasting effects.

Conclusion

This study highlights a significant sexual dimorphic response to IF. Our results indicate that F344 males improved memory, decreased growth rate, and inhibited mTOR expression in both the brain and skeletal muscles in response to 10 weeks of every other day IF. In contrast, there were no changes in body weight, memory, or mTOR expression in F344 females undergoing the same IF regimen. Thus, amplifying the importance of considering rat strain and sex as variables in studies of intermittent fasting, as males and females may experience distinct metabolic and cognitive effects.

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Chapter 3 - Intermittent Fasting elicits sex specific central and peripheral metabolic adaptations

Abstract

Background: Examine sex specific metabolic responses in young Fischer-344 rats subjected to either ad-libitum (AL) feeding or intermittent fasting (IF) diets. Our prior work identified IF-induced alterations in body weight and food intake in males but not in females. In the present study, we hypothesized that tissue-specific metabolic adaptations and body composition shifts contribute to these differences. **Methods:** Thirty-six, two month old Fisher-344 rats were randomly assigned to four groups: ad libitum (AL) males, IF males, AL females and IF females. AL group had 24/7 access to food and water, while the IF group had full access to food on every other day basis. Rats were introduced to a grip strength test (grip strength meter), a memory test (Novel Object Recognition), and body composition (Lunar Piximus densitometer (GE Lunar Corporation)). Skeletal muscles were collected for future analysis. Group differences were analyzed using two-way ANOVA per Prism Graph Pad. **Results:** Lean and fat masses were lower in males ($p < 0.0001$) but not in females. Food intake was lowered in both IF groups. Recognition memory improved in IF females ($p = 0.0313$). Atrogin1 and MurF1 expressions were lower in male soleus (vs AL males, $p_{\text{Atrogin}} = 0.0273$, $p_{\text{MurF1}} = 0.0222$). IF did not alter Atrogin1 or MurF1 expression in females. Atrogin-1 and MuRF1 are key skeletal muscle atrophy-related ubiquitin proteins that regulate protein degradation and muscle wasting. CPT1A expression was higher in the male hippocampus (vs IF females, $p = 0.0168$). CPT1A is a rate-limiting enzyme in mitochondrial fatty acid oxidation and reflects lipid utilization. **Conclusion:** Fat and lean masses were lower in IF males, whereas IF females preserved body composition. In addition, fatty acid

oxidation was higher in IF males compared to IF females, indicating that IF elicited sex specific peripheral and central adaptations in Fischer-344 rats.

Background

Calorie restriction (CR) and intermittent fasting (IF) have emerged as powerful metabolic interventions shown to improve metabolic health, delay aging, and enhance brain function across experimental models, including rodents, non-human primates, and humans (1–4). Nearly a century ago, McCay and colleagues demonstrated that animals maintained on a CR diet exhibited significantly extended lifespan compared to ad libitum (AL)-fed controls (2). More recently, Acosta-Rodriguez et al. (2017) highlighted the need to reevaluate interpretations of CR studies, noting that animals subjected to CR often engage in prolonged, self-imposed fasting periods (5). These findings suggest that the temporal pattern of food intake and not caloric reduction alone may contribute substantially to the physiological benefits traditionally attributed to CR.

Consistent with this concept, Takahashi and colleagues demonstrated that extending fasting duration markedly enhances the longevity benefits of CR. In their study, CR alone increased lifespan by approximately 10% in mice, whereas combining CR with a circadian-aligned feeding–fasting protocol increased lifespan by up to 35% (68). These findings indicate that fasting duration and feeding timing play critical roles in extending health span and may exert effects beyond those achieved by caloric reduction alone.

IF, which involves time-restricted eating with or without an overall reduction in caloric intake, has therefore gained increasing interest in both clinical and experimental settings (3). IF has been shown to promote healthy aging through mechanisms including enhanced autophagy

and mitochondrial function (6–8), while also improving obesity, insulin resistance, dyslipidemia, and inflammation in both humans and animal models (7, 9, 11). Importantly, fasting regimens that follow a 24-hour feeding–fasting cycle minimally disrupt circadian rhythms, as they align with the natural light–dark cycle, thereby preserving metabolic and hormonal homeostasis.

Alternate-day fasting (ADF), a commonly used IF paradigm, induces robust alterations in body weight and body composition (41, 62). For example, Wilson et al. demonstrated that ADF produces sex-specific changes in body composition and energy metabolism in high-fat diet–fed C57BL/6 mice (41). Yoshii et al. further showed that restricting food access to 3 hours per day for six weeks reduced body fat while preserving muscle mass in male mice (21). Based on these findings and our previous pilot study (62), we selected a 24-hour ADF paradigm to capture robust sex-specific metabolic adaptations in skeletal muscle.

Rat models exhibit age-dependent physiological changes that closely resemble those observed during human aging, making them well suited for investigating the metabolic effects of IF in young animals (55, 63). Our prior work demonstrated that short-term fasting induces sex-specific changes in body weight and food intake in young (2–3 month old) Fischer-344 non-transgenic rats (62). In the present study, we extend these findings by evaluating sex-specific alterations in body composition in young adult rats subjected to IF.

There is increasing evidence that dietary interventions, including IF, elicit sex-specific metabolic responses (12, 62). Ramadan diurnal intermittent fasting (RDIF), characterized by complete abstinence from food and drink from dawn to sunset for one month, is one of the most extensively studied fasting paradigms in humans. While some studies report no significant improvements in body composition or metabolic health parameters (e.g., waist circumference, lipid profiles, blood pressure, or fasting glucose), others demonstrate beneficial and sex-

dependent effects (13–15). Sex-specific eating behaviors during fasting may be influenced by sexually dimorphic ghrelin signaling and differences in neural reward processing (16, 17). Ghrelin, a gastrointestinal hormone that stimulates hunger and modulates stress and reward pathways, is typically present at higher circulating levels in females than in males, although its orexigenic effects may be attenuated in females under certain conditions (18). Importantly, fasting-induced caloric restriction differs from traditional CR in the timing of food availability, which can differentially influence hunger-related hormonal fluctuations. For instance, Lucia et al. reported that circulating ghrelin levels were significantly higher following a 17-hour fast compared to a 12-hour fast (66). Additionally, the mesocorticolimbic reward system has been shown to be more responsive in females than in males, potentially contributing to greater susceptibility to hunger and altered feeding behavior during fasting (19).

Sexually dimorphic responses to IF may also be shaped by differences in sex hormones, body composition, and energy utilization strategies. Estrogen plays a critical role in appetite regulation, insulin sensitivity, and glucose homeostasis, contributing to enhanced satiety and protection against body weight gain in females (20–22). In contrast, testosterone promotes increased muscle mass in males, resulting in higher basal energy requirements (23); however, fasting has been shown to reduce circulating testosterone levels in males (24). Shifts in body composition can substantially alter systemic metabolism, thermogenesis, and muscular strength.

Skeletal muscle mass during fasting is tightly regulated by proteostatic pathways, including the ubiquitin–proteasome system. Muscle-specific E3 ubiquitin ligases such as Atrogin-1 and Muscle RING Finger 1 (MuRF1) are key mediators of fasting-induced muscle atrophy and may be differentially regulated in males and females. Thus, IF-induced changes in

muscle mass may reflect a balance between catabolic signaling, mitochondrial adaptations, and suppression of proteolytic pathways.

Beyond peripheral metabolic regulation, IF also exerts significant effects on central energy metabolism. Our previous studies demonstrated that prolonged fasting significantly reduced protein synthesis in the cerebral cortex of male rats via suppression of mammalian target of rapamycin (mTOR) signaling (62). mTOR is a master regulator of cellular energy homeostasis, promoting protein synthesis and cellular growth under nutrient-replete conditions while inhibiting anabolic processes during nutrient deprivation (71). During fasting, suppression of mTOR signaling shifts cellular metabolism toward a catabolic state while preserving metabolic flexibility (62, 71). In addition to mTOR signaling, regulators of mitochondrial fatty acid oxidation, such as carnitine palmitoyltransferase 1A (CPT1A), play critical roles in neuronal and astrocytic bioenergetics. CPT1A is the rate-limiting enzyme responsible for transporting long-chain fatty acids into mitochondria for β -oxidation and is essential for maintaining energy balance during nutrient scarcity (70). Recent evidence indicates that astrocytic CPT1A activity supports mitochondrial fatty acid oxidation and contributes to improved cognitive function (69). Given known sex differences in brain energy utilization and neuroplasticity, IF may differentially impact cognitive outcomes in males and females, potentially conferring cognitive benefits in females independent of changes in muscle strength or body composition.

Despite extensive investigation, there remains no consensus regarding the effects of IF on body composition in rodent models, with studies reporting both preservation and loss of muscle mass in males (25, 26). Our previous work showed that males subjected to IF reduced overall food intake and body mass, whereas females maintained food intake and body mass to AL-fed controls (62). Collectively, these findings underscore the importance of identifying mechanisms

underlying IF-induced, sex-specific changes in body weight, body composition, and metabolic signaling. We hypothesized that females subjected to IF would exhibit an energy conservation phenotype characterized by preservation of lean mass and neurometabolic resilience, whereas males would experience significant reductions in fat and lean mass accompanied by distinct skeletal muscle and central metabolic adaptations. To test this hypothesis, we examined sex-specific alterations in peripheral regulators (Atrogin-1, MuRF1) in skeletal muscle and adipose tissue, as well as central energy regulators (CPT1A and mTOR signaling) in the hippocampus, alongside functional assessments of strength and cognition.

Methods

Ethical approval

All methods carried out in this experiment were performed in accordance with the guidelines for the Care and Use of Laboratory Animals under the approval of the Institutional Animal Care and Use Committee of Kansas State University (protocol code 4466 and date of approval Oct 9, 2020).

Experimental design

Thirty-six 2-month-old female and male Fischer-344 rats were randomly assigned into AL or IF groups and housed 2-4 animals per cage, on a 12:12 light: dark cycle in the Kansas State University (K-State) Comparative Medicine Group facility, accredited by AAALAC International (000667), OLAW (A3609-01) and USDA (48-R-0001) assured. The AL group (male, n=10; female, n=6) had 24/7 access to food and water while the IF group (male, n=10; female, n=10) had 24/7 access to water and full access to food on an every-other-day basis (i.e.,

alternate day fasting). Based on preliminary data, it is suggested that 8 female and 8 male animals in each group be used to detect significant differences in the primary outcomes with a power of 0.8 at a $p < 0.05$ level (72). Rats were maintained within a 68 -79°F temperature range throughout the study. Food was taken or given at the beginning of the animals' dark cycle each day. The feeding intervention was 4 weeks in duration, and weekly food intake was measured for four weeks. Rats were fed the 5001 Rodent Lab Diet. After four weeks, animals were tested on memory, grip strength, and body composition. After four weeks, tissues were collected for mechanistic analysis.

Novel Object Recognition (NOR)

Novel object recognition (NOR) is a measure of cognition, specifically recognition memory. It relies on the concept that an animal will explore a novel object before a known object (38). The four-day protocol included pre-habituation, habituation, training and testing phases. During the pre-habituation and habituation days, the animal is allowed to explore the empty testing arena for 5 minutes. On the training day, the animal is placed in the arena with two identical objects and allowed 5 minutes to explore them. On testing day, one of the objects is replaced with a new, novel object. The time each animal spends exploring the novel object is recorded and reported as their level of memory/cognition, which was occurred during their fast state. Trials were recorded and analyzed with a video tracking system (EthoVision, Noldus). Memory was assessed using the Novel Object Recognition task (NOR). This was determined by $\text{time spent on novel object} / (\text{time spent on novel object} + \text{time spent on familiar object})$. This can range from 0 to 1, with 0.5 representing the chance performance. In the novel object recognition task, a value of 0.5 represents equal exploration of novel and familiar objects. Values greater

than 0.5 indicate a preference for the novel object, whereas values below 0.5 indicate a preference for the familiar object (29).

Grip Strength

Skeletal muscle strength was assessed with a grip strength meter (10) (BIOSEB V 3.48). To do this, the animal was removed from its home cage by gripping the base of the tail between the thumb and forefinger. Then the animal was gently lowered over the top of the grid so that its four paws could grip the grid. The animal was allowed to attach to the grid properly, with all four limbs, before being pulled away. The torso was kept horizontal as the animal was pulled back steadily until the grip was released. When the animal released the grid, the force was recorded as the strength of the grip. This protocol was repeated over four consecutive days and the highest value was analyzed.

Body Composition with Densitometry

The shifts in body composition were quantified using the densitometry analysis. The Lunar-Pixi densitometer ((GE Lunar Corp, 30200) allows for the determination of total body fat and lean muscle mass in live animals (excluding the head). In this current study, each animal was scanned with the densitometer. Under 2-3% isoflurane anesthesia, whole-body scans were taken to quantify the amount of fat mass and muscle mass. This quantifies the shifts induced via IF and informs if there were significant alterations in the effects of IF in males and females. Our experiment included brief exposure to isoflurane prior to decapitation and tissue collection. Previous work has confirmed that extended (30 minutes) isoflurane exposure can lead to increased mTOR phosphorylation in the frontal cortex with no changes in the

hippocampus. However, it is unlikely that our brief exposure (<5 min) would impact the brain (73,74).

Tissue Harvest

After the 4-week intervention, animals were placed under anesthesia with 2-3% isoflurane. Reflexes were tested with tail and toe pinches to ensure they were fully anesthetized prior to decapitation. Brain tissues (frontal cortex, whole hippocampus), soleus and extensor digitorum longus (EDL), were isolated and weighed following decapitation. These harvested tissues were snap-frozen in liquid nitrogen and stored at -80°C until further homogenization procedure.

Homogenization and Western Blotting Procedure

Frontal cortex (20mg), hippocampus (20mg) and skeletal muscle samples (20 mg) stored at -80°C were homogenized using the RIPA buffer with protease inhibitor (1 mg: 25 µL) (28). Thick, rounded glass rods and heavy-walled test tubes were used for manual homogenization. The homogenized samples were gently agitated for 2 hours at 4°C, then centrifuged at 17000 Xg RCF for 20 minutes. Protein concentration was measured using Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA), following the manufacturer's instructions. Jess Protein Simple automated system (Bio-Techne, Minneapolis, MN) was used to quantify protein expression. Total sample protein was quantified with the jess total protein detection kit. Total protein was used to correct the target proteins. This is an accurate method to replace the need for housekeeping protein. The following antibodies were used to detect target proteins. Atrogin1 (Novus, NBD1-36955, goat 1:100), MuRF1 (ECM Bioscience, MM3161, mouse1:50), mTOR (cell signaling, 7C10, rabbit

1:100), p-mTOR (cell signaling, D9C2R, rabbit 1:10) CPT1A (Abcam, ab128568, rabbit1:100) and TSC-1 (Abcam, ab200728, mouse 1:10).

Statistical Analysis

Group differences were identified via two-way ANOVA with Prism GraphPad software (version 9.5.0 (730)). Pairwise group differences were analyzed using Tukey's post hoc multiple-comparison test. Protein expression data collected via Compass software (Biotechne, version 5.0) generated the area under the curve values (AUC). Over time, body mass and food intake data were analyzed via mixed-effect analysis. Novel object exploration time was determined by dividing the total object exploration time by the exploration of the novel object. Most of the statistical significance was set as $p < 0.05$, with two-tailed hypothesis testing. A one-sample *t*-test was used to determine whether the discrimination index differed significantly from chance level (0.5), indicating preference for the novel object. Data is presented as mean standard error. Over time, body mass and food intake data were analyzed via mixed-effect analysis.

Results

Food intake and body mass were lower in Fischer-344 male rats

Food consumption was lower in male IF groups compared to AL males. Food intake was reported as an average per animal cage intake and not an animal-specific food intake. Due to group housing, we were not able to generate individual animal intake. In addition, due to low cage numbers per group, food intake can only be reported descriptively and not statistically. The average daily food intake in the male AL group was 17.6 g (SD=1.01), and the male IF group consumed less on average at 11.1 g (SD=0.10) (calculated from the total 4-week consumption

amount). In this current study, body weights were monitored weekly and presented as baseline (week 0) and post-4-week intervention (week 4). Males reported a significant difference in body weight between male AL and male IF ($p < 0.0001$). Females reported a significant difference in body weight between the Female AL and female IF group ($P = 0.0132$)

Figure 3-1 Body weight shifts and food intake in control and IF animals

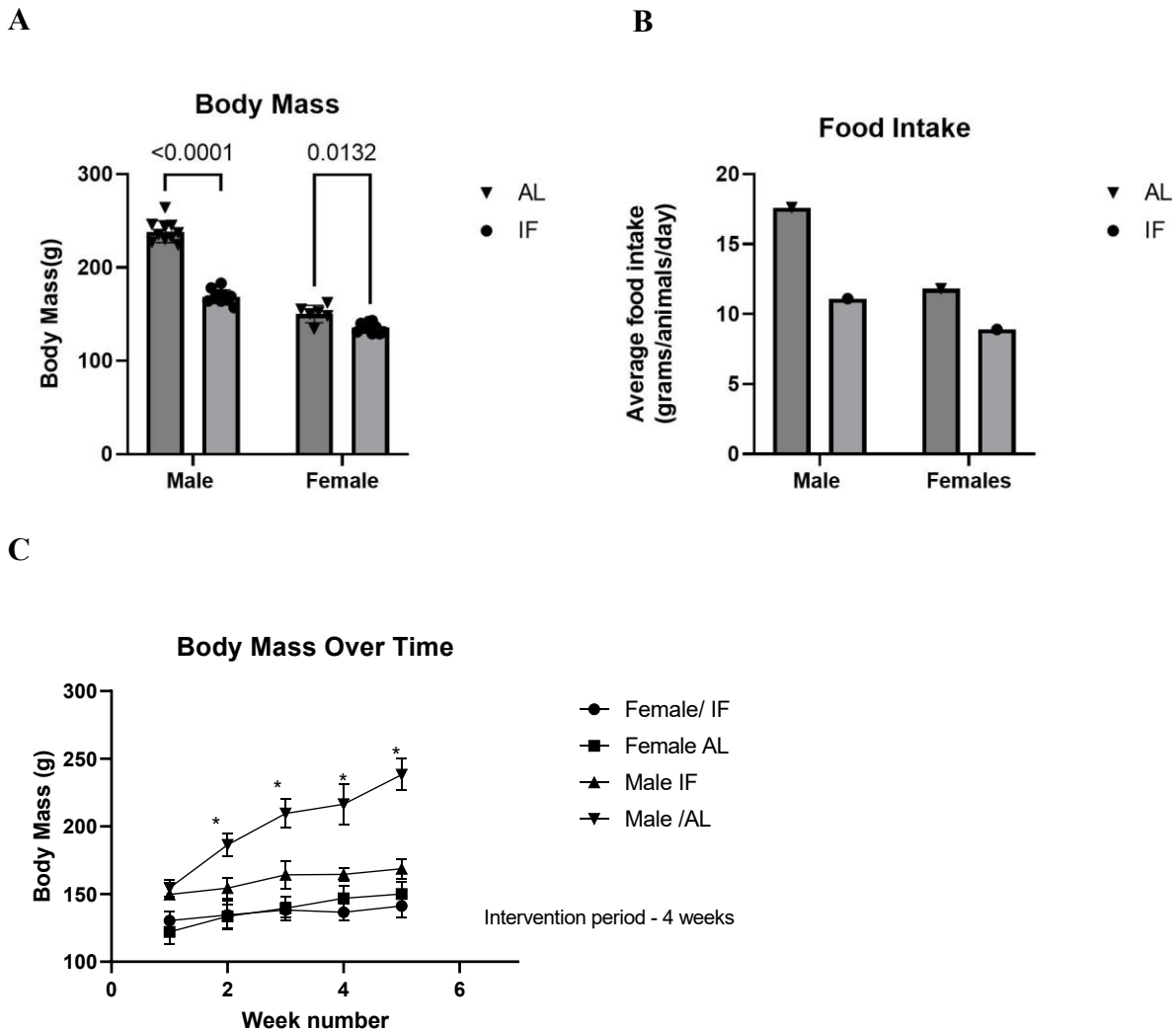


Figure 3.1: Body weight shifts and food intake in control and IF animals (A-D). Intermittent fasting lowered the growth rate over time in male rats (vs AL males, $p < 0.0001$) and female rats (vs AL females, $p < 0.0132$). (Interaction: $p < 0.0001$, Feeding $p < 0.0001$, Sex $p < 0.0001$). (Figure 3.1 A). Statistical analysis was performed within the Two-way ANOVA test. Average food

intake per animal was lower in both female and male IF groups (vs AL counterparts) (Figure 3.1 B). Weekly body mass was lower in Male IF (vs AL male, * $p < 0.05$). Data are presented as mean (Figure 3.1C) $\pm$ SE.

Females maintained body composition during IF

We investigate whether one month of IF intervention impacts lean and fat mass in young Fischer-344 rats (Figure 3.2). As indicated in Table 3.1/Figure 3.2. In comparison with male AL rats, lean muscle mass and fat mass were significantly lowered in male IF rats. However, fat and lean masses were not altered in our IF females compared to their AL counterparts. In addition, EDL mass (vs. AL males, $p < 0.0001$), and soleus mass (vs. AL males, $p < 0.0001$) were significantly lower in IF males. In contrast, females displayed no significant differences in the EDL or soleus muscle mass between the IF and AL groups (Table 1).

Lean mass (Figure 3.2B): In male IF groups, lean mass was significantly lower, with the mean value dropping from 149.6g $\pm$ 12.53g to 116.4g $\pm$ 5.53g ($p < 0.0001$). Conversely, Females did not alter their lean mass as a result of IF; they maintained an average of 90.20g $\pm$ 7.68g (vs AL 95.25g, $\pm$ 13.68g) throughout the study.

Fat mass (Figure 3.2A): In males, IF groups, Fat mass was significantly lower compared to AL counterparts; the mean fat mass dropped from 34.29 $\pm$ 4.18 g to 23.23 g $\pm$ 1.99g ($p < 0.0001$). In contrast, the females IF group did not alter fat mass; they maintained an average of 23.46g $\pm$ 3.42g (vs AL 24.58g, $\pm$ 1.79) throughout the one month of this study.

Figure 3-2 Females maintained body composition during IF

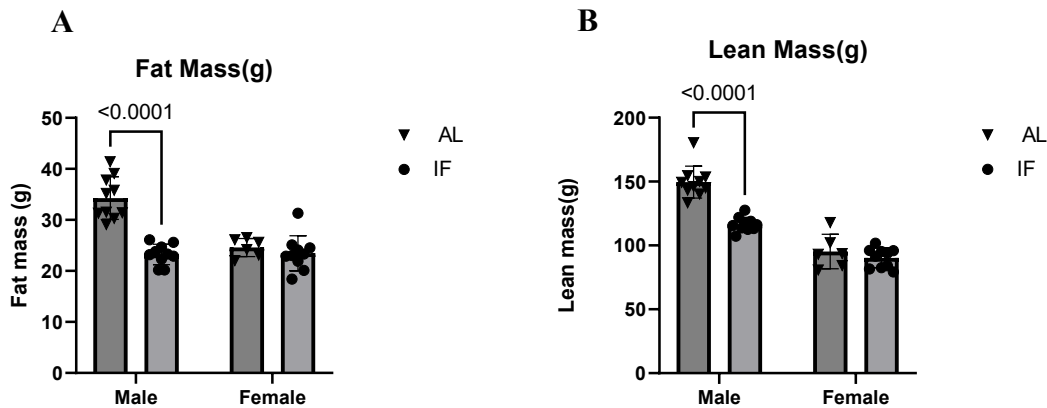


Figure 3.2: Body composition shifts in control (AL) and treatment (IF) groups (A, B). Fat mass (vs. AL males, $p < 0.0001$), and lean mass (vs. AL males, $p < 0.0001$) were significantly lower in IF males. Lean muscle mass: Interaction $p = 0.0002$, Feeding $p < 0.0001$, Sex $p < 0.0001$. Fat mass; Interaction $p = 0.0001$, Feeding $p < 0.0001$, Sex $p < 0.0001$. Neither lean mass nor fat mass was changed in females as a result of IF. Statistical analysis performed using a two-way ANOVA with $p < 0.05$ considered statistically significant. AL female ($n = 6$), IF female ($n = 10$), AL male ($n = 10$), IF male ($n = 10$). Data are presented as mean $\pm$ SE Sample sizes.

Table 3-1 Sexual dimorphic effects of IF on body weights, composition, food intake and muscle masses

	n		Week 0 weight (g)	Week 4 weight (g)	Δ weight (g)	Food intake (g)	Lean mass (g)	Fat mass (g)	Fat %	Soleus mass (mg)	EDL mass (mg)
Male AL	10	Avg	154.5	238.3	+83.8	17.6	149.6	34.3	14.4	85.9	93.0
		SD	6.33	11.74	9.64	1.02	12.53	4.18	1.66	4.94	9.42
Male IF	10	Avg	149.7	168.7*	+19.0*	11.1	116.4*	23.2*	13.8	66.8*	70.6*
		SD	3.56	7.39	6.87	0.10	5.53	1.99	1.28	4.89	3.13
Female AL	6	Avg	122.0	150.2	+28.2	11.8	95.3	24.6	16.37	54.8	62.8
		SD	8.98	9.30	2.93	0.36	13.43	1.79	4.92	6.47	6.00
Female IF	10	Avg	130.4	141.3	+10.9*	8.9	90.2	23.5	16.6	54.7	59.8
		SD	6.52	8.76	5.08	0.18	7.68	3.42	2.13	4.69	4.92

Table 3.1: The alterations of food intake, body weight shifts and body composition shift in females and males following 4 weeks of IF treatment. The average food intake (grams/animal/day) lowered in both females and males. Females did not display IF-induced declines in body weight. Males increased their body mass compared to their female counterparts (* $p < 0.0001$). Body composition shifts were measured using a Lunar Piximus rodent densitometer. Fat mass (vs. AL males, $p < 0.0001$), and lean mass (vs. AL males, $p < 0.0001$) were significantly lower in IF males. Neither lean mass nor fat mass was changed in females as a result of IF. EDL mass (vs. AL males, $p < 0.0001$), and Soleus mass (vs. AL males, $p < 0.0001$) were significantly lower in IF males. Neither EDL mass nor Soleus mass was changed considerably in females in response to IF. (Data are presented as mean (Avg) and standard deviation (SD) *indicates $p < 0.0001$ vs. AL group of the same sex).

Relative tissue masses in males and female animals following one month of IF intervention

Relative tissue masses in male and female animals under ad libitum (AL) feeding and intermittent fasting (IF) conditions. Top row: brain mass expressed as percentage of body mass (left), hippocampus mass as percentage of body mass (middle), and hippocampus mass as percentage of brain mass (right). Bottom row: extensor digitorum longus (EDL) muscle mass and soleus muscle mass expressed as percentage of body mass. Data are presented as mean $\pm$ SE. IF male rats have higher relative brain and hippocampus mass (brain and hippocampus $p < 0.0001$ vs AL males), relative brain mass was higher in females ($p = 0.0028$), while relative hippocampus mass remained unchanged. Relative EDL mass was higher in IF males ($p = 0.0308$) and relative soleus mass was higher in both females ($p = 0.0084$) and males ($p = 0.0075$) compared with AL controls.

Figure 3-3 Relative tissue masses in male and female animals

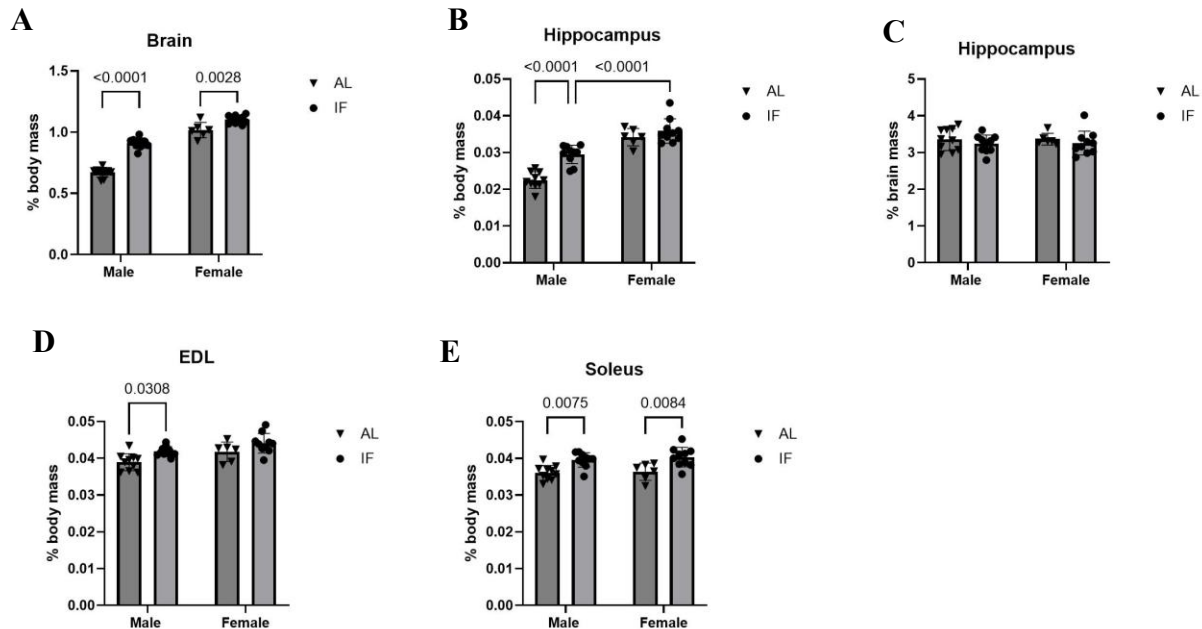


Figure 3.3 Relative brain, hippocampus, EDL, and soleus masses in male and female animals under ad libitum (AL) and intermittent fasting (IF). Brain (interaction $p < 0.0001$, sex $p < 0.0001$, feeding $p < 0.0001$), hippocampus (interaction $p = 0.0067$, sex $p < 0.0001$, feeding $p < 0.0001$), EDL (interaction $p = 0.6726$, sex $p = 0.0025$, feeding $p = 0.0018$), and soleus (interaction $p = 0.7526$, sex $p = 0.5140$, feeding $p < 0.0001$) masses are expressed as percentage of body mass, while hippocampus mass is also shown relative to total brain mass. Data are presented as mean $\pm$ SE.

One month of IF resulted in better memory performance in female rats.

Memory was assessed using the Novel Object Recognition (NOR) task. Preference: The results demonstrated that preference for novel objects improved in IF females. One-sample t-test against a 50% chance showed that memory performance was better in IF females. This was determined

by dividing the total object exploration time by the exploration of the novel object. In the novel object recognition task, a value of 0.5 represents equal exploration of novel and familiar objects. Values greater than 0.5 indicate a preference for the novel object, whereas values below 0.5 indicate a preference for the familiar object (29).

Figure 3-4 Female IF rats displayed better recognition memory

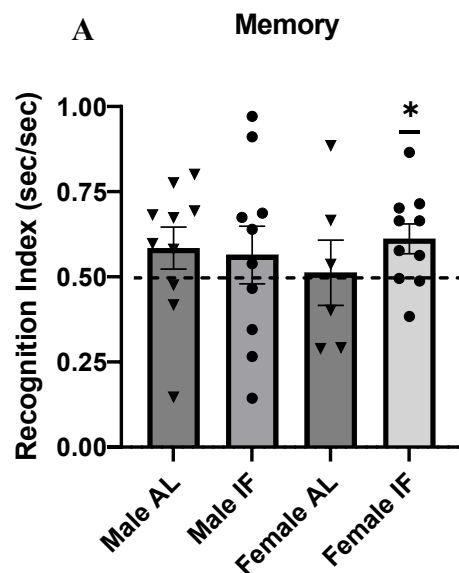


Figure 3.4 Recognition memory in treatment (IF) and control (AL) groups. A one-sample *t*-test against 50% preference showed that recognition memory in IF females was significantly above 0.50, indicating intact recognition memory in IF females. * $p=0.0313$. A one-sample *t*-test was used to determine whether the discrimination index differed significantly from chance level (0.5), indicating preference for the novel object. Data are presented as mean $\pm$ SE Sample sizes.

One month of IF did not alter grip strength in male and female rats

Grip strength was assessed using a grip strength meter. There were no significant improvements or impairments between IF and AL groups following the grip test. All groups exhibited similar strength, indicating that one month of intermittent fasting did not affect strength in young F344 rat animals.

Figure 3-5 Grip Strength was not impacted by IF



Figure 3.5 Grip strength in treatment and control group. Grip strengths were not significant among groups. Group differences were identified via two-way ANOVA with Prism GraphPad software. Data are presented as mean $\pm$ SE Sample sizes

One month of IF lowered Atrogin-1 and MuRF1 in the male Soleus muscle

To determine whether IF alters skeletal muscle atrophy pathways, we quantified the protein expression of Atrogin-1 and Muscle Specific RING finger protein 1 (MuRF1) in the soleus muscle following one month of IF intervention. IF significantly lowered the expression of both Atrogin 1 and MuRF 1 in male rats compared with AL fed males (Fig. 3.6A, 3.6B, Atrogin 1 $p=0.0273$, MuRF1 $p=0.0222$), indicating a suppression of muscle atrophy. In contrast, no

significant differences in Atrogin 1 or MuRF 1 expression were observed between IF and AL females (Fig. 3.6C, 3.6D). These findings demonstrate a sex-specific response to IF, with males exhibiting lower expression of muscle atrophic protein in the Soleus following one month of fasting.

Figure 3-6 IF decreases Atrogin-1 and MurF1 in the male Soleus muscle.

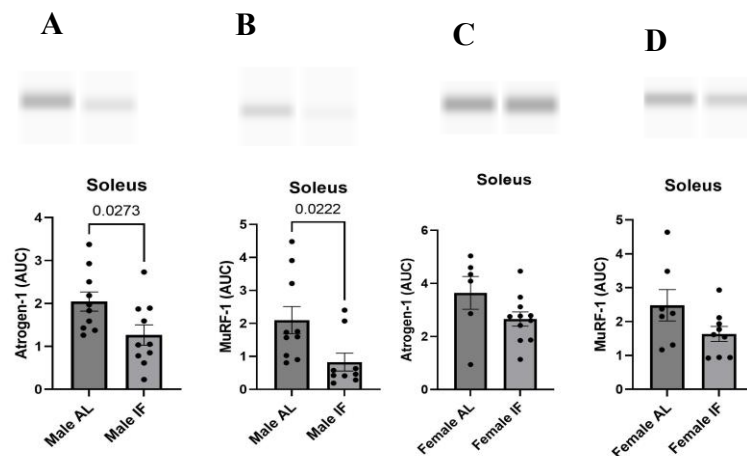


Figure 3.6: Protein expression of Atrogin 1 and Muscle Specific RING finger protein 1 (MurF1) in soleus following one month of IF. Atrogin 1 and MuRF 1 expressions were lower in male soleus (vs AL males, $p_{\text{Atrogin}}=0.0273$, $p_{\text{MurF1}}=0.0222$). IF did not alter Atrogin-1 or MuRF1 expression in females. Data are presented as mean $\pm$ SE Sample sizes.

One month of IF shows sex specific alterations protein synthesis (via mTOR) and fatty acid metabolism in the hippocampus

To assess whether IF modulates fatty acid metabolism and protein synthesis pathways in the brain, we examined the expression of CPT1A, TSC-1, and mTOR. CPT1A expression was higher in IF males compared with IF females, indicating enhanced capacity for mitochondrial fatty acid oxidation in the male hippocampus. Additionally, males exhibited significantly higher

expression of TSC-1, a key upstream regulator of mTOR signaling, compared to females under both AL and IF conditions, suggesting sex differences in the mTOR pathway. Despite these changes, neither sex nor IF impacts total mTOR.

Figure 3-7 Sex specific alterations in protein synthesis (via mTOR) and fatty acid metabolism

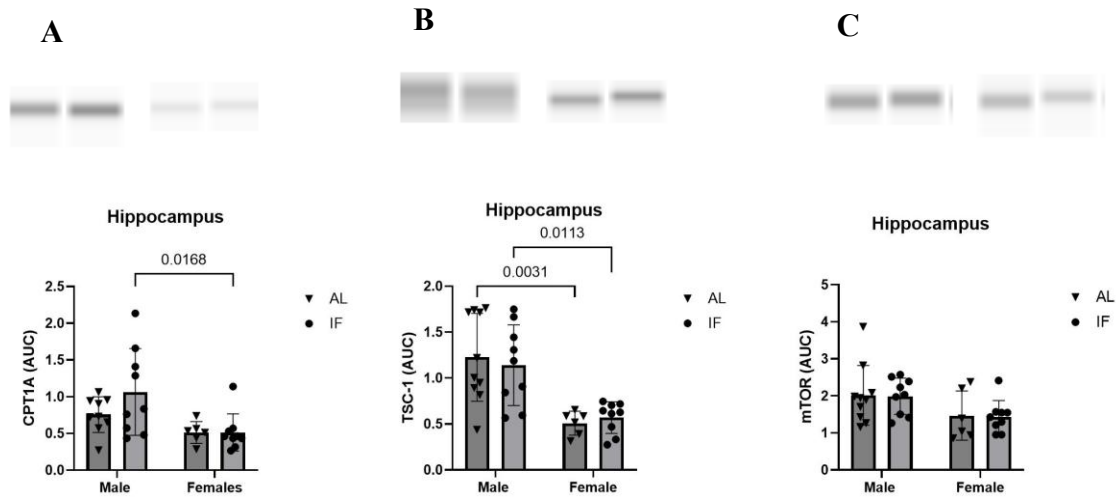


Figure 3.7: protein expression of carnitine palmitoyl transferase 1 (CPT1A), tuberous sclerosis complex 1 (TSC-1), mammalian target of rapamycin (mTOR), and phosphorylated mTOR in the hippocampus following one month of IF. CPT1A expression was higher in the male hippocampus (vs IF female hippocampus, $p=0.0168$; interaction $p=0.2466$, sex $p=0.0046$, feeding $p=0.2446$) Males also exhibited higher hippocampal TSC1 expression compared to female counterparts under both AL and IF fed stages (Figure 3.7 B, male AL vs female AL $p=0.0031$, male IF vs female IF $p=0.0113$; interaction $p=0.5656$, sex $p<0.0001$, feeding $p=0.9251$) The total mTOR expression was not altered among groups (Figure 3.7 C). Data are presented as mean $\pm$ SE.

Discussion

Summary of key findings

The present study investigated the sex-specific metabolic and cognitive effects of a 4-week intermittent fasting (IF) intervention in young adult, healthy Fischer-344 rats. Our findings demonstrate pronounced sexual dimorphism in response to IF. After four weeks of IF, Male rats exhibited a lower absolute fat mass and lean mass compared to ad libitum (AL) controls, whereas females-maintained body composition shifts. Skeletal muscle mass was preserved in females, as indicated by unchanged extensor digitorum longus (EDL) and soleus muscle weights. Importantly, recognition memory performance was intact in IF females without accompanying changes in muscle strength. At the molecular level, IF induced sex- and tissue-specific alterations in metabolic regulators, including muscle atrophy markers, and hippocampal CPT1A. Collectively, these findings indicate that 4 weeks of IF elicits distinct peripheral and central metabolic adaptations in males and females and suggest that metabolic flexibility and energy conservation strategies underlie these divergent responses. To our knowledge, this is among the first studies to simultaneously examine sex-specific muscular, and cognitive adaptations to short-term IF in young rats.

Intermittent fasting drives distinct, sex-specific body composition adaptations

In the present study, male rats displayed significant reductions in bodyweight, and both fat and lean mass following 4 weeks of IF, whereas females preserved body composition despite reduced average food intake. Importantly, these differences in lean muscle mass remained evident after normalization to body weight, indicating that the observed alterations were not attributable to reductions in total body mass, but rather reflect sex-specific changes in lean

muscle mass in response to IF. These findings support sex-specific responses to fasting and align with prior studies demonstrating that IF promotes lipolysis and protein catabolism, particularly in males (32–35). In contrast, Randall et al. reported that middle-aged male C57BL/6J mice subjected to repeated fasting cycles exhibited metabolic shifts (e.g., increased ketone production and reduced glucose levels) without changes in body mass (44).

Females have been reported to exhibit higher fasted lipolytic activity than males (36, 37). Fischer-344 x Brown Norway rats (6 and 25 months) subjected to a 5-week IF regimen significantly reduced relative lean muscle mass without altering fat mass (26). Skeletal muscle and the liver serve as a nutrient reservoir during fasting; however, IF without a sustained caloric deficit has been shown to preserve lean mass despite reductions in body weight (25). This pattern was observed exclusively in females in our study. Estrogen may contribute to this phenotype by regulating appetite, substrate utilization, and metabolic homeostasis, but this factor was not analyzed in our study. Similarly, Estrogen receptor signaling plays a key role in feeding behavior, and ovariectomy promotes weight gain and metabolic dysregulation in females (67), and this factor was not analyzed here either. Moreover, estrogen enhances lipid oxidation during periods of reduced carbohydrate availability (19, 20), allowing females to maintain energy balance during fasting. Baseline metabolic differences may further influence IF responsiveness. Previous work demonstrated that calorie-restricted females exhibit chronic reductions in $\dot{V}O_2$ and $\dot{V}CO_2$, indicative of metabolic downregulation, whereas males do not (45). In that study, females also showed superior preservation of metabolically active tissues under energy restriction (45).

Suppression of Muscle Atrophy Signaling Despite Lean Mass Loss in Males

Fasting is commonly associated with activation of muscle atrophy pathways. Preclinical studies have shown that prolonged fasting increases expression of muscle-specific E3 ubiquitin

ligases, including Atrogin-1 and MuRF1, leading to muscle fiber atrophy (47). Previous findings evident that master regulator of mitochondrial biogenesis, PGC-1 α , is known to suppress FOXO-mediated transcription of atrogenes, thereby protecting against muscle wasting (47).

In the present study, IF males exhibited reduced lean mass alongside significant decreases in Atrogin-1 and MuRF1 expression in the soleus muscle. Although PGC-1 α was not reported in this study, these findings may suggest a compensatory response aimed at limiting proteolysis during energy deprivation rather than preventing initial muscle loss. These findings indicate that short-term IF may activate protective mechanisms that restrain further muscle degradation even as body mass declines.

Central Metabolic Adaptations and Implications for Cognition

Energy metabolism is closely linked to neurodegenerative disease risk, including Alzheimer's disease (AD) (30, 56–58). In the present study, IF females exhibited improved recognition memory without changes in bodyweight, body composition or muscle strength, indicating a dissociation between peripheral metabolic adaptation and cognitive benefit.

IF increased hippocampal CPT1A expression in males relative to females, suggesting enhanced mitochondrial fatty acid oxidation capacity; however, IF did not elevate CPT1A relative to AL controls in either sex. These findings indicate that short-term IF modifies sex differences in brain energy metabolism without uniformly increasing fatty acid oxidation. Notably, IF did not alter mTOR or phosphorylated mTOR levels in the hippocampus, contrasting with our prior work showing reduced mTOR signaling following longer fasting durations (62). This suggests that the duration of fasting is a critical determinant of central protein synthesis regulation.

Enhanced cognitive performance in IF females may therefore arise from improved metabolic efficiency, synaptic plasticity, or neuromodulatory signaling independent of fatty acid oxidation or mTOR suppression. Prior studies demonstrate that IF can reduce neuroinflammation, enhance neurogenesis, and improve cognitive function with longer intervention durations (64, 65). For example, Lee et al. reported that IF-induced reductions in adipose inflammation attenuated hippocampal neuroinflammation (65). Whether prolonged IF further enhances cognitive resilience in females warrants investigation. Given the established link between metabolic disease, obesity, and dementia risk (59–61), preservation of metabolic homeostasis during IF may confer neuroprotective benefits. The observation that IF improved recognition memory in females without altering body composition suggests that peripheral metabolic stability may support brain energy demands during fasting.

Limitations

Several limitations should be considered. Animals were group housed, preventing individual food intake measurements; intake was estimated at the cage level. Sample size in the female AL group was limited ($n = 6$), potentially reducing statistical power. Based on preliminary data, suggest 10 females and 10 males in each group to detect significant differences in the primary outcomes with a power of 0.8 at $p < 0.05$ level. Stress responses associated with prolonged fasting may also affect behavioral outcomes; future studies incorporating stress hormone and anxiety assessments are warranted. Some molecular analyses included reduced sample sizes due to tissue loss, resulting in underpowered comparisons in select assays. Finally, the absence of a pair-fed AL control group limits our ability to fully dissociate caloric intake from fasting effects.

Conclusions

This study demonstrates that IF induces pronounced sex-specific metabolic and cognitive adaptations in young Fischer-344 rats. Females maintain body composition, and muscle mass while exhibiting intact recognition memory. In contrast, males experience significant reductions in bodyweight, fat and lean mass accompanied by peripheral molecular adaptations in skeletal muscle. These findings highlight sex as a critical biological variable in fasting responses and provide a mechanistic foundation for future investigations into IF as a modifiable intervention for metabolic and neurodegenerative disorders.

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Chapter 4 - Effects of acute IF on protein synthesis and inflammation in aged brain tissues and skeletal muscle

Abstract

Background: Intermittent fasting (IF) has emerged as a promising intervention for improving metabolic health and mitigating age-related neurovegetative diseases. Current evidence suggests that inflammatory cytokines may contribute to neuronal loss and cell death in the brain; however, the effects of acute IF on inflammation in the aged population remain poorly understood. This study investigated the impact of three days of IF on protein synthesis in aged (18-month-old) male and female rats. We hypothesize that acute IF would modulate protein synthesis and attenuate inflammation while preserving cognitive functions. Methods: Twenty-seven 18 month F344 rats were assigned to either an ad-libitum (AL) feeding group or an IF group subjected to alternate-day fasting for three days. Spatial learning and memory were assessed using the Morris Water Maze. Following behavioral testing, brain and skeletal muscles were isolated and snap frozen in liquid nitrogen for molecular analysis. After homogenization, protein expression of Mammalian target of rapamycin (mTOR), phosphorylated mTOR (p-mTOR), and tumor necrosis factor alpha (TNF- α) was quantified in both the brain and skeletal muscles. Prism GraphPad was used to determine group differences (one-way ANOVA) and correlations (Pearson) with a significance level set at $\alpha = 0.05$. Results: Three days of IF did not significantly alter absolute body mass, brain mass or muscle mass in aged rats. Cognitive performance was preserved in aged rats. mTOR and p-mTOR expression remained unchanged in both the brain and skeletal muscles across sexes. Notably, TNF- α expression was lower in fasted female brain tissues compared to AL females ($p=0.0038$), while no significant changes were observed in the male rats. TNF- α expression was significantly lower in the skeletal muscle of fasted males ($p<0.0079$ vs

AL males). Conclusion: Acute IF preserves protein synthesis (via mTOR) and differentially modulates inflammatory cytokines in aged females and males, and these findings suggest that short-term IF may initiate anti-inflammatory adaptation in late age. Understanding the IF-induced inflammatory pathways may provide insight into optimizing dietary intervention for neuroprotection during aging. Future studies will investigate these mechanisms in Alzheimer's disease rat models.

Background

Intermittent fasting (IF), which is time-restrictive food intake, has emerged as a promising non-pharmacological strategy to improve cognitive impairments and mitigate neurodegenerative pathologies in rat models (1,2). Our previous pilot study, along with prior literature, has shown that IF is associated with better memory performance in young rats (3,4). There are several forms of dementia, including vascular dementia, Alzheimer's disease, and Lewy body dementia. Alzheimer's Disease (AD) is the most common form of dementia, characterized by progressive memory loss and cognitive decline, which are closely linked to impaired glucose metabolism and insulin signaling. These metabolic disturbances disrupt key nutrient-sensing pathways that regulate cellular metabolism in the brain (5,6).

One of the central regulators of cellular metabolism is the mammalian target of rapamycin (mTOR), a serine-threonine kinase, a protein complex present in all mammalian cells that plays a critical role in regulating protein synthesis and degradation (7). In a normotensive individual, activation of the mTOR pathway occurs in the presence of nutrient and insulin signaling. When nutrients are available, mTORC1 phosphorylates and promotes growth, proliferation, and protein synthesis. In contrast, during fasting, under a nutrient-deficient state or

when insulin signaling is absent, the mTOR pathway is suppressed and promotes autophagy, a key homeostatic process responsible for the degradation and recycling of cytosolic components (8,9). In AD, mTOR is often hyperactivated and that has been linked to the accumulation of pathological proteins such as amyloid beta (6). In addition, excessive protein synthesis (including amyloid-beta and tau) alongside inhibited autophagy (allowing the toxic aggregate buildup) contributes to the cognitive decline in AD (6,10). Our previous observations demonstrated lower mTOR expression in the brain cortex of young animals. The current study investigates whether acute IF can modulate mTOR expression in aged rats. While several effects of IF (anabolic and catabolic processes) have been measured in young and adult rats (9,18), limited data exist in aged rat models, which are characterized by increased vulnerability to catabolic stress such as muscle atrophy. The current study examines healthy F344 rats to model physiological aging in the absence of AD.

Neuroinflammation is another key pathological hallmark of age-related neurodegenerative disease. Proinflammatory cytokines, including TNF- α , were elevated in the AD brains and contribute to the neuronal necroptosis, an inflammatory form of cell death in the hippocampus (11). TNF- α plays a dual role in regulating functions in the CNS, supporting neurogenesis, cell survival, and death (12,13) while also promoting neuronal toxicity and inflammation under pathological conditions, possibly through the activation and release of cytokines from glial cells (14). Prior studies have shown that IF exerts a strong anti-inflammatory effect in both preclinical and clinical models (15,16). Clinical trials report that several months of fasting have reduced pro-inflammatory cytokines Interleukin-1beta (IL-1beta), Interleukin 6 (IL-6), and TNF- α expression in healthy subjects (16). Similarly, IF reduced inflammation (via TNF- α) in rodents under distressed conditions (15). Although evidence

suggests that IF can attenuate systemic and central neuroinflammation, no studies have yet evaluated the immediate impact of IF on the aged brain tissues and skeletal muscle. We hypothesized that acute IF rescues memory while reducing inflammation (via TNF- α) and modulating protein synthesis pathway (via mTOR) in aged Fisher-344 rats. We will also examine sex as a biological variable to access sex dependent effects in aged rats.

Methods

Ethical approval

All methods carried out in this experiment were performed in accordance with the guidelines for the Care and Use of Laboratory Animals under the approval of the Institutional Animal Care and Use Committee of Kansas State University (protocol code 4466 and date of approval Oct 9, 2020).

Experimental design

Twenty-eight, aged (18-month) female and male Fisher-344 rats were randomly assigned into two groups: ad-libitum (AL) and Intermittent fasting (IF) (Male AL=6, Male IF=6, Female AL=7, Female IF=8) and housed 2-3 animals per cage, on a 12:12 light: dark cycle in the Kansas State University (K-State) comparative medicine group facility. Sample size in each group was limited, potentially reducing statistical power. Based on preliminary data suggest 8 females and 8 males in each group to detect significant differences in the primary outcomes (mTOR, memory) that exist power of 0.8 at $p < 0.05$ level. The ad-libitum (AL) group had unrestricted access to food and water while the IF group was fasted on an every-other-day basis (i.e., alternate day fasting) for 3 days (i.e. two-24 hour fasting periods with one-24 hour AL

between). Memory was assessed via the Morris Water maze. Following memory tests, animals were euthanized, and tissue masses were weighed and documented. The brain tissues (hippocampus and cortex) and skeletal muscles were sectioned and snap-frozen in liquid nitrogen for future pathological analysis. After the homogenization process, western blots were performed using the Jess Protein Simple automated western blotting system (Bio-Techne, Minneapolis, MN). All procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC).

Morris Water Maze

Spatial learning and memory were assessed using the Morris Water Maze. Briefly, the tank was filled with water to 1 inch above the platform. Nonfat dry milk was used to make the water opaque. Under a red light, at a water temperature of 25°C, and following an acclimatization period, the animal was placed in the pool. The animal was allowed 60 seconds to find the hidden platform using the visual cues learned during the acclimatization period. The time-to-platform was recorded as the outcome measure, and shorter times are indicative of better learning of the platform's location. The animal was directed to the platform if it was not able to find the platform within 60 seconds on their own, and the data were censored by recording these trials as a time-to-platform of 60 seconds, as video tracking was not available during the experiment. Two training experimental trials were performed on the same day.

Tissue Harvest

The hippocampus plays a critical role in spatial navigation, episodic memory, learning, working memory, and decision-making. Also, it would be necessary to understand any adaptive changes in type I, soleus and type II, EDL fibers because of their differential adaptive capabilities. Type I

fibers are more fatigue-resistant, while type II fibers are quicker to fatigue but can generate force more quickly¹⁹. We would eagerly like to explore how dietary regimens such as fasting change the fiber composition and function. Therefore, to help understand such mechanisms, we investigate molecules that regulate energy homeostasis and inflammation within the skeletal muscles and brain tissues. All animals were weighed throughout the study and at the end of the study. Following anesthetized protocol and decapitation, the whole brain and skeletal muscle tissues were removed to provide molecular evidence of IF-induced neuroprotection and mechanisms. Brain (right and left hippocampus) and skeletal muscle tissue (extensor digitorum longus (EDL), and soleus) were excised, and blotted to remove excess fluid and weights were documented before snap freezing in liquid nitrogen for future protein analysis.

Homogenization procedure

Snap-frozen brain (Frontal cortex and hippocampus) and skeletal muscle tissue samples that were stored at -80 °C were homogenized using the RIPA (radio-immunoprecipitation assay) buffer. Brain cortex and skeletal muscle were cut and weighed to 20 mg and transferred to a 2 mL glass dounce homogenizer. RIPA buffer was added (1mg:25µL) and samples were homogenized (4-6 passes with the Dounce pestle for brain tissue and 8-10 passes for skeletal muscle). Thick, rounded glass rods and heavy-walled test tubes were used for manual homogenization. Whole hippocampus was homogenized using a dounce homogenizer. The homogenized samples were transferred into an intermediate microcentrifuge tube and incubated on a rocker (4°C) for 2 hours. Homogenized samples were centrifuged at 17000 xg RCF for 20 minutes at 4°C. Then, the supernatant was transferred into a microcentrifuge tube and protein

concentration was determined with a Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA). Samples were stored at -80°C for future western blotting analysis.

Western blotting procedure

Following homogenization, western blots were completed via Jess Protein Simple automated western blotting (Bio-Techne, Minneapolis, MN), Jess Protein Simple automated system (Bio-Techne, Minneapolis, MN) was used to quantify protein expression. Total sample protein was quantified with the Jess total protein detection kit. Total protein was used to correct the target proteins. Antibodies: Specific proteins of interest include mTOR (Santa Cruz, sc-517464, Rabbit 1:100), p-mTOR (Invitrogen, PA5104899, Mouse 1:10), and TNF α (Novus, D151805, Rabbit 1:10).

Data Collection and Statistical Analysis

Group differences were identified via two-way ANOVA with Prism GraphPad software (version 9.5.0 (730)). Pairwise group differences were analyzed using Tukey's post hoc multiple-comparison test. Protein expression data collected via Compass software (Biotechne, version 5.0) generated the area under the curve values (AUC). Data is presented as means and SE.

Results

Alterations of body weight shifts and tissue masses in aged rats

As expected, the acute IF intervention did not significantly impact body mass or the mass of isolated brain, hippocampi, and skeletal muscles (Table 4.1). In all but the hippocampi, male masses were significantly greater than females (Table 4.1).

Table 4-1 Alterations of body weights and tissue masses in aged healthy rats

	n		Week 0 weight (g)	Week 4 weight (g)	Δ weight (g)	Brain mass(g)	Hip mass (mg)	EDL mass (mg)	Soleus mass (mg)
Male AL	6	Avg± SD	432±51.74 *	429±52.09 *	3±7.15	1.89±0.09*	62.68±9.28*	158±17.60*	160.6±28.49*
Male IF	6	Avg± SD	417.5±18.49	395.5±17.12	22±2.28	1.87±0.02	64.18±9.00	145.4±6.27	148.53±8.35
Female AL	7	Avg± SD	235.8±11.68	234.71±10.76	1.14±1.77	1.7±0.03	57.94±4.78	87.46±5.37	81.31±6.01
Female IF	8	Avg± SD	241.1±12.12	227.625±10.28	14.5±2.06	1.72±0.05	62.25±4.05	86.57±5.31	87.46±8.51

Table 4.1: The Alterations of the body weights, brain weights, and Skeletal muscle masses in control and treatment groups. Pre and post body weights and post body weights were higher in males ($p < 0.0001$ vs AL and $p < 0.0001$ vs IF) compared to females. Brain weights were higher in males compared to female counterparts ($p < 0.0001$) and skeletal muscle weights were significantly higher in male groups than in females ($p < 0.0001$). Hippocampus mass was not significant among groups. Group differences were identified via two-way ANOVA with Prism GraphPad software (version 9.5.0 (730)). * indicates significant differences between males and females. Data are presented as mean $\pm$ SD.

Acute IF did not impact memory in aged rats

Statistical analysis of the escape latencies revealed that there was no significant improvement in the learning and memory functions after acute IF. The rats continued to exhibit similar latencies to reach the hidden platform compared to their AL counterparts. Memory was not significantly altered in either male or female groups and remained unchanged following fasting.

Figure 4-1 Learning and memory in healthy aged rats following IF

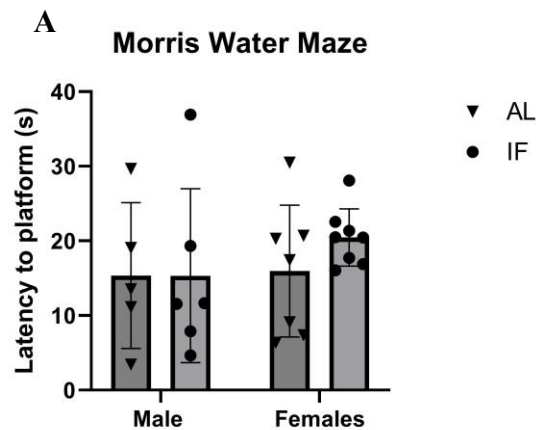


Figure 4.2: Effects of IF on spatial memory in healthy aged rats. Neither sex nor IF impacts cognitive performance in aged rats. Spatial memory was assessed via the Morris water maze. (Figure 4.1 A) Data are presented as mean $\pm$ SE.

Table 4-2 Alterations of food intake in aged healthy rats

	DAY 1 (g)	DAY 2	DAY 3	Intervention Avg
F-AL	13.3 $\pm$ 3.43	11.7 $\pm$ 1.70	12.3 $\pm$ 1.54	12.4 $\pm$ 0.80
F-IF	0.0	17.1 $\pm$ 0.42	0.0	5.7
M-AL	19.5 $\pm$ 3.28	20.3 $\pm$ 3.21	19.5 $\pm$ 1.80	19.8 $\pm$ 0.48
M-IF	0.0	25.0 $\pm$ 0.94	0.0	8.3

Table 4.2 Daily food intake over three days and average intake in aged female and male rats following IF. During the fed stage, both male and female fasted animals consumed more food compared to their AL counterparts. The overall daily average food consumption lowered in IF compared to AL counterparts. Data are presented as mean $\pm$ SD

mTOR expression in the aged brain tissue following acute IF

Aging is typically accompanied by a decrease in protein synthesis, particularly in the hippocampus, which correlates with memory impairment. As a marker of protein synthesis, we measured phosphorylated mTOR and total mTOR in hippocampal samples. As indicated in Figure 4.3A, in comparison with AL females and males, the mammalian target of rapamycin (mTOR) was not altered in our IF female and male hippocampus (Fig. 4.3A). Neither sex nor IF impact phosphorylated mTOR (p-mTOR) expression in the brain (4.3B). These findings suggest that acute IF does not significantly impact protein synthesis in the hippocampus tissues of aged female and male rats.

Figure 4-2 mTOR expression in the aged brain tissues following acute IF

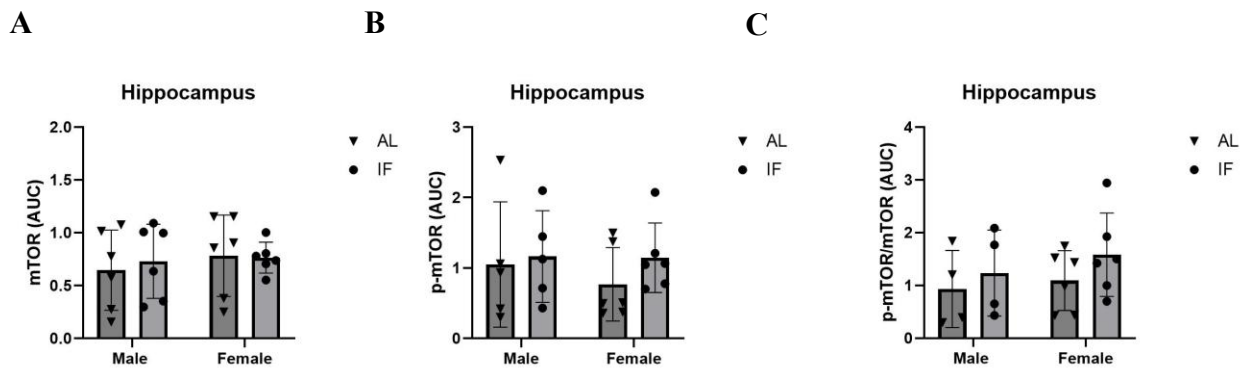


Figure 4.3: The alterations of hippocampal mammalian Target of Rapamycin (mTOR) and Phosphorylated mTOR expression in AL and IF groups. Protein synthesis (via mTOR) remained unchanged in male and female groups following IF. Similarly, hippocampal expression of P-mTOR was not significantly altered among groups. p-mTOR/ mTOR ratio was not significant among groups. Data are presented as mean $\pm$ SE.

TNF- α expression in the aged brain tissue following acute IF

Figure 4.4 displays Inflammatory protein Tumor Necrosis Factor (TNF- α) expression in the cortex and hippocampus. As indicated in Figure 4.4A, TNF- α expression was lowered in the fasted brain tissues ($p=0.0038$ vs AL cortex tissues). In the brain cortex, TNF- α expression was not altered in male IF rats compared to AL counterparts.

Figure 4-3 TNF- α expression was lower in the IF brains

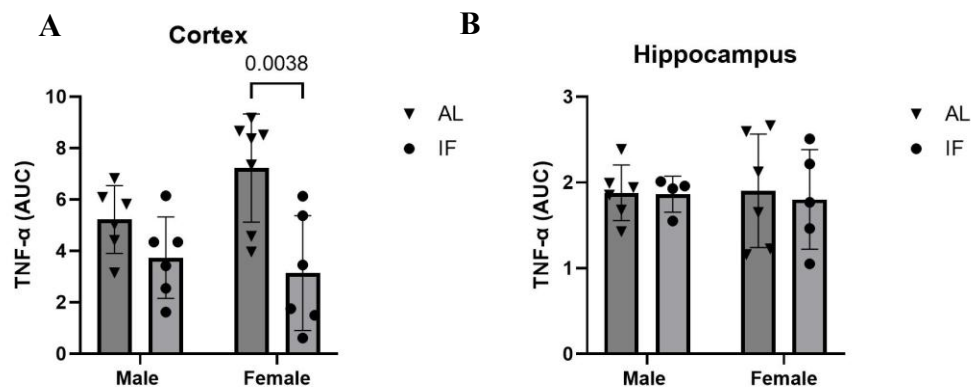


Figure 4.4 shows the cortical and hippocampal protein expression of TNF- α . In the cortex, TNF- α expression was lower in the female IF cortex compared to the female AL cortex ($p=0.0038$) (variation: Interaction $P=0.0951$, Feeding: $P=0.3558$, Sex: $P=0.0012$). There were no significant alterations within the male groups. In the hippocampus, there were no significant alterations. Data are presented as mean $\pm$ SE.

IF did not alter protein synthesis in slow-twitch and fast-twitch muscles

Figure 4.5 compares the effects of three days of IF on muscle protein synthesis (via mTOR). We investigated whether acute IF intervention changes protein synthesis via mTOR and phosphorylated mTOR (p-mTOR) in our aged rats (Fig A-Fig D). Our previous pilot study,

which was 2 months of IF intervention, revealed that mTOR expression was significantly altered in male Fisher-344 rats. To understand the impact of IF on glycolytic and oxidative muscles, we have measured protein quantification in slow-twitch (soleus) and fast-twitch muscles (EDL). Protein quantification analysis showed that there were no significant effects on protein synthesis (via mTOR or p-mTOR) after acute IF intervention. p-mTOR/mTOR ratio was not significant among groups.

Figure 4-4 mTOR expression in the aged skeletal muscles following IF

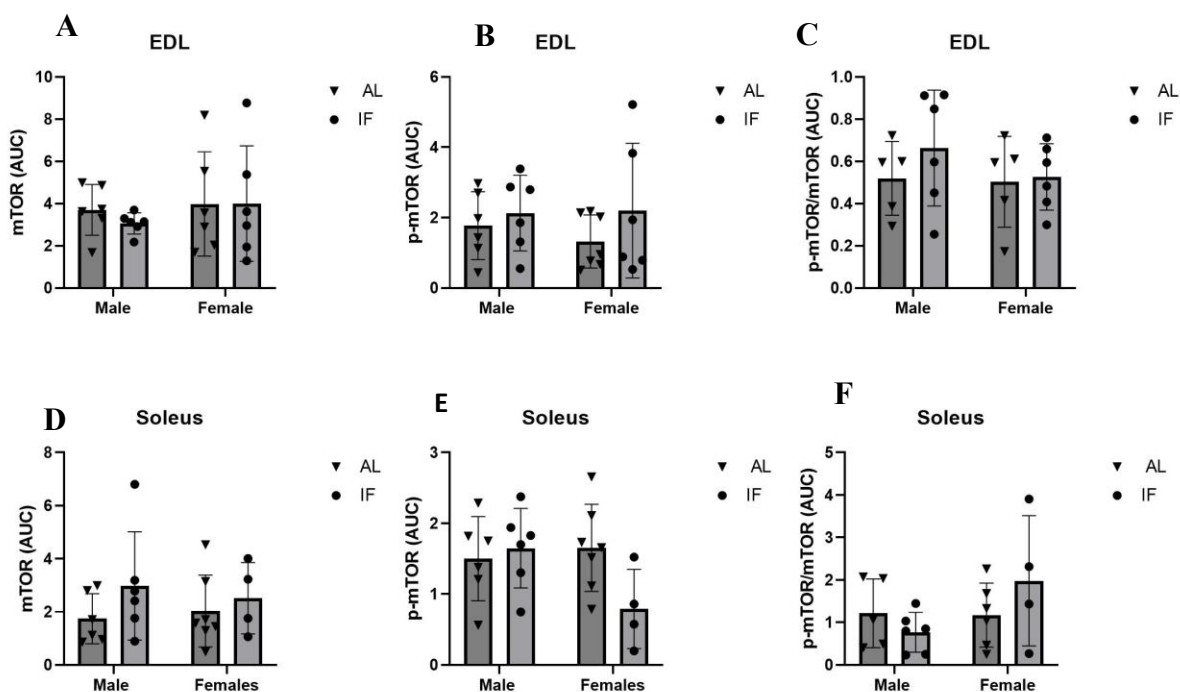


Figure 4.5: The skeletal muscle protein expression of mammalian target of rapamycin (**mTOR**) and phosphorylated mTOR (p-mTOR) (Fig.4.5 A-B, Fig.4.5 D-E). Soleus and EDL expression of p-mTOR/mTOR ratio (Fig.4.5 C, F). Neither mTOR nor P-mTOR protein expression was altered among groups. Neither sex nor IF impacts protein synthesis in the fast and slow-twitched muscles. Neither Soleus nor EDL expression of the p-mTOR/mTOR ratio was significantly altered within groups. Data are presented as mean $\pm$ SE.

IF significantly altered inflammation in the skeletal muscles

Our study found that IF significantly alters the inflammatory response in skeletal muscle via TNF- α , a key cytokine involved in the inflammatory response. We also examined the potential differential effects on fast and slow-twitch muscles. Fast-twitch fibers are involved in powerful contractions, while slow-twitch muscles are endurance-oriented and fatigue-resistant. Our results show that TNF- α expression was lower in fast-twitch (EDL) muscle fibers ($p=0.0079$ vs AL males) in IF males.

Figure 4-5 TNF- α expression was lower in IF males

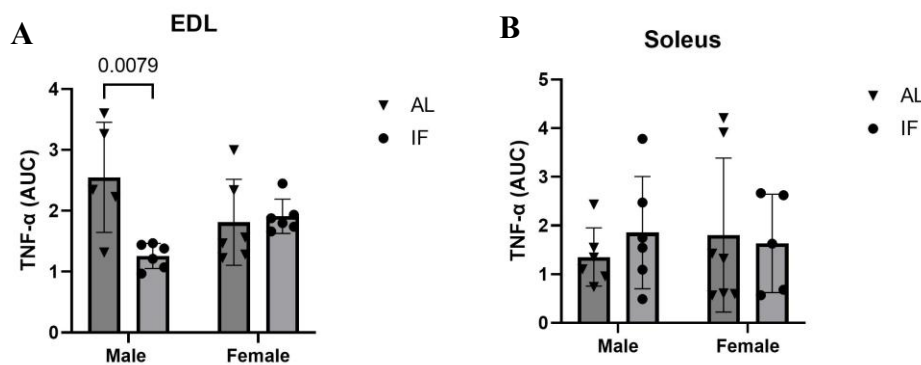


Figure 4. 6 Tumor necrosis factor alpha expression in EDL and Soleus muscles of aged females and male rats. In the EDL, TNF- α expression was lower in IF males compared to AL males ($p=0.0079$), while females showed minimal changes. (Variation: Interaction $P=0.0098$, Feeding $P=0.8628$, Sex $P=0.0235$). In the soleus, TNF- α expression was not altered across groups. Data are presented as mean $\pm$ SE.

Discussion

Summary of key findings

The current study investigated the effects of acute IF on skeletal muscle/ brain protein synthesis (via mTOR) and inflammation in aged F344 rat models. First, we noted that acute IF intervention did not impact body weight or brain and skeletal muscle masses in males or females. In addition, the fasted females consume an average of 5.7g of food per day/per animal (Vs AL females $12.4 \pm 0.80\text{g}$), and fasted males consume an average of 8.3 g of food per day/per animal (vs AL males $19.8 \pm 0.48\text{g}$).

While acute IF had no effect on body mass, it significantly lowered cortical inflammation in females, as measured by lower expression of TNF- α in the brain cortex. No IF-induced inflammatory effect was seen in males or in hippocampal tissues. The observed regional reduction in TNF- α may reflect differences in astrocytic cytokine signaling between brain regions, as cortical and hippocampal astrocytes can differentially regulate the release of inflammatory cytokines. Accordingly, 3 days of IF lowered inflammatory markers in the cortex (41). Protein synthesis was not altered in the brain tissue as a result of IF (via mTOR and p-mTOR).

Several diet restriction strategies, such as IF and CR, have enhanced protein breakdown while reducing protein synthesis in the brain and skeletal muscle (9,18). However, in the present study, the mTOR expression remained the same following acute IF intervention (2 fasted days and 1 feeding day). While IF did not affect muscle protein synthesis, it did significantly lower skeletal muscle inflammation in males as measured by lower TNF- α protein expression in the EDL. These results also suggest that longer IF duration is required for altered protein synthesis in the brain and skeletal muscle.

IF-induced effects on skeletal muscle protein synthesis

In contrast to our prior studies reporting mTOR expression was lower in young animals, no significant changes in protein synthesis (via mTOR) were observed in aged animals (19). It has been shown that sustained activation of mTOR in skeletal muscle inhibits autophagy, a process involved in the degradation of cellular components (18). Although autophagy was not measured in this study, it is possible that protein synthesis remained unchanged in aged rats, thereby maintaining skeletal muscle mass (20). Multiple pathways indicate that several dietary restrictions and starvation patterns decrease mTORC1 expression while increasing autophagy markers in animal studies (18). However, Z Erlangga et al. (21) observed that plasma UNC -51 like kinase 1 (ULK-1), which is an autophagy-activated kinase, increased during the first week of IF and depleted to a basal rate a few days after the intervention. Yoshii et al. demonstrate that IF reduces body fat while preserving skeletal muscle through regulating protein synthesis and autophagy. For example, IF enhances insulin sensitivity and increases the phosphorylation of ribosomal protein S6, a downstream target of mTOR (20). In skeletal muscle, IF suppressed autophagy during fasting, as evidenced by reduced light chain 3-II to light chain 3-I ratio, a marker for autophagosome formation (20). In the current study, consistent with the lack of changes in protein synthesis (via mTOR) observed in skeletal muscle, IF did not alter protein synthesis in the brain tissues.

IF induced effects on the brain protein synthesis

It has been shown that higher food consumption and insulin receptor activation activate the mTOR signaling pathways, which leads to increased protein synthesis (22). In the present study, the total food consumption during the fed stage was higher in the IF groups compared to

the AL groups. Even though they had lower average food consumption throughout the intervention, their protein synthesis (via mTOR) remained consistent following IF. In contrast, our previous finding revealed that in young rats introduced to two months of IF showed lower mTOR expression in the brain cortex; (19) however, we did not see any alterations in aged rats. Similarly, Chapter 5 indicates that 12-month-old healthy rats introduced to 8 weeks of IF significantly reduced mTOR expression in the brain cortex. This evidence suggests that aged animals may need longer IF duration to regulate the mTOR pathway. Prior studies have shown that the mTOR signaling plays a critical role in the aging brain, with pharmacological reduction of mTOR activity in animal models of AD shown to rescue amyloid beta-induced cognitive decline (23). Future studies should investigate the minimal and safe duration of IF required to effectively modulate mTOR pathways in aged rat models, as aging increases vulnerability to neurodegenerative diseases such as AD.

Previous studies have identified that dietary restrictions significantly increase ketone bodies, such as (BHB) (24), which can pass through the blood-brain barrier and can increase the expression of brain-derived neurotrophic factor (*bdnf*), as *bdnf* is involved in neurogenesis and cognitive function (25,26). In the present study, the total average food consumption was lower in IF groups; however, cognitive performance was not altered following IF.

IF-induced effects on the brain and neuroinflammation

Although no significant differences in TNF- α expression were observed in male brain tissues, IF reduced cortical inflammation in females. Previous studies have shown evidence that elevated levels of amyloid beta were linked to neuroinflammation in female mice with AD, which can also exacerbate cognitive declines (27). A previous study found that females

introduced to a high-fat diet increased activation of glial cells (both astrocytes and microglia) and glucose intolerance compared to AD males (27), and these activated astrocytes and microglia enhance proinflammatory cytokines such as TNF- α (28). Interestingly, our acute IF study has demonstrated that TNF- α levels were lower in the female brain cortex. Diabetic patients have higher systemic TNF- α expression, which can be correlated with serum glucose and insulin resistance (14,29). Especially in mouse models of obesity, one of the diseases that can cause inflammation is type 2 diabetes, which can also link to cognitive declines in transgenic AD mouse models (27). For example, Navaratna et al. suggest that cerebrovascular BDNF protein was reduced in the cortical endothelium in 6-month-old diabetic rats (30). Investigating inflammatory pathways in the female brain is particularly important, as females are more susceptible to metabolic diseases such as obesity, diabetes, and neurodegenerative disorders such as AD. Similar to current findings, in males, Erlangga et al. showed that two weeks of IF did not change the TNF- α expression in postmortem brains; however, one week after the two weeks of IF-initiated period, TNF- α was significantly reduced (44% reduction) compared to basal TNF- α expression (21). Previous studies have also found that IF significantly reduces IL-6 (Interleukin 6) and TNF- α expression in the plasma while improving cognitive functions (memory) in male mice (15). Acute IF induced adaptive inflammatory alterations in the female brain, whereas in males, it was associated with reduced inflammation in the skeletal muscle.

IF induced effects on the peripheral tissues, neuroinflammation

Our results show that TNF- α expression in skeletal muscle was lower in IF males compared to AL males, suggesting an IF-induced response to neuroinflammation. These findings indicate that short-term fasting can modulate muscle inflammation in aged males. A previous

study supports the role of TNF- α in muscle physiology. For example, previous studies showed a significant negative correlation between TNF- α and lower-body muscle performance (31). and its contribution to muscle catabolism, which ultimately reduces the muscle mass and strength in both healthy aged men and women (32). Evidence also suggests that fasting can impact inflammatory responses in females. For example, a study by Dannecker et al. reported that an 8-hour fast in untrained women did not alter exercise-induced markers of muscle damage, such as pain, strength loss, or muscle swelling; however, IF significantly reduced circulating TNF- α and increased nitric oxide levels, indicating reduced inflammation and enhanced vascular functions (33). Similar to physical exercise, IF may act as a mild physiological stressor (eustress) that promotes adaptive responses (15). Although exercise can increase pro-inflammatory markers such as interleukin-6 (IL-6) in muscles (34), it can also induce anti-inflammatory adaptations, including a reduction in TNF- α and an increase in IL-10. Notably, prior work in post-myocardial infarction rat models introduced to exercise training has reported lower pro-inflammatory cytokine expression (TNF- α) and higher anti-inflammatory cytokines (IL-10) expression in Type 1 soleus muscles (35). In contrast, our findings suggest a greater inflammatory response in the EDL, highlighting potential differences between fasting and exercise-induced adaptations in skeletal muscle inflammation.

The minimal inflammatory changes observed in aged female skeletal muscle may be due to sex hormones (36), as estrogen has been shown to suppress pro-inflammatory cytokines in skeletal muscles. In the aged females, hormone replacement therapies significantly reduce pro-inflammatory cytokines such as TNF- α and IL-6 in muscle biopsies, which are also associated with a reduction in skeletal muscle damage (36). While TNF- α expression in the adipose was not measured in the present study, the TNF- α pathway may be associated with fat mass. K Speaker

et al. saw correlations between TNF- α expression and fat mass (37). In the stressed Fisher rats, the 24-hour fast has reduced the TNF- α expression in white fat. Fasted control rats (without the stress induced by electric shock) reduced all basal cytokines in the plasma and adipose tissue (15). Together, these data support that fasting may modulate inflammation in both central and peripheral tissues.

Limitations

One of the limitations of the present study was that rats were not individually housed, and average energy intake was calculated as cage intake. When it comes to clinical applications, the basal metabolic rate (BMR) of rodents is 6.4 times faster compared to humans. Different eating patterns, duration of fasting, and circadian cycle may alter the molecular outcome in clinical applications in response to acute fasting (39,40). The relatively small sample size may have limited the statistical power of the present study. Power analysis based on preliminary data suggests that approximately 10 male and 10 female animals per group would be required to detect significant differences in primary outcomes, such as mTOR signaling and memory performance, with a statistical power of 0.80 at $\alpha = 0.05$.

Conclusion

This study highlights the importance of sex specific responses to IF, specifically in central and peripheral neuroinflammation. This study suggests that the brain inflammatory response to acute IF may be more pronounced in aged females. In contrast to young Fisher 344 animals introduced to two months of IF (19), the mTOR pathway was not altered in aged animals, suggesting a longer IF duration might be necessary to reverse the mTOR pathway or the

inability of aged animals to respond. The increased susceptibility of females to metabolic disease and neurodegenerative diseases underscores the relevance of targeting brain inflammation as a key mechanism in therapeutics. The observed alterations in TNF- α expression in IF male skeletal muscle were noteworthy. Future studies should further investigate how intermittent fasting influences central and peripheral inflammatory (both adipose and skeletal muscle) pathways in AD models.

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Chapter 5 - Intermittent Fasting alleviates neuroinflammation in TgF344-AD rats

Abstract

Background: Alzheimer's Disease (AD) is a progressive neurodegenerative disease, and emerging evidence suggests that adipose-derived inflammatory mediators may contribute to AD pathology and behavior by promoting neuroinflammation in the brain. Intermittent Fasting (IF) has gained attention as a non-pharmacological intervention capable of improving metabolic and brain health; however, its effects on neuroinflammatory pathways in AD remain unclear. We hypothesize that IF would rescue memory in TgF344-AD rats (APP/PSEN1) by reducing inflammatory adipokines and attenuating brain inflammation through lipocalin-2 expression. It has been reported that mammalian target of rapamycin- Adenosine Monophosphate Protein Kinase (mTOR–AMPK) is upregulated in AD, and we will examine the effects of IF on the (mTOR–AMPK) axis in both healthy and TgF344-AD rats. Methods: Twelve-month-old male TgF344-AD (n=26) and wild-type control rats (n=25) were assigned to ad libitum (AL) feeding or alternate day fasting for eight weeks. The IF group had full access to food on an every other-day basis for 8 weeks. After the 8 weeks of intervention, memory and locomotor activity were assessed using the Morris Water Maze and the Open Field Test. Following behavioral testing, brain and adipose tissues were isolated and snap frozen in liquid nitrogen for future analysis. lipocalin-2 (LCN-2), tumor necrosis factor alpha (TNF- α) expression were quantified in tissues. Prism GraphPad was used to determine group differences (one-way ANOVA) and correlations (Pearson) with $\alpha < 0.05$. Results: Body weight gain was higher in AD rats ($p < 0.05$). Memory performance in the MWM and distance traveled in the open field test were unchanged following IF intervention. In TgF344-AD animals, LCN-2 expression was lower in IF males compared to

fasted AD animals ($p=0.0318$); however, no significant changes were observed in adipose tissue. TNF- α expression was not altered by IF in either the AD or the control groups. Additionally, mTOR expression was lowered in healthy IF males ($p=0.0460$) but not in TgF344-AD rats introduced to IF. Conclusion: IF induced lower cortical neuroinflammation, without affecting adipose inflammation. We also observed differential effects of IF on mTOR expression in the brain, with no changes in mTOR expression in AD brains and lower mTOR expression observed in the healthy cortex. This study highlights IF as a potential non-pharmacological strategy to modulate inflammatory pathways in AD brains and nutrient-sensing pathways in aged brains.

Background

Alzheimer's Disease (AD) affects about 6.5 million Americans age 65 and older, and the number could extend up to 13.8 million by 2060. AD ranks as the fifth leading cause of death among older Americans, and while mortality from stroke and heart disease declined between 2000 and 2019, mortality associated with AD increased by 145% (1). AD is characterized by memory loss, apathy, impaired communication, disorientation, confusion, difficulty speaking, walking, and swallowing. There is an urgent need to improve the treatment options for individuals with AD, which requires a better understanding of the underlying mechanism that is currently unknown. Increasing evidence suggests that neuroinflammation play central role in AD pathophysiology (2).

Neuroinflammation as a Central Driver of AD Pathology

Neuroinflammation is now recognized as a key driver of AD pathology. Activated microglia and astrocytes produce proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), which disrupt neuronal functions (3). In AD, amyloid accumulation drives chronic activation of these microglia, resulting in continuous production of pro-inflammatory cytokines (4).

Beyond the brain, adipose tissue function as an energy storage and active endocrine organ that secretes adipokines and other inflammatory mediators that are capable of influencing central inflammation. Obesity can alter the metabolic status of adipose tissue, and adipose tissue can become highly inflamed (5). For example, excess adipose mass is associated with increased levels of proinflammatory markers in the systemic circulation (IL-6, C-reactive protein) (6). In addition, lipocalin-2 (LCN-2), a neutrophil gelatinase associated lipocalin, is an adipokine that is secreted from adipocytes and has been shown to increase neuroinflammation and associate with neuronal cell damage in the brain (7). In addition, LCN-2 is known to increase blood-brain barrier (BBB) permeability, which allows inflammatory substances to cross the BBB (8). However, further research is needed to better understand the role of LCN-2 in and BBB disruption in AD.

Intermittent Fasting as a Modulator of Inflammation and mTOR Signaling

Intermittent fasting (IF), a time restricted fasting, has emerged as a promising intervention in brain health with multiple neuroprotective benefits, such as ameliorating memory deficits (9) and a reduction in anxiety-like behavior (10) in rodent models. Additionally, IF reduces disease pathologies such as amyloid plaque accumulation (11). Some proposed

mechanisms underlying these benefits include metabolic switching, altering nutrient sensing pathways, and modulating inflammatory pathways.

IF has been shown to mitigate brain inflammation. It has been shown that IF reduces astrocytic LCN-2 protein expression in the mouse brain, which has been associated with brain astrogliosis, an increased astrocyte reactivity (12). A study by Wu et al. demonstrates that IF ameliorates AD pathology and cognitive deficits in the AD model, which is associated with lipid droplets associated with microglia (11). These microglia with fewer lipid droplets have been associated with enhanced phagocytosis, which may contribute to enhanced clearance of Amyloid β (11). This provides support for the use of dietary intervention against AD pathology. However, the beneficial effects of IF on neuroinflammation in AD animals have yet to be fully examined.

Additionally, in a healthy individual, nutrient and insulin signaling activate mTORC1, promoting growth and protein synthesis and maintaining basal autophagy, a key homeostatic process responsible for the degradation and recycling of cytosolic components (13). Conversely, during fasting, mTORC1 signaling is suppressed and promotes autophagy (13,14). In a normotensive individual, activation adenosine monophosphate protein kinase (AMPK), a sensor of low cellular energy, leads to inhibition of mTORC1, thereby reducing anabolic processes while promoting catabolic processes such as autophagy (15). However, in AD, hyperactivation of mTOR signaling has consistently been observed (16). Our own work in Fischer-344 rats introduced to ADF demonstrated that cortical mTOR reduction occurred independently of tuberous sclerosis complex (TSC -1), an upstream regulator of mTOR, suggesting an alternative mechanism of regulation. In this current study, we will examine fasting differently after the brain mTOR-AMPK axis in TgF344-AD vs healthy rats.

We hypothesize that intermittent fasting rescues memory in aged Fisher-344 AD rats by reducing inflammatory adipokines while attenuating brain inflammation via lipocalin-2 expression. In addition, we aim to examine the effects of fasting on the mTOR-AMPK axis in the brain.

Methods

Ethical approval

All methods carried out in this experiment were performed in accordance with the guidelines for the Care and Use of Laboratory Animals under the approval of the Institutional Animal Care and Use Committee of Kansas State University (protocol code 4466 and date of approval Oct 9, 2020).

Experimental design

Fifty-two 12-month-old male wild type F344 rats (n=26) and TgF344-AD (n=26) were randomly assigned into Ad-libitum (AL)(n=26) or intermittent fasting (IF)(n=26) groups. Prior studies suggest 10 females and 10 males in each group to detect significant differences in the primary outcomes (mTOR, memory) that exist power of 0.8 at a $p < 0.05$ level (33) In male IF rats, we observed IF-induced changes in body weight, food intake along with the alterations in mTOR expression. First, we wanted to evaluate male AD rats and future studies will extend this work to female AD models to assess potential sex specific responses. The TgF344-AD rat line expressing mutant human amyloid precursor protein and presenilin-1 transgenes (TgFischer-344 rats are bred in house and genotype is confirmed via Transgenyx). These rats housed 2-3 animals per cage, on a 12:12 light: dark cycle in the Kansas State University (K-State) Comparative Medicine Group facility, accredited by AAALAC International (000667), OLAW (A3609-01)

and USDA (48-R-0001) assured.) The AL group had 24/7 access to food and water, while the IF group had 24/7 access to water and full access to food on an every-other-day basis (i.e., alternate day fasting). Rats were maintained in a 68 -79°F temperature range throughout the study. Food was taken or given at the beginning of the animals' dark cycle each day. The feeding intervention was 8 weeks in duration, and weekly food intake was measured for eight weeks. Rats were fed the 5001 Rodent Lab Diet. After eight weeks of diet and 14 months of age, animals were tested on memory and distance traveled in the open field test. The animals were placed under anesthesia with 3–4% isoflurane in an induction box. Absence of response to tail and toe pinch reflexes ensured they were fully anesthetized. Animals were decapitated with a large rodent guillotine. Brain tissues and adipose tissue were collected for molecular analysis.

Morris Water Maze (MWM)

Spatial learning and short-term memory were assessed using the Morris Water Maze (17). Briefly, this MWM protocol consisted of a four day protocol. The MWM protocol was slightly modified from previously published procedures to accommodate video based tracking and improve the accuracy of behavioral measurements. These adjustments did not alter the fundamental principles of the MWM task. During the learning and test trials, the pool (180 cm diameter) was filled with water to a level that was 1 inch above the platform, and nonfat dry milk was used to make the water opaque (days 2-4). Once the water temperature reached 25°C, the animals were placed in the pool and allowed to find the hidden platform for 2 minutes, using visual clues. Four learning trials were conducted consecutively for days 1-3, and the time to reach the platform was recorded. During the trials, animals were allowed to swim freely and locate the hidden platform. The animals were directed to the platform if they were not able to

find it within 120 seconds on their own. On the fourth day, a test trial was performed. In the probe trial test, the platform was removed, and the time they spent in the target quadrant (where the previous platform was located) was recorded. % time spent within the target quadrant was recorded and used for analysis. All of the trials were recorded using the Etho-vision video tracking system (EthoVision XT Version 17.0.1622).

Open Field Test (OFT)

Locomotor activity in the open field was assessed using the Open Field test (18). OFT was used to quantify distance travel as a measure of cage activity and to observe that any alterations in body weight and composition shifts were not affected by differences in activity level between groups. The apparatus was placed in a lighted, quiet testing room, and the testing was conducted during their light cycle. Rats were placed in the open field for over 10 minutes, and the distance traveled was recorded using the Etho Vision video tracking system (EthoVision XT Version 17.0.1622).

Tissue Harvest

After the 8-week intervention, animals were placed under anesthesia with 2-3% isoflurane. Reflexes were tested with tail and toe pinches to ensure they were fully anesthetized prior to decapitation. The brain frontal cortex (20mg) and the whole hippocampus were collected following decapitation. Subcutaneous white fat pads (SWAT) were harvested from the inguinal region, and visceral white adipose tissue (EWAT) was collected from the perigonadal area. These harvested tissues were snap-frozen in liquid nitrogen and stored at -80°C until further homogenization.

Homogenization and Western Blotting Procedure

Brain tissues (whole hippocampus 50-60 mg, frontal cortex 20 mg) stored at -80°C were homogenized using the RIPA buffer with protease inhibitor (1 mg: 25 µL) (20). The homogenized samples were gently agitated for 2 hours at 4°C, then centrifuged at 17000 x g RCF for 20 minutes. Thick, rounded glass rods and heavy-walled test tubes were used for manual homogenization. Adipose samples were centrifuged at 4025 x g RPM for 15 minutes. Then triton-X-100 was added to a final concentration of 1% (V/V) and the samples were mixed and incubated for 60 min and spun again at 12000 RPM for 5 min. Supernatant was collected. Protein concentration was measured using a Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA), following the manufacturer's instructions. Protein quantification was completed with the Jess Protein Simple automated system (Bio-Techne, Minneapolis, MN). Total sample protein was quantified with the Jess total protein detection kit (total protein reconstruction agent 1 & 2 REF 043-824-C). Total protein was used to correct the target proteins. This is an accurate method to replace the need for a housekeeping protein. The following antibodies were used to detect target proteins: LCN -2, (Thermo Fisher, 26991-1AP, rabbit, 1:10), TNF- α (Novus, D151805, rabbit, 1:10), AMPK (Thermo Fisher, 10929-2-AP, rabbit, 1:100), mTOR (Santa Cruz, sc-517464, mouse, 1:100).

Statistical Analysis

Group differences were identified via two-way ANOVA with GraphPad software (version 9.5.0 (730)). Pairwise group differences were analyzed using Tukey's post hoc multiple-comparison test. Protein expression data collected via Compass software (Biotechne, version 5.0) generated the area under the curve values (AUC). Over time, body mass and food intake data were

analyzed via mixed-effect analysis. Statistical significant was set as $p < 0.05$, with two tail hypothesis testing. A one-sample t-test was used to determine whether the percentage of time spent in the target quadrant differed significantly from chance level (25%), indicating spatial memory performance. Data are presented as mean $\pm$ standard error. All statistical significance was set as $p < 0.05$.

Results

IF reduced body weight gain in AD animals to a level similar to wild type controls

Figure 5.1A shows the body weight changes over the 9-week study for all experimental groups. ($p < 0.001$). Compared with AL-fed controls, TgF344-AD animals showed a greater body weight gain under AL feeding conditions. Under fasting conditions, AD animals exhibited lower body weight gain over time. The data points indicate weekly body mass. Due to group housing, individual animal intake was not available, and group housing was preferred to allow social interactions. Data analysis for body weight showed a significant reduction in the male fasted control group compared to AL-fed controls ($p < 0.0001$).

Figure 5-1 IF induced alterations in body weight shifts

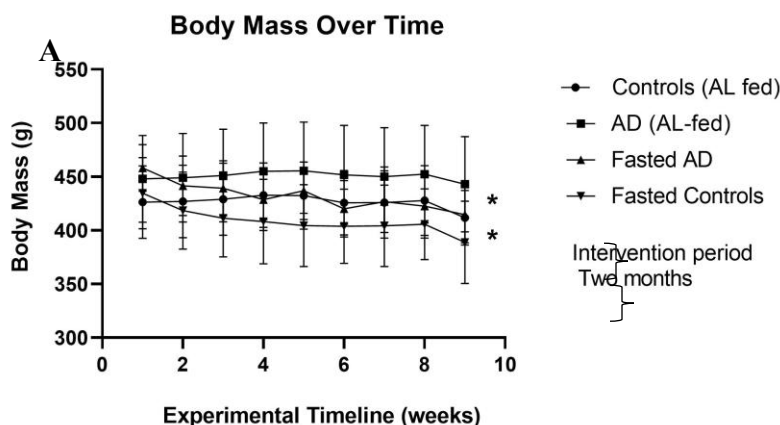


Figure 5.1: Body weight shifts in AD, fasted AD, and controls and fasted controls groups following two months of IF treatment (Figure 5.1A-C). Body mass over time showed a genotype effect, with AD animals maintaining a higher body weight compared to AL-fed controls throughout the experimental period (* $p < 0.001$). The control AL-fed group maintained higher body weights compared to fasted controls (* $p < 0.001$). While both groups exhibited relatively stable body mass across weeks, AD animals consistently remained heavier. IF. Data are presented as mean $\pm$ SE.

IF did not alter deficits in memory in TgF344-AD rats

Figure 5.2 shows the effects of IF on rats' memory and locomotor activity. The percentage of time spent in the target quadrant was analyzed, with chance performance set at 25%. Animals spent less time in the target quadrant compared to the chance level 25%, indicating a memory impairment. In the current study, AD animals exhibit a significant reduction in performance, indicating impaired spatial memory. Notably, fasting did not further exacerbate the cognitive impairments in AD animals. The Open field test was conducted to determine whether differences in body weight shifts were influenced by changes in locomotor activity. Total distance moved during the Open Field Test did not significantly differ among groups, indicating the shifts in body weights were not due to alterations in activity levels.

Figure 5-2 Effects of IF on spatial memory and locomotor activity

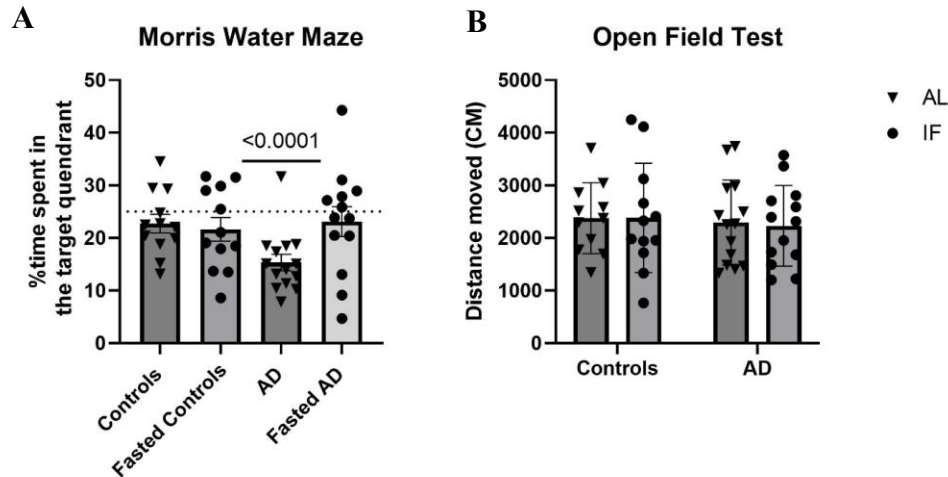


Figure 5.2: Effects of IF on spatial memory and locomotor activity in wildtype and Tg-F344 AD rats. Memory performance was not significant among groups; however, AD animals spent significantly less time in the target quadrant compared to chance level (25%). Total distance moved (cm) within the open field was not significant among groups (Figure 5.2B), indicating that locomotor activity was not altered by genotype or feeding condition. Data are presented as mean $\pm$ SE.

Cortical inflammation was lower in TgF344-AD rats

Figure 5.3 shows the effects of IF on brain inflammation. Elevated LCN-2 expression was associated with reactive astrocytes and neuroinflammation, and its elevation is associated with astrogliosis in AD pathology. In the cortex, lipocalon-2 (LCN-2) expression was lower in fasted AD animals compared to AL- fed controls ($p=0.0318$), indicating that fasting attenuated these adipose derived pro inflammatory cytokines in the brain. In contrast, TNF- α levels did not differ

significantly between fasted groups and AL-fed groups in the cortex. Similarly, in the hippocampus, neither LCN-2 nor TNF- α levels were altered by IF. Together, these findings suggest that two months of IF intervention selectively attenuates brain inflammation in AD animals.

Figure 5-3 Cortical and hippocampal protein expression of lipocalin-2 (LCN-2)

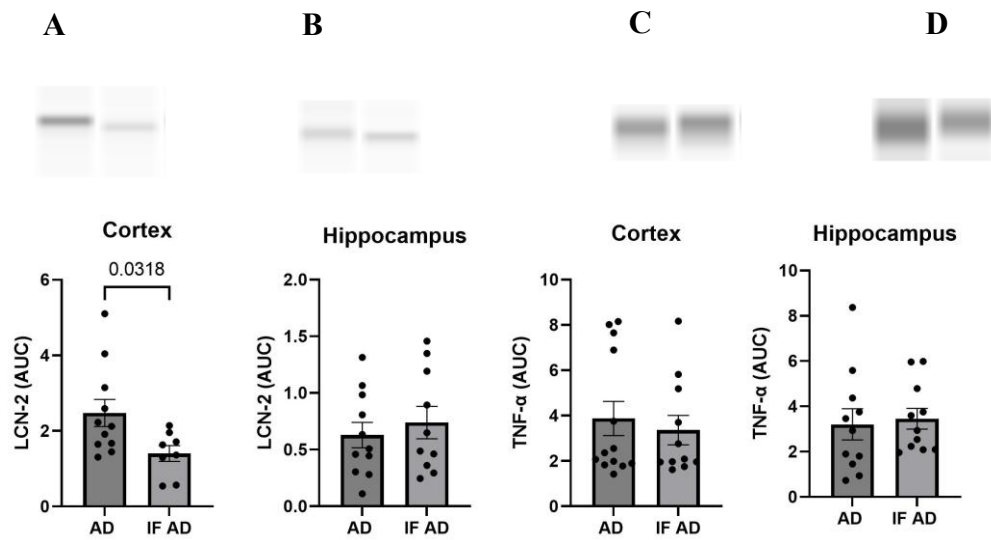


Figure 5.3: Cortical and hippocampal protein expression of lipocalin-2 (LCN-2), Tumor Necrosis Factor Alpha (TNF- α) (Figure 5.3A-D). Cortical LCN-2 expression was significantly lower in male fasted AD animals compared to AL-fed AD animals (Figure 5.3A) ($p=0.0318$). Hippocampal LCN-2 showed no significant differences (Figure 5.3B). In the brain tissues, TNF- α protein expression was not significantly altered among groups (Figure 5.3C-D). Data are presented as mean $\pm$ SE.

IF did not impact inflammation in the adipose tissue in TgF344-AD rats

Figure 5.4 shows the effects of IF on adipose inflammation in TgF344-AD rats. LCN-2 expression was quantified in subcutaneous white adipose tissue (SWAT) from the inguinal region and epididymal white adipose tissue (EWAT) from the visceral fat deposition. Comparison between fasted AD rats and AL-fed AD controls showed no significant differences in LCN-2 expression in either SWAT or EWAT. These findings indicate that two months of IF with a normal diet did not significantly alter LCN-2 mediated inflammation in subcutaneous or visceral adipose tissue in TgF344-AD rats.

Figure 5-4: Effects of IF on LCN-2 expression in adipose tissue

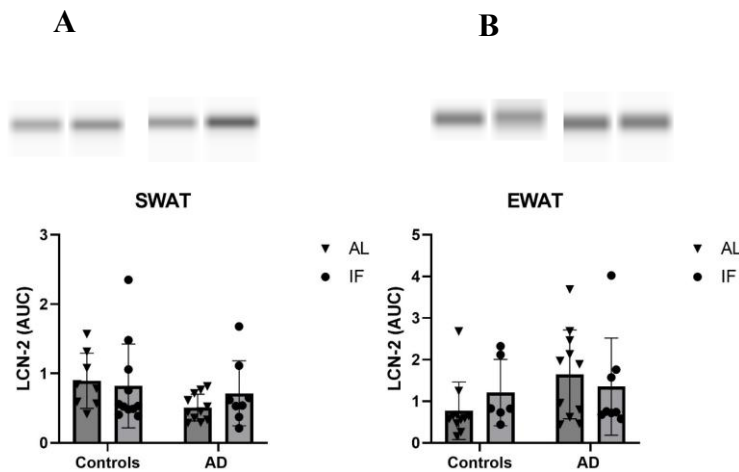


Figure 5.4: Effects of IF on LCN-2 expression in adipose tissue of wild type and TgF344-AD rats. LCN-2 expression was quantified in subcutaneous white Fat (SWAT) (interaction $p=0.3433$, AD/WT $p=0.0996$, feeding= 0.6524) (Figure 5.4A) and epididymal white adipose tissue (EWAT) (interaction $p=0.2729$, AD/WT $p=0.1300$, feeding $p=0.8234$) (Figure 5.4B). No significant differences were observed between fasted and AL-fed TgF344-AD rats. Data are presented as mean $\pm$ SE.

Brain mTOR expression was lower in fasted control rats

Figure 5.5 displays the mammalian target of rapamycin (mTOR) and its upstream regulator, Adenosine monophosphate protein kinase (AMPK) expression in the frontal cortex and hippocampus (A-D). In the cortex, mTOR expression was lower in fasted control males compared to AL-lib fed controls ($p=0.0460$); however, mTOR was not altered in fasted AD animals compared to AD animals (5A). Cortical AMPK levels were not significantly different across groups, although modest variability was observed. In the hippocampus, neither AMPK nor mTOR expression showed statistically significant differences among fasted rats compared to AL-fed control rats (5B,5C). Together, these findings indicate that in healthy animals, IF alters mTOR expression with no or limited effects on AMPK, but this effect was not observed in 14-months old AD animals.

Figure 5-5 Cortical and hippocampal protein expression of mTOR and AMPK

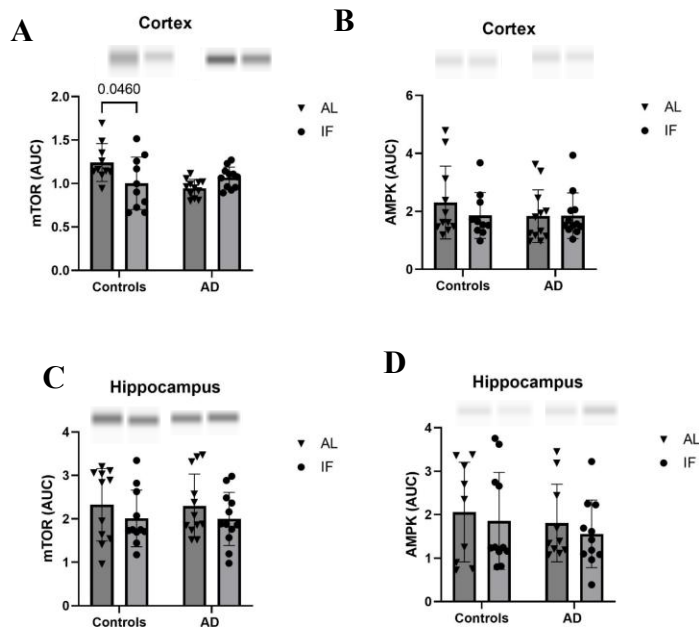


Figure 5.5: Cortical and hippocampal protein expression of mammalian target of rapamycin (mTOR) and Adenosine Monophosphate Protein Kinase (AMPK). In the cortex, mTOR expression differed significantly among groups ($p=0.0460$) (interaction $p=0.0046$, AD/WT $p=0.0611$, feeding $p=0.3370$) with lower mTOR expression observed in fasted controls compared to AL-fed controls, while no significant effect was observed within AD animals (Figure 5.5A). Cortical AMPK expression was not significantly altered by diet or disease status (Figure 5.5B). In the hippocampus, neither mTOR nor AMPK protein expression levels differed significantly among groups. Data are presented as mean $\pm$ SE.

Pearson Correlation analysis in TgF344-AD animals

A positive correlation was found between LCN-2 expression in EWAT and body weight ($r=0.74$). Both hippocampus and brain mass have a positive correlation with post body weights ($r(\text{hip})=0.69$, $r(\text{brain})=0.61$). Inflammatory markers showed several correlations across tissues. TNF- α levels in the hippocampus were positively correlated with LCN-2 levels in the hippocampus ($r=0.54$).

Figure 5-6 Heat map of Pearson correlation in male TgF344-AD rats

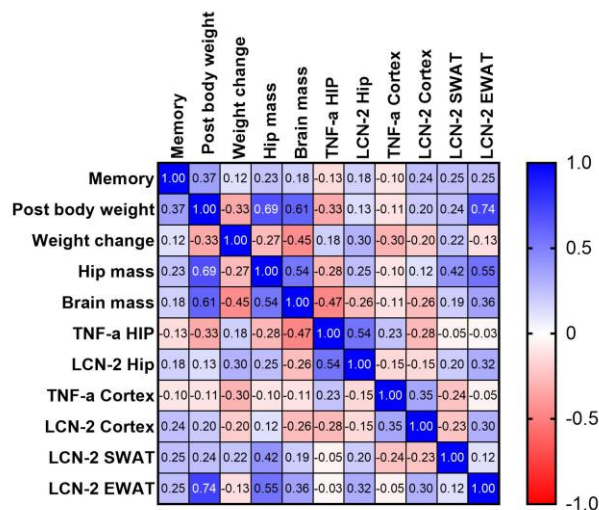


Figure 5.6: Heat map of Pearson correlation in male TgF344-AD rats. Blue indicates positive correlation with shade, noting the strength, and red indicates a negative correlation. (Hippocampus, EWAT- epididymal white fat (visceral fat), SWAT- subcutaneous white fat)

Discussion

Summary of findings

In the present study, we observed that TgF344-AD rats exhibited significantly higher body mass compared to wild-type controls. A two month IF intervention lowered the rate of weight gain in male TgF344-AD rats. In addition, LCN-2 expression was lower in the frontal cortex of TgF344-AD rats, while no significant alterations were observed in subcutaneous white adipose tissue. The downstream regulator of LCN-2, TNF- α expression, was not affected by fasting in the brains of either AD or control rats. In fasted healthy males, mTOR expression was lower in the brain cortex compared to AL-fed controls; however, this effect was not observed in fasted TgF344-AD rats. In AD rats, a positive correlation was observed between body weight and inflammation (via LCN-2) in the visceral white fat.

IF drives body weight alterations in TgF344-AD rats

In the present study, we observed that TgF344-AD rats exhibited significantly higher body mass compared to wild-type controls, and IF attenuated this weight gain in male TgF344-AD rats. In the TgF344-AD rat model, physiological body weight measurements have shown that AD rats exhibit greater body mass than wild type rats at baseline (12 months: AD male 492g vs WT male 475g) (21). Our study also indicated that food consumption was higher in

AL-fed AD animals compared to fasted AD animals (n=6). Average food intake (grams/animal/day) was $17.85 \text{ g} \pm 1.14 \text{ g}$ in AL-fed AD rats and was lowered to $14.91 \text{ g} \pm 1.27 \text{ g}$ in fasted AD animals. This increased food intake in AL-fed AD rats likely contributes to the greater body weight observed in these adult rats. In addition, the strong positive association ($r=0.74$) between body weight and inflammation in visceral adipose tissue suggests that weight gain or increased adiposity may promote peripheral inflammation.

Similar to our findings, a study by Knight et al. demonstrated that two-month-old 3XTg AD mice had increased food intake (17%) and greater body weight gain; however, in contrast to our findings at 12 months of age, middle-aged AD mice consumed even more food (30%) while showing lower body weight than wild-type controls. The body weight was lower than that of wild-type controls. This reduction in body weight was accompanied by a significant increase in metabolic rate, indicating greater oxygen consumption (22).

Furthermore, studies have shown that AD mice fed a high-fat diet showed abnormal feeding behavior and increased their calorie intake over time, which is associated with insulin resistance in their brain (23). Consistent with the IF-induced body weight shifts observed in our study, limiting food intake in transgenic AD mice has been shown to prevent weight gain and obesity in these mice. In humans, obesity during midlife is associated with an increased risk of dementia later in life (24). IF has also been shown to reduce body fat and body mass in clinical trials. For example, after 12 months of a restricted feeding protocol in which calories were consumed within an 8 hour window, healthy individuals showed significantly lower body mass and fat mass compared to those consuming a normal diet (25).

Cognitive and behavioral effects of IF in Alzheimer's Disease

In the present study, AD animals showed memory deficits, as evidenced by significantly reduced % time spent in the target quadrant relative to the chance level (25%) in the Morris Water Maze test. However, this effect was not observed in fasted control rats. Previous clinical studies have shown that healthy elderly participants who underwent 30% calorie restriction for three months exhibited improvements in their verbal memory performance. These improvements were correlated with reductions in fasting insulin and inflammatory markers, suggesting that dietary restriction may support cognitive functions in aging individuals (26).

Preclinical studies found that Young APP/PS1 double transgenic mice subjected to 5 months of IF intervention ameliorated memory deficits induced by AD. In this study, AD mice displayed longer escape latencies during training trials, and they spent less time in the target quadrant (resident time) compared to wild-type mice. AD mice treated with the ADF exhibited a remarkable increase in the resident time in the target quadrant. Furthermore, mice treated with alternate-day fasting showed a reduction in the number of A β plaques in the cerebral cortex (27). Similarly, A study by Suzan et al. reported that male Wistar rats subjected to a 12-week IF ameliorated high fat diet induced memory disturbances (via Y-maze test) and brain tissue damage. In contrast to our finding, these IF- treated rats also showed significant improvement in total distance traveled in the open field (28). In the present study, both healthy and AD rats showed no significant alterations in the open field following IF intervention. This suggests that the observed alterations in body weights were independent of locomotor activity. Overall, the present findings may support growing evidence that fasting may support cognitive function under pathological conditions. Therefore, to better understand the mechanism underlying the

behavioral alterations observed with fasting, we next examined inflammatory mediators within the brain.

Central but not peripheral inflammatory changes following IF in AD

In the present study LCN-2 expression was lower in the cortical regions of fasted TgF344-AD rats. LCN-2 is an adipose derived pro inflammatory cytokine that is upregulated in neuroinflammation (29). Elevated LCN-2 levels in the brain have been associated with astrocyte activation and microglial activation (12). A study by Lee et al. investigated the association between adipose and hippocampus in high fat diet fed mice (three week old male C57BL/6). Following IF treatment, they observed a significant reduction in LCN-2 and Galectin-3 (GAL-3) in adipose tissue and within the astrocytes. This study also demonstrated that IF preserved blood brain barrier integrity and ameliorated deficits in spatial memory (12). These findings suggest that IF may mitigate obesity associated with cognitive impairment by suppressing inflammatory cytokines.

Similarly, in the present study, TgF344-AD rats subjected to two months of IF showed lower LCN-2 expression in the cortex. Interestingly, this reduction occurred independently of changes in LCN-2 expression in the subcutaneous adipose tissue. These findings highlight LCN-2-mediated neuroinflammation as a potential mechanism linking brain cognitive functions. Increased adipose inflammation reported in previous studies may be due to changes in diet, as the Tg F3444-AD animals in the present study consumed a normal diet rather than a high-fat diet (12). A strong positive correlation associated with LCN-2 expression in visceral white fat and body weight ($r=0.74$), suggesting that increased adiposity may promote peripheral inflammation in AD rats, which is further supported by the greater body weight gain observed in AD animals.

Clinical studies further support the role of LCN-2 in AD. For example, Plasma LCN-2 expression was elevated in individuals with preclinical AD compared with healthy controls and was associated with cerebrospinal fluid amyloid β levels. Collectively, these findings suggest that lipocalin-2 may be involved in AD pathogenesis (30). TNF- α is a key proinflammatory cytokine that is known to induce LCN-2 in the brain. TNF- α activates microglial cells and stimulates LCN-2 production, and this positive feedback between TNF- α and LCN-2 has been reported in AD (31). Consistent with this relationship, Pearson correlation analysis in the present study showed a positive correlation between hippocampal TNF- α and LCN-2 expression ($r=0.54$), suggesting coordinated inflammatory regulation of these inflammatory mediators within the brain. However, IF did not alter TNF- α expression in the current study, highlighting that the fasting induced reduction in LCN-2 may occur independently of TNF- α mediated microglial signaling in the brain.

In addition to its role in neuroinflammation, recent preclinical evidence suggests that LCN-2 also plays an important role in metabolic regulation and appetite control. In diabetic mice, circulating LCN-2 levels were elevated and associated with altered feeding behavior, and inhibition of LCN-2 has been shown to improve metabolic parameters such as blood glucose, and lowering insulin resistance in type-2 diabetes. Blocking LCN-2 increases AL feeding and restores feeding patterns following fasting, highlighting LCN-2 as a key regulator of energy balance in addition to its role as an inflammatory mediator (32). In this study, the higher food consumption observed in AD rats may be related to dysregulation of LCN-2 in the brain.

IF alters mTOR signaling in healthy controls but not in TgF344-AD rats

AD is associated with profound alterations in cellular metabolism, including dysregulation of both anabolic and catabolic signaling pathways. One of the central regulators of these processes is the mammalian target of rapamycin (mTOR) pathway, and hyperactivation of mTOR pathways has been widely reported in AD (16). A common non-pharmacological intervention to utilize this pathway is intermittent fasting. In the present study, it has been shown that mTOR expression was lower in fasted healthy control rats compared to AL controls, whereas no such effect was observed in AD rats. Our previous work demonstrated that two months of IF lowered mTOR protein expression in the brain cortex of young healthy rats. ($p=0.006$) (33). Similarly, in the present study, 12 month old fasted control rats showed a lower cortical mTOR expression following two months of IF. These findings also suggest that alterations in the mTOR pathway by IF can be brain region specific, as both studies observed no difference in the hippocampal mTOR expression but a significant reduction in the cortex.

In normotensive brains, activation of AMPK, a sensor of low cellular energy, typically inhibits mTORC1, thereby reducing anabolic processes while promoting catabolic processes (15). Preclinical studies indicate that AD brains exhibit concurrent activation of both phosphorylated AMPK and MTOR, which is also associated with increased tau pathology and oxidative damage (16). This co activation suggest distribution of the usual inhibitory link between AMPK and mTOR, thus allowing mTOR to remain hyperactive despite AMPK signaling and potentially contributing to impaired catabolic processes. A study by Babygirija et al. demonstrated that in 3xTg-AD mice (expressing human gene mutations APP, pSEN1, and MAPT), calorie restriction combined with fasting reduced phosphorylation of mTOR substrates, including eukaryotic translation initiation Factor 4E Binding protein 1 (P-4EBP1), a downstream regulator of mTOR (34). Higher levels of naturally secreted A β were also associated with

mTOR hyperactivity in 3xTg-AD mice, and mTOR activity can be normalized by reducing amyloid accumulation (35). Importantly, the mTOR inhibitor drug rapamycin induced autophagy before the formation of A β plaques and tau tangles in 3xTg-AD mice (36). In the healthy brain, autophagy mediates the degradation of proteins and organelles during nutrient starvation, whereas nutrient sufficiency suppresses excessive activation and maintains autophagy flux, a catabolic process regulated by mTOR.

As expected, we didn't observe mTOR inhibition in our Tg344-AD animals with fasting. According to Cohen et al. In the TgF344-AD model, age-dependent accumulation of amyloid plaques in the hippocampus and cortex appeared between 6 and 26 months of age. Our IF (14 months of age) intervention ended at 14 months of age, just before the onset of approximately 40% neuronal loss reported at 16 months (37). The absence of mTOR reduction in 14 month old, fasted AD rats may be due to their already advanced AD pathology. However, we did not quantify the amyloid beta level or neuronal loss in this current study. Overall, these findings suggest that early intervention may be critical for reducing mTOR expression in AD and potentially mitigating pathological impairments.

Limitations

One of the major limitations of this study is that both AD animals and control animals were group housed, which prevented the measurement of individual food intake and the calculation of average food consumption per animal/ per cage. Food intake was therefore analyzed at the cage level for two cages containing fasted AD animals (n=6) and two cages containing AL-fed AD rats (n=6). Additionally, the absence of a pair-fed AL control group limits our ability to fully dissociate caloric intake from fasting effects.

Conclusions

Overall, the present study demonstrates that IF induced physiological and cognitive effects in the TgF344-AD rat model. IF attenuates body weight gain in TgF344-AD animals, suggesting dietary restriction may help counter metabolic dysregulations associated with AD. At the molecular level, IF reduced cortical inflammatory signals, as evidenced by decreased LCN-2 expression, suggesting a potential role of IF in modulating neuroinflammation in TgF344-AD rats. Interestingly, these changes occurred independently of alterations in adipose-derived inflammatory markers and changes in the hippocampus. In addition, IF altered mTOR expression in healthy controls but not in TgF344-AD rats. Together, these findings highlight IF as a promising non-pharmacological intervention that may influence metabolic health, neuroinflammation, and cognitive outcomes in AD. With these benefits, IF could be a feasible lifestyle intervention to improve brain health while improving quality of life in individuals with AD.

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Chapter 6 - Conclusions

This dissertation provides evidence supporting intermittent fasting (IF) as a non-pharmacological strategy to improve brain health and modulate molecular pathways associated with AD. Across multiple experimental models, including young, aged, and healthy vs TgF344-AD rats, this work demonstrates that the neuroprotective effects of IF are highly dependent on sex, age, and genotype. Furthermore, this dissertation also investigated the mechanisms underlying both peripheral and central responses to IF.

In young healthy animals, IF induced sex-specific adaptations in both central and peripheral tissues. Male rats exhibited shifts in body weight and body composition following short-term IF, accompanied by molecular adaptations in the brain, skeletal muscle, and adipose tissue. These changes included decreased mTOR expression in both the brain and skeletal muscles. In contrast, IF females preserved body composition and showed minimal molecular adaptation. Despite these differences, both young males and females showed IF-induced cognitive benefits. IF males showed enhanced spatial memory compared to AL controls, while IF female animals showed a preference for novel objects during the novel object recognition task, indicating that the cognitive benefits of IF may occur in both males and females.

In aged animals, IF primary impact on the neuroinflammatory process. Short-term IF reduced cortical inflammatory markers in females without altering protein synthesis (via mTOR), suggesting that the neuroprotective effects of acute IF may be primarily driven by attenuation of neuroinflammation. Short-term IF in aged rats showed lower cortical lipocalin -2 (LCN-2) expression, an adipose-derived inflammatory mediator that causes neuroinflammation in the brain. Notably, these anti-inflammatory effects occurred independently of changes in adipose

tissue inflammation, suggesting that IF may directly modulate central brain inflammatory pathways. In contrast to healthy controls, IF did not significantly alter mTOR expression in transgenic AD animals, indicating differed response to IF under AD.

Collectively, these findings suggest that in healthy rats, IF primarily modulates nutrient-sensing pathways such as mTOR, indicating that early intervention, prior to the onset of AD pathology, may be critical to alter anabolic processes such as protein synthesis. In aged and AD models, IF exerts its neuroprotective benefits by attenuating inflammatory pathways that are key contributors to disease progression. Overall, this work supports IF as a promising and accessible intervention to promote brain health. This study also emphasizes the need to consider critical variables such as fasting duration, sex, and healthy vs AD status when evaluating its therapeutic potential for brain health.

