THESIS

BRAIN PROTEIN SYNTHESIS RATES AND ENERGY SENSING IN SULFUR-AMINO ACID RESTRICTED MICE

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ABSTRACT

BRAIN PROTEIN SYNTHESIS RATES AND ENERGY SENSING IN SULFUR-AMINO ACID RESTRICTED MICE

Protein homeostasis (proteostasis) is the maintenance of the cellular proteome through protein synthesis, folding, trafficking, and degradation. Loss of proteostasis is considered one of the hallmarks of aging and is a driver for age-related neurodegenerative diseases. Dietary sulfur amino acid restriction (SAAR), a life-/healthspan extending treatment, activates mechanisms that maintain proteostasis in the liver. However, it is unknown if dietary SAAR activates mechanisms promoting proteostatic maintenance in the brain. To address this knowledge gap, wild-type male C57Bl/B6 mice were fed one of two levels of SAAR (expressed as % kcal); 0.18% methionine and 0% cysteine (Low SAAR) or 0.12% methionine and 0% cysteine (High SAAR), or a matched control diet sufficient (0.85-0.88%) in methionine. Deuterium enriched water was used to measure rates of newly synthesized proteins and DNA (as a marker of cellular proliferation). Brains were collected at days 1, 3, 7, 14, 21, and 35 of treatment. Mitochondrial, cytosolic, and mixed fractions of frontal cortex were analyzed for rates of protein synthesis and cell proliferation using GC/MS. A one phase association was used to determine the rate of the rise of newly synthesized protein and DNA to capture the kinetic parameter k (1/d). Phosphorylated and total protein content for AMP-protein kinase (AMPK), ribosomal Protein S6

(RPS6) and eukaryotic Initiation Factor 2 (eIF2) were measured at day 1 (acute) and day 35 (long-term) via western blot. Mitochondrial protein synthesis rates were significantly greater in the Low SAAR diet compared to the matched control diet, but did not differ in the cytosolic and mixed fractions. Protein synthesis rates in all fractions of the High SAAR diet were not different from control. There was no significant difference in cell proliferation rates between the SAAR diets and their control matched diets. However, in the High SAAR diet, as reflected by greater protein synthesis to DNA synthesis ratios, more newly synthesized proteins were allocated toward mitochondrial proteome maintenance rather than cell proliferation compared to control. At day 1, eIF2 activation tended to greater ($p=0.0922$) in the Low SAAR diet compared to control, but was not different at day 35 in the Low SAAR or High SAAR diets. AMPK activation did not differ in the Low SAAR or High SAAR diet compared to their controls at day 1 and day 35. RPS6 activation was not significantly different at day 1 or day 35 in either SAAR diet compared to their controls. This is the first study to simultaneously assess rates of protein synthesis and cell proliferation in the frontal cortex during dietary SAAR, as well as assess activation of key energy sensing proteins. The results from this study show that despite restriction of the sulfur amino acids, rates of protein synthesis were maintained in the cytosolic and mixed fractions with Low SAAR, while the rate of mitochondrial protein synthesis was greater than the control group with Low SAAR but not High SAAR. Dietary SAAR also promoted allocation of more newly synthesized proteins towards maintenance of the existing proteome, with less for new cell proliferation.

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CHAPTER I: INTRODUCTION

Chronic disease is defined as a physical or mental condition that persists for more than one year, requires continued medical attention, and restricts function in daily life (Goodman, Posner et al. 2013). Nearly half of all adults aged 50 and older in the United States are living with at least one age-related chronic disease (Parekh, Goodman et al. 2011), including neurodegenerative diseases (NDs) such as Alzheimer's disease (AD) and Parkinson's Disease (PD). Living with a ND can greatly decrease the quality of life of the individual and their families (Batista and Pereira 2016). The consequences of living with a ND can include loss of personal independence, greater financial burden, increased stress to family and caregivers, and loss of life.

Aging is the primary risk factor for developing age-related NDs. However, lifestyle factors such as nutrition have a strong contribution to the development of NDs. High consumption of foods high in fat and sugar, which are commonly found in the American diet, has been linked to a greater risk of developing a ND (Kanoski and Davidson 2011, Graham, Harder et al. 2016). With an increase in life-expectancy, the number of ND cases is expected to rise (Fernández-Ruiz 2019). Much research has been geared towards reducing symptoms and pathologies associated with NDs, however, there is no cure for these diseases. AD, which is the most common ND, and AD-related dementias have become a funding priority for the federal government with upwards of $3.1 billion of funding in 2020 (Alzheimer's Association 2020).

Despite major advances in AD research, many treatments have not advanced past the clinical trial phase (Oxford, Stewart et al. 2020). One major factor contributing to the failure of many clinical trials is the stage of the disease at the point of treatment initiation (Oxford, Stewart et al. 2020). AD, like many other NDs, is a disease that develops throughout many years before the onset of symptoms. When treatment is started, AD-associated pathology may have already caused irreversible damage, rendering that treatment ineffective. Rather than countering a disease that may have already caused irreversible damage, a more effective method would be to prevent or delay the onset of the disease. One approach is to focus on increasing healthspan, the period of life spent in good health, free from the chronic diseases and disabilities of aging (Kaeberlein 2018), which would delay NDs and other diseases. Lifestyle choices such as nutrition are powerful variables that can be modulated to prolong healthspan. Moreover, healthy nutrition can exert whole-body changes, contributing to disease resistance (Marsman, Belsky et al. 2018, Moore, Hughes et al. 2018, Di Renzo, Gualtieri et al. 2019). Therefore, identifying interventions that target both diet and the process of aging, ultimately prolonging healthspan, are critical for the prevention of chronic diseases such as AD.

In particular, a dietary intervention called sulfur-amino acid restriction (SAAR) has gained interest for its life/healthspan-extending effects. SAAR extends longevity and delays age-related pathologies in animal models including rats (Zimmerman 2003), mice (Miller, Buehner et al. 2005), *C. elegans* (Cabreiro, Au et al. 2013), and *Drosophila* (Lee, Kaya et al. 2014). SAAR's effects on longevity may be due to a variety of systemic improvements in the organism. For example, SAAR has been shown to improve glucose homeostasis (Luo, Yang et al. 2019), increase energy expenditure (Malloy, Krajcik et al. 2006), decrease bodyweight (Malloy, Krajcik

et al. 2006), decrease levels of inflammatory markers (Sharma, Dixon et al. 2019), and activate stress-resistance genes (Wang, Wang et al. 2019). Despite the vast amounts of animal studies, the literature on the use of dietary SAAR as an intervention to improve health in humans is limited due to the small number of studies. However, the few studies show great promise in improving health. For instance, a 16-week methionine restricted diet increased fat oxidation in obese adults with metabolic syndrome, but did not change body composition, which may be attributed to the lack of restricting cysteine along with methionine (Plaisance, Greenway et al. 2011). Another study showed that six days of a SAAR diet increases gene activity of enzymes involved in lipid breakdown in overweight or obese women (Olsen, Øvrebø et al. 2020).

One potential, less-extensively studied mechanism contributing to SAAR's life/healthspan extension, is maintenance of protein homeostasis (proteostasis). Cellular proteostasis is maintained by a network of processes including protein synthesis, folding, transport, repair, and degradation (Morimoto and Cuervo 2009). Loss of proteostasis is a driver of age-related diseases including NDs (Cheng, North et al. 2018). Maintaining proteostasis is crucial for brain health, and potentially for the prevention of age-related NDs (Martínez, Khatiwada et al. 2018, Kurtishi, Rosen et al. 2019). We have previously shown that in mice, dietary SAAR activates energy sensing proteins involved in the maintenance of proteostasis within liver tissue (Pettit, Jonsson et al. 2017, Jonsson, Margolies et al. 2021). However, the impact of dietary SAAR on brain energy sensing and proteostasis has not been investigated. Given our previous findings that SAAR improved mechanisms of liver mitochondrial proteome maintenance, we hypothesized that SAAR will activate energy sensing pathways to maintain mitochondrial proteostasis in the brain. Given that most dietary SAAR studies use a range of

0.12% to 0.18% SAAR, this study will compare two levels of dietary SAAR on rates of protein synthesis and activation of energy sensing pathways in the brain over a 35-day period. The data collected from this study will provide valuable information about how dietary SAAR impacts mechanisms to maintain proteostasis in the brain and whether it could be a potential treatment improve healthy brain aging.

PURPOSE OF STUDY

The purpose of this study is to identify how brain proteostasis is influenced by dietary sulfur-amino acid restriction (SAAR). Specifically, the study is designed to investigate the effects of SAAR on protein synthesis, cell proliferation, and markers of energy sensing in murine brain.

STATEMENT OF PROBLEM

A driver for age-related neurodegenerative disease is loss of proteostasis. The loss of proteostasis can result in neuronal death. Maintaining proteostasis and the ability to adapt to stress is essential for healthy brain aging. Dietary SAAR improves proteostatic mechanisms in liver. However, it is not known whether those mechanisms are activated within the brain and if their activation is due to/associated with increased activity of energy sensing pathways.

HYPOTHESIS

We hypothesize that dietary SAAR will decrease rates of brain protein synthesis in the cytosolic and mixed sub-cellular fractions, while the mitochondrial proteome will be maintained. We also hypothesize that SAAR will have greater activation of proteins involved in cellular adaptation to stress.

CHAPTER II: LITERATURE REVIEW

AGE-RELATED NEURODEGENERATIVE DISEASES ARE ON THE RISE

Aging is the primary risk factor for developing neurodegeneration and conditions such as mild-cognitive impairment and Alzheimer's Disease (AD) (Zonderman, Fozard et al. 2000). The consequences of neurodegenerative diseases (NDs) are far-reaching. Progressive loss of cognitive function or motor function are some of many consequences associated with age-related neurodegeneration. Loss of cognitive function can lead to the loss of personal independence and susceptibility to other mental conditions such as anxiety and depression (Geda, Roberts et al. 2008). In the United States, AD is the most common ND and healthcare costs for treatment exceed $305 billion and is expected to continue rising (Wong 2020). The global population of persons living with NDs has doubled from 1990 to 2016 (Nichols, Szoeke et al. 2019) and is estimated to triple by the year 2050 (Nichols and Vos 2020). The continued rise in the number of persons with ND is a major societal challenge that needs immediate intervention.

EFFECTS OF ADVANCING AGE ON THE BRAIN

Aging is the progressive decline in physiological function of an organism that increases susceptibility to disease and death (López-Otín, Blasco et al. 2013). The related metabolic and structural changes that occur in the brain with advancing age are termed "brain aging" within this document. Brain aging is associated in a decline in cognitive performance that impacts the ability to reason, produce and recall memories, and execute motor function (Harada, Natelson

Love et al. 2013). The volume and weight of the brain declines at a rate of 5% every decade after the age of 40 (Svennerholm, Boström et al. 1997). The age-associated loss of brain volume and weight have been attributed to decreased gray and white matter volumes of the parietal, temporal, and prefrontal regions of the brain (Raz, Ghisletta et al. 2010). In fact, the prefrontal cortex experiences the largest decline in volume as aging progresses (Fjell, Westlye et al. 2009). Importantly, the prefrontal cortex has important roles in executive function which include decision making and reasoning (Kolb, Mychasiuk et al. 2012). Age-related changes in the brain lead to and are further exaggerated in the most common NDs. For example, patients with AD have reduced gray matter and white matter volume in the prefrontal region (Canu, McLaren et al. 2011) and those with Parkinson's Disease (PD) have altered white matter integrity in the frontal lobe (Karagulle Kendi, Lehericy et al. 2008).

As aging progresses, interactions between brain regions become dysregulated (Andrews-Hanna, Snyder et al. 2007), with decreased neural activity in some regions and compensatory overactivation of others (Park and Reuter-Lorenz 2009). Persistent localized overactivation of a brain region, particularly the hippocampus, could predispose that region to developing neurodegenerative-related pathologies (Bakker, Gregory et al. 2012). Demyelination of axons also occurs as aging progresses, contributing to the loss in brain volume and connectivity within brain regions (Bartzokis, Beckson et al. 2001).

The anatomical and physiological changes that occur as the brain ages may be explained by a set of interconnected drivers of aging (López-Otín, Blasco et al. 2013, Mattson and Arumugam 2018). One of the drivers of brain aging is mitochondrial dysfunction. Neurons rely on oxidative phosphorylation to meet their high energetic demands (Zheng, Boyer et al. 2016).

One of the byproducts of oxidative phosphorylation is reactive oxygen species (ROS). Persistently high production of ROS can damage neuronal structures. Antioxidant defenses exist to counteract ROS. However, neurons have naturally low levels of antioxidants (Fernandez-Fernandez, Almeida et al. 2012) and antioxidant levels decline as age progresses (Iskusnykh, Zakharova et al. 2022). Furthermore, the high concentration of polyunsaturated lipids within the brain make it more susceptible to ROS damage (Rauchová, Vokurková et al. 2012).

Mitochondrial dysfunction is interconnected with another driver of brain aging; neuroinflammation. One of the most notable events driving neuroinflammation that occur with aging is the activation of microglia and astrocytes (Kwon and Koh 2020). In a healthy state, microglia and astrocytes release pro-inflammatory cytokines including IL-6, IL-1β, and TNF to protect from dangerous stimuli and to remodel synapses (Weinhard, Di Bartolomei et al. 2018). However, in an aged brain, microglia and astrocytes are chronically activated, which may contribute to metabolic dysfunction and degeneration (Vandenbark, Offner et al. 2021), as well as sustained inflammatory signaling that can cause neuronal injury. Moreover, neurons within the central nervous system are post-mitotic cells that have limited capabilities to recover from injury (Huebner and Strittmatter 2009).

Because the drivers of aging are tightly interconnected, improvement or deterioration of one driver can profoundly impact others. For example, ROS produced from mitochondria of inflammatory cells such as astrocytes can damage proteins (Sheng, Hu et al. 2013). Because of limited protein repair mechanisms (Chondrogianni, Petropoulos et al. 2014), damaged proteins must be degraded and replaced to maintain a functional proteome. However, an accumulation of damaged proteins may overwhelm degradation mechanisms (Cuanalo-Contreras, Mukherjee

et al. 2013), thus resulting in the loss of proteostasis. Additionally, protein aggregates can activate pro-inflammatory signals (Currais, Fischer et al. 2017, Bevan-Jones, Cope et al. 2020).

In contrast, a shared characteristic of many lifespan-extending interventions or long-lived animal models is the maintenance of proteostasis. A global reduction of protein synthesis has been observed in many lifespan-extending interventions and long-lived animal models, which may relieve the burden of protein folding and degradation mechanisms (Drake, Bruns et al. 2014, Drake, Bruns et al. 2015). Interestingly, mitochondrial proteostasis is maintained in life-extending interventions or long-lived animal models, which is indicative of a prioritization of the mitochondria (Hamilton and Miller 2017). However, proteostasis is regulated, in part, by energy sensing pathways, which also become dysregulated during the aging process (Mattson and Arumugam 2018). Therefore, it is important to consider interventions that can improve multiple drivers of aging to maximize the improvement of other drivers of aging. The next sections will focus on impaired energy sensing and loss of proteostasis due to their strong role in the development of NDs (Mattson and Arumugam 2018, Hohn, Tramutola et al. 2020) .

IMPAIRED ENERGY SENSING IS A DRIVER OF BRAIN AGING AND ND

Brain cell types including neurons have high energetic demands that must be constantly maintained (Attwell and Laughlin 2001). Therefore, any fluctuation in energy balance must be rapidly counteracted to maintain neuronal function and survival (Ronnett, Ramamurthy et al. 2009). AMP-activated protein kinase (AMPK) is a master regulator of energy homeostasis and stress-adaptation. AMPK becomes activated in conditions of low ATP availability such as caloric restriction and amino acid deprivation (Steinberg and Carling 2019). AMPK regulates

metabolism of carbohydrates, lipids, and proteins to maintain a sufficient pool of nutrients necessary to maintain cellular metabolism under conditions of nutrient stress (Hardie, Ross et al. 2012). Activated AMPK is responsible for the induction of stress adaptation signaling including amplification of the antioxidant response (Zimmerman, Baldinger et al. 2015), mitochondrial biogenesis (Zong, Ren et al. 2002), and autophagy (Egan, Kim et al. 2011).

Furthermore, key energy sensing proteins including the mechanistic target of rapamycin (mTOR) and eukaryotic initiation factor 2 (eIF2), are involved in the dynamic regulation of energy homeostasis. Activation of mTOR promotes anabolic pathways to increase protein synthesis and cellular proliferation, and inhibits pathways involved in catabolism including autophagy (Saxton and Sabatini 2017). eIF2 functions as an amino acid sensor within the cell. Under conditions of low nutrient status, mTOR is inhibited and eIF2 becomes activated to preserve nutrients necessary to maintain cellular function (Jonsson, Margolies et al. 2019). However, dysregulated activity of AMPK, mTOR, and eIF2 has been observed in NDs. Dysregulated nutrient sensing is considered a driver of brain aging (Mattson and Arumugam 2018). The ability for AMPK, mTOR, and eIF2 to direct strong transcriptional programs in response to nutrient stress make them viable targets to improve brain aging and reduce age-related cognitive decline.

For instance, one study found that human brains with AD have an abnormal accumulation of phosphorylated AMPK in neurons, particularly in neurons that contain neurofibrillary tangles, which are aggregates of hyperphosphorylated Tau and are considered a hallmark of AD (Vingtdeux, Davies et al. 2011). Furthermore, β-amyloid oligomers, which are protein aggregates of β-amyloid and considered another hallmark of AD, activate AMPK and its

chronic activation promotes the loss of dendritic spines (Mairet-Coello, Courchet et al. 2013). Furthermore, inhibition of AMPK activity by compound C decreases amyloid-β associated inhibition of long-term potentiation (Ma, Chen et al. 2014). Interestingly, differences in the protein content of the two α-subunits needed to regulate AMPK activity have been observed in post-mortem brain samples of AD patients, who have greater AMPKα1 isoform protein content while AMPKα2 protein content was less than their age-matched controls. In the same study, cognitive impairment and synaptic density were rescued by repressing AMPKα1 activity in a mouse model of AD (Zimmermann, Yang et al. 2020). These studies indicate that dysregulated AMPK activity could be a driver of age-related NDs.

AMPK is a direct inhibitor of mTOR. Despite hyperactivation of AMPK, which should inhibit mTOR, mTOR hyperactivity has been observed in PD (Zhu, Yang et al. 2019) and AD (Hodges, Reynolds et al. 2018). This observation depicts a major dysregulation of the energy sensing systems within the brain during a diseased state (Diagram 1). However, hyperactivation of mTOR can occur before the onset of AD, reflected by an abnormal accumulation of activated mTOR in post-mortem brain tissue of patients with mild-cognitive decline (Tramutola, Triplett et al. 2015). Furthermore, post-mortem brains from patients with AD have significantly greater protein content of ribosomal protein S6 (RPS6), a protein directly downstream of mTOR that is necessary for protein synthesis (An, Cowburn et al. 2003). An upregulation of protein synthesis through mTOR contributes to the accumulation of tau, which is considered another hallmark of AD (Caccamo, Magrì et al. 2013). Moreover, the degradation of protein aggregates through autophagy is inhibited by mTOR activation, further worsening the toxic accumulation of β-amyloid oligomers (Yang, Stavrides et al. 2011), which can lead to neuronal death (Yang, Wang

et al. 2017). However, AMPK is a potent activator of autophagy, and hyperactive AMPK contributes to synaptic loss through an autophagy-mediated process (Domise, Sauvé et al. 2019). Therefore, the relationship between AMPK and mTOR during age-related NDs needs to be explored further.

When activated by stresses such as low amino acid conditions, eIF2 reduces protein synthesis and activates the integrated stress response (ISR). The ISR coordinates stress-adaptive responses to maintain cellular homeostasis by reducing protein synthesis and activating cytoprotective genes (Jonsson, Margolies et al. 2019). Interestingly, in post-mortem brain tissue of AD patients, there is aberrant hyperactivation of eIF2 (Ojo, Reed et al. 2021). Chronic activation of eIF2 and the ISR have been shown to disrupt memory consolidation and lead to neuronal apoptosis (Bond, Lopez-Lloreda et al. 2020).

Major dysregulation of the key energy sensing pathways, AMPK, mTOR, and eIF2, is evident during NDs (Diagram 1). Specifically, the hyperactivation of AMPK, mTOR, and eIF2, may be due to negative feedback from multiple processes going awry during the diseased state. Therefore, delaying the development of NDs by restoring normal signaling through energy/nutrient sensing during a pre-diseased state through pharmacological or dietary interventions may be a feasible option. Metformin, for example, activates AMPK and reduces oxidative stress in hippocampal neurons of Wistar rats, which leads to an improvement in cognition during aging (Gorgich, Parsaie et al. 2021). Another study shows that metformin activates AMPK in hippocampus and improves cognitive function in mice (Li, Chaudhari et al. 2019). Rapamycin, an inhibitor of mTOR, decreases neurodegeneration and accumulation of β-amyloid and phosphorylated Tau in a rat model of accelerated aging (Kolosova, Vitovtov et al.

2013). Additionally, inhibition of eIF2 through transfection decreases levels of β-amyloid accumulation in 5XFAD mice by reducing levels of the β-site APP cleaving enzyme-1, which is the rate-limiting enzyme involved β-amyloid production (O'Connor, Sadleir et al. 2008). Restoring energy/nutrient sensing, through the use of pharmacological interventions or other forms, can heavily influence and improve other drivers of aging, including the loss of protein homeostasis.

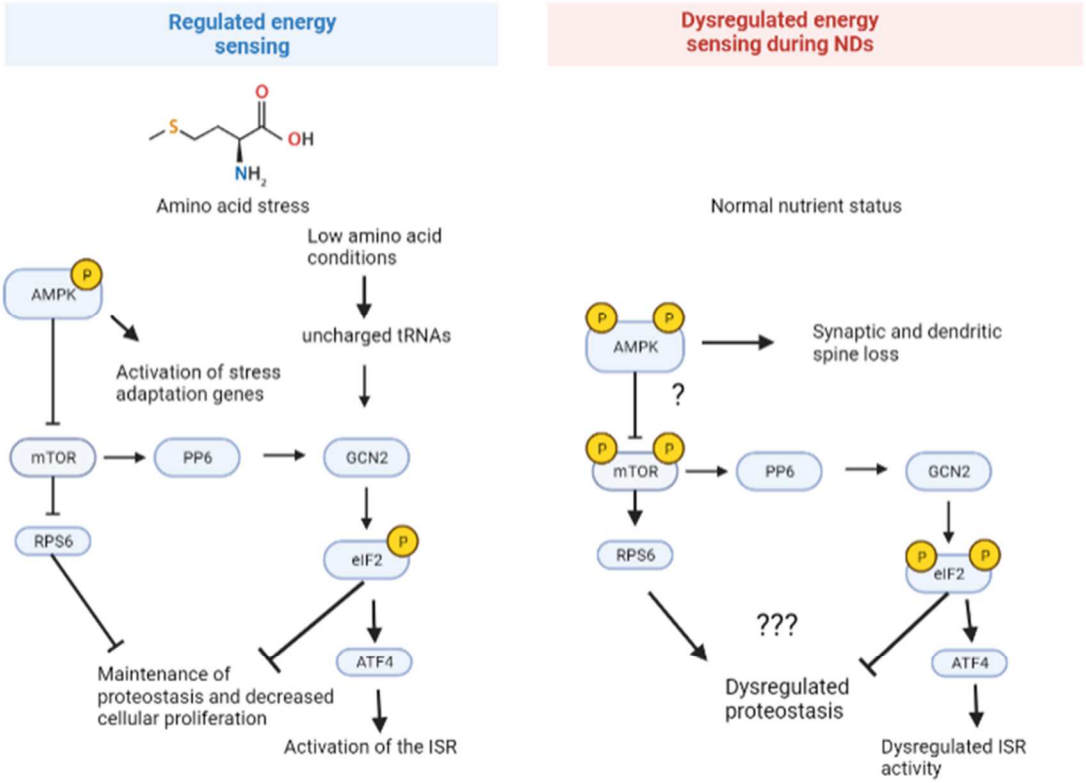

Figure 1. Illustration of key energy sensing pathways during a healthy and diseased state. In a healthy state, nutrient stresses such as amino acid deprivation, activates AMPK and induces the transcription of genes involved in stress-adaptation and inhibits mTOR. Inhibition of mTOR shuts off pathways involved in growth including protein synthesis and cellular proliferation and promotes autophagy to maintain proteostasis. Additionally, the AMPK/mTOR pathway is indirectly connected to the eIF2 pathway. During low amino acid conditions, the amino acid sensor, GCN2, binds to uncharged tRNAs. Activated GCN2 then phosphorylates eIF2 which reduces protein synthesis and activates the Integrated Stress Response (ISR) through ATF4 to promote stress resistance. During NDs, in the absence of nutrient stresses, these pathways are dysregulated. Hyperactivation of AMPK and mTOR is seen during NDs, despite AMPK being an inhibitor of mTOR. Hyperactivation of mTOR is associated with increased protein synthesis and protein accumulation and impaired autophagy, which removes damaged proteins. Paradoxically, eIF2 is also hyperactivated during NDs, which shows a major impairment of energy sensing pathways.

LOSS OF PROTEIN HOMEOSTASIS IS A KEY PLAYER IN BRAIN AGING AND ND

Maintenance of the proteome, which is essential for cell function, occurs via a dynamic system that is constantly responding to cell-intrinsic and extrinsic factors, including energy sensing. A network of mechanisms are required to maintain protein homeostasis or "proteostasis," including protein synthesis, folding, trafficking, and degradation (Webster, Gildea et al. 2020). Protein synthesis is the production of new proteins that are involved in various biological processes such as signaling and structure formation. Protein synthesis or translation is highly prone to errors, with 15% of all translated proteins containing at least one error in their amino-acid sequence, which can lead to irregular protein folding and/or aggregation. Furthermore, damaged proteins begin to accumulate through a decline in chaperoning and degradation systems as age progresses, as depicted by mathematical modeling and experiments in *C. elegans* (Santra, Dill et al. 2019). Moreover, the loss of proteostasis is a common feature of NDs including AD and PD (Yerbury, Ooi et al. 2016). Protein aggregation of β-amyloid or phosphorylated Tau, which are two hallmarks of AD, can become toxic to cells and cause neuronal death (Trojanowski, Goedert et al. 1998, Murphy and Levine 2010). In PD, the clearance of aggregates of the protein alpha-synuclein is impaired, which is strongly associated with the death of dopaminergic neurons involved in the motor control of movement (McKinnon, De Snoo et al. 2020).

Processes to maintain proteostasis become overwhelmed or dysfunctional as age progresses. Chaperones, which are molecules that assist with proper folding of nascent peptides, become overburdened with the increasing amount of age-related protein misfolding

(Santra, Dill et al. 2019). Additionally, the concentration of chaperones declines with advancing age (Yang, Huang et al. 2014), further limiting the capacity to handle protein misfolding.

Mechanisms to degrade misfolded or damaged proteins decline as age progresses. For example, the ubiquitin-proteasome system (UPS), which tags damaged or misfolded proteins for degradation, has a reduction in activity in several regions of the brain including the prefrontal cortex and hippocampus (Dulka, Pullins et al. 2020). The decline in proteasomal activity has been attributed to various factors including decreased levels of ubiquitin (Almási, Murlasits et al. 2021), or interactions with oligomerized proteins that inhibit the formation of the proteasome (Thibaudeau, Anderson et al. 2018). Reduced or aberrant proteasomal activity is a major player in PD (Cook, Stetler et al. 2012) and AD (Ciechanover and Kwon 2015).

Protein synthesis must occur in order to replace proteins that have been damaged or degraded. However, in the context of brain aging and NDs, protein synthesis declines, which is inconsistent with increased mTOR activity observed in NDs A decline in protein synthesis in frontal cortex, cerebellum, and brainstem has been observed in rats three months and older, demarcating differences in protein synthesis once neural tissue has been fully developed (Dwyer, Fando et al. 1980). Similarly, old rats compared to young rats exhibit significant reductions in brain protein synthesis (Fando, Salinas et al. 1980). A decline in protein synthesis is also present in age-related NDs. Post-mortem brains from patients with AD have greater phosphorylation of eIF2, which reduces global protein synthesis in response to cellular stress (Oliveira, Lourenco et al. 2021). Furthermore, aggregation of β-amyloid and phosphorylated-Tau interfere and reduce ribosomal protein synthesis, which may interfere the process of replacing damaged proteins with healthy proteins (Evans, Benetatos et al. 2019).

LIFESPAN-EXTENDING INTERVENTIONS AND PROTEIN HOMEOSTASIS

Interventions shown to extend lifespan in the laboratory, such as caloric restriction and low-protein diets, share the common trait of activation of mechanisms to maintain proteostasis. Caloric restriction, for instance, is a dietary intervention that reduces total calories without malnutrition, resulting in greater longevity in various experimental models (Speakman and Mitchell 2011). Diets low in protein and higher in carbohydrates (LPHC) are also associated with an extension in longevity (Le Couteur, Solon-Biet et al. 2016). Both of these lifespan-extending diets activate AMPK and inhibit mTOR (Rühlmann, Wölk et al. 2016, Ma, Wang et al. 2018). As mentioned previously, AMPK activation can induce transcriptional programs involved in stress-resistance, while mTOR inhibition conserves energy by reducing energetically costly processes such as protein synthesis. For instance, mice fed a calorically restricted diet for 10 months had an improvement in cognition compared to a control diet, and immunofluorescence of hippocampal tissue showed lower mTOR and RPS6 expression (Dong, Wang et al. 2016). Another study found that mice deficient in Apolipoprotein E undergoing caloric restriction for 20 or 64 weeks, have increased activation of AMPK and inhibition of mTOR compared to *ad-libitum* mice, which decreased Tau phosphorylation and improved cognitive performance in Morris water maze test (Rühlmann, Wölk et al. 2016). Another lifespan-extending intervention, rapamycin treatment, inhibits mTOR and has been shown to reduce cortical β-amyloid levels and improve memory deficits in 12 month-old hAPP(J20) mice (Van Skike, Hussong et al. 2021). Additionally, inhibition of mTOR activity in hippocampus of mice treated with a LPHC diet was associated with stronger neuron integrity (Wahl, Solon-Biet et al. 2018). These studies show

that experimental targeting of energy sensing pathways promotes maintenance of proteostasis, and may serve as an intervention to improve the brain aging process.

THE SULFUR AMINO ACIDS: METHIONINE AND CYSTEINE

Methionine and cysteine are two of the four sulfur-containing amino acids. Methionine is an essential amino acid that cannot be endogenously synthesized and, therefore, must be obtained through the diet. Cysteine is a non-essential amino acid that can be synthesized from methionine or obtained from the diet. Common food sources of methionine and cysteine are red meats, fish, and eggs. Methionine holds important roles in initiation of protein synthesis, methylation of DNA, polyamine synthesis, and redox sensing (Lim, Kim et al. 2019).

Methionine's Role in Initiation of Protein Synthesis

In mammals, methionine is required to initiate protein translation. During initiation of protein translation, the 40S ribosomal subunit is bounded by factors eIF-1, eIF-1A, and eIF-3 (Asano 2000). Initiator methionyl tRNA binds to factor eIF-2 (Asano 2000). Then, a group of factors of eIF-4 bring a strand of mRNA to the ribosome. Once the strand of mRNA has been brought to the 40S ribosomal subunit, eIF-3 associates with eIF-4G and opens the strand of mRNA. The 40S ribosomal subunit, in conjunction with methionyl tRNA and all the eIFs, begins to scan the mRNA strand in search for the AUG codon, which codes for methionine. Once the methionine start codon is found, the 60S ribosomal subunit attaches to the 40S ribosomal subunit and forms the 80S initiation complex. The process of protein translation continues with subsequent elongation and termination.

Role in Methylation and Oxidant Defense

Methionine is the precursor to S-adenosylmethionine (SAM). SAM is the main methyl donor to DNA, lipids, and proteins, and is a ubiquitous substrate used in many metabolic reactions (Obeid 2013). Through the action of methionine adenosyltransferase (MAT), methionine receives adenosine to its sulfur-containing region, which produces SAM. Once SAM donates its methyl groups, S-adenosylhomocysteine (SAH) is produced. SAH is an inhibitor of SAM, so it must be further broken down into homocysteine, or remethylated back to methionine (Caudill, Wang et al. 2001). Homocysteine is produced when SAH is broken down through the action of SAH hydrolase. In the transsulfuration pathway, cystathionine-B-synthase synthesizes cystathionine from homocysteine. Cystathionine-y-lyase then hydrolyzes cystathionine to produce cysteine. Cysteine is a precursor to hydrogen sulfide (H2S), which is a major signaling molecule and is involved in protection from oxidative stress (Xie, Liu et al. 2016). Furthermore, methionine is a precursor to glutathione, which is a major antioxidant within the body. In addition to being a precursor to important biomolecules, methionine itself is a strong anti-oxidant. Methionine is highly prone to oxidation due to its sulfur-containing residue. Once oxidized, methionine becomes methionine sulfoxide, which can be converted back to methionine by methionine sulfoxide reductases. Overexpression of methionine sulfoxide reductases can protect tissue from oxidative damage and extend lifespan in various animal organisms (Cabreiro, Picot et al. 2008)

DIETARY SULFUR AMINO ACID RESTRICTION (SAAR) EXTENDS LONGEVITY

In 1993, a seminal study was published describing an extension of longevity by reducing dietary methionine content. In this study, Orentreich and colleagues randomly assigned 60 Fischer 344 male rats to one of two diets: one that contained a standard amount of methionine, 0.86% of total dietary kcals, or a restricted amount of methionine, 0.17%. Orentreich and colleagues tracked the lifespan of the rats and fascinatingly, methionine restricted rats died at a much slower rate than rats on the standard 0.86% methionine diet. Overall, methionine restricted rats lived roughly 200 days longer (1059 vs 818 days) and had a maximum lifespan of 1252 days compared to 1116 days for the standard diet (Orentreich, Matias et al. 1993). The results of Orentreich's study were verified by another study that restricted methionine and increased mean and maximum lifespan of Fischer 344 rats by 42% and 44% (Richie, Leutzinger et al. 1994). Extension of lifespan through methionine restriction was further expanded to other experimental models including other strains of rats (Zimmerman 2003), mice (Miller, Buehner et al. 2005), *Drosophila* (Lee, Kaya et al. 2014), and *C. elegans* (Cabreiro, Au et al. 2013). Lifespan-extension with methionine restriction is eliminated if cysteine is present in the diet (Elshorbagy, Valdivia-Garcia et al. 2011); that is, the experimental diet needs to have a restricted methionine content and be devoid of cysteine. The dietary restriction of methionine and cysteine is referred to as sulfur amino acid restriction (SAAR).

Interestingly, long-lived animal models have altered methionine metabolism. For example, Ames dwarf mice, which live around three to four years, roughly 50% longer than other mouse strains, have increased activity of MAT and SAH that could potentially confer stronger antioxidant properties (Uthus 2003). The long-lived naked mole rat, which can live

from 10 to 30 years, exhibits a youthful physiology throughout its lifespan until death and has lower levels of circulating methionine (Lewis, Rubinstein et al. 2018). These studies illustrate that longevity may be extended through altered sulfur amino acid metabolism.

MECHANISMS OF SAAR-INDUCED LIFESPAN/HEALTHSPAN EXTENSION

An important observation is that dietary SAAR extends both longevity and healthspan. The extension of healthspan is attributed to the many systemic changes that dietary SAAR induces. In mice and rats, SAAR increases energy expenditure (Hasek, Stewart et al. 2010), reduces body weight, and improves glucose tolerance through reductions in fasting insulin, insulin-like growth factor, and leptin (Orentreich, Matias et al. 1993, Miller, Buehner et al. 2005, Lees, Król et al. 2014). Furthermore, SAAR increases fatty acid oxidation (Malloy, Perrone et al. 2013), which may explain the reduction in adiposity (Hasek, Boudreau et al. 2013). It should be noted that different levels of dietary restriction have different metabolic outcomes. For example, mice that undergo 0.25% SAAR are heavier in weight compared to mice on a 0.17% SAAR diet. Mice on a 0.34% SAAR diet do not have any changes in weight compared to mice on a control diet.Conversely, a diet lower than 0.12% methionine induced rapid weight loss and caused early death in mice (Forney, Wanders et al. 2017).

Decreased markers of oxidative stress are also observed in rats undergoing SAAR, which are not due to increased glutathione levels (Maddineni, Nichenametla et al. 2013), as one study has reported previously. However, other studies have reported a decrease in hepatic and blood glutathione levels, which is paradoxical for a life-/healthspan extending intervention. Despite the decrease in glutathione, there is an increase in glutathione reductase and thioredoxin

reductase, as well as a decrease in ROS production by mitochondria, which might compensate for the reduced glutathione concentrations (Tamanna, Kroeker et al. 2019). Interestingly, there is a 5- to 10-fold increase in systemic fibroblast growth factor 21 (FGF21) hours after initiation of a SAAR diet (Wanders, Stone et al. 2016). FGF21 is an important hormone that is released during states of nutrient stress and is involved in glucose homeostasis, lipid metabolism, and overall energy metabolism (Tezze, Romanello et al. 2019). FGF21 deficient mice undergoing dietary SAAR do not exhibit the commonly seen increase in energy expenditure and improvements in insulin sensitivity in response to SAAR (Wanders, Forney et al. 2017). However, dietary SAAR reduces markers of hepatic and white adipose tissue inflammation independently of FGF21 (Sharma, Dixon et al. 2019), suggesting that there are multiple mechanisms driving the healthspan extending benefits of SAAR.

SAAR AND PROTEOSTASIS

Improved mechanisms to maintain proteostasis is a shared characteristic of lifespan-extending interventions (Hamilton and Miller 2017). A key mediator of proteostatic processes is eIF2, which becomes activated under low amino acid conditions to halt global protein synthesis in order to avoid depletion of the amino acid pool (Anthony, McDaniel et al. 2004). Activation of eIF2 is observed in hepatic tissue under SAAR (Wanders, Stone et al. 2016, Nichenametla, Mattocks et al. 2018). Furthermore, activation of hepatic eIF2 results in the preferential translation of mRNAs involved in the metabolic changes observed during SAAR (Jonsson, Margolies et al. 2019). Importantly, SAAR activates the protein kinase R–like endoplasmic reticulum (ER) kinase (PERK), which holds an important role in the induction of protein degradation pathways (Jonsson, Margolies et al. 2019). Additionally, SAAR reduces SAM levels,

which is negative regulator of mTOR (Gu, Orozco et al. 2017). Inhibition of mTOR reduces global rates of protein synthesis and is a characteristic of lifespan-extending interventions and long-lived animal models (Drake, Bruns et al. 2014). Previous studies have shown that dietary SAAR decreases hepatic protein synthesis in mixed and cytosolic fractions, while maintaining mitochondrial protein synthesis (Pettit, Jonsson et al. 2017, Nichenametla, Mattocks et al. 2018, Jonsson, Margolies et al. 2021).Therefore, we may conclude that mTOR and its downstream targets including RPS6 are inhibited.

When rates of protein synthesis are expressed relative to rates of cellular proliferation, we can gauge where newly synthesized proteins are being allocated. Despite rates of hepatic mitochondrial protein synthesis being maintained during dietary SAAR, rates of cellular proliferation decrease, suggesting preferential allocation of newly synthesized proteins towards maintaining the mitochondrial proteome (Hamilton and Miller 2017, Jonsson, Margolies et al. 2021), which is consistent with other lifespan-extending interventions (Miller, Drake et al. 2014, Jonsson, Margolies et al. 2021). Mitochondria are necessary to produce ATP to maintain cellular bioenergetic demands. A loss or decline of the mitochondrial proteome impairs cellular function and worsens other drivers of aging. Therefore, maintaining mitochondrial integrity is essential for health.

GAPS IN KNOWLEDGE

Cognitive decline is strongly associated with brain aging and age-related NDs. Dietary SAAR delays age-related cognitive decline in 15 and 18 month old mice by improving synaptic structure and increasing mitochondrial biogenesis. Additionally, in the same study, dietary

SAAR decreased markers of oxidative stress and neuroinflammation (Ren, Wang et al. 2021). An improvement in cognition during dietary SAAR may be due to activation of proteostatic mechanisms. However, there are no studies to date investigating proteostasis and energy sensing in frontal cortex during dietary SAAR. Dietary SAAR decreases rates of protein synthesis in cytosolic and mixed fractions of hepatic tissue, while the mitochondrial proteome is maintained. SAAR also decreases rates of cellular proliferation in hepatic tissues, suggesting that newly synthesized proteins are allocated towards proteome maintenance. SAAR activates energy sensing proteins such as eIF2 and AMPK in hepatic, adipose, and endothelial tissue (Bárcena, Quirós et al. 2018, Longchamp, Mirabella et al. 2018, Wang, Wang et al. 2019). Furthermore, different levels of dietary SAAR are used in the literature, which impacts metabolic phenotype in mice, but it is unknown if proteostasis and energy sensing are affected by different levels of dietary SAAR. This study will reduce the gap in knowledge by comparing two levels of dietary SAAR to their control diets on rates of protein synthesis and cell proliferation in the frontal cortex, as well as activation of key energy/nutrient sensing proteins.

CHAPTER III: METHODS

ANIMAL CARE

80 male wild-type C57BL/6J mice aged 3-6 months old were housed at Rutgers University in standard shoebox cages on a 12-hour light/dark cycle and building temperature maintained at 23°C. All animal protocols complied with NIH Care and Use of Laboratory Animal standards and were approved by the Rutgers University Institutional Animal Care and Use Committee.

ANIMAL DIET COMPOSITION

Four experimental diets were used in this study. Two diets with 18% fat (percentages are reported as kcal of total diet) were either methionine-sufficient (0.88%), referred to as regular-fat control (Low SAAR Control; product code 510072; Dyets, Inc.) or contained restricted amounts of methionine (0.18%) and no cysteine (Low SAAR; product code 510071; Dyets, Inc.). The two other diets contained 60% fat and were either methionine-sufficient (0.85%; High SAAR Control; product code A11051306; Research Diets, Inc.) or restricted amounts of methionine (0.12%) and no cysteine (High SAAR; product code A11051305; Research Diets, Inc.). All diets were isocaloric and isonitrogenous, maintained by increasing glutamic acid content when methionine content was decreased. The diets were given in pelleted form.

STUDY DESIGN

Mice were divided into the four experimental diet groups: Low SAAR Control, Low SAAR, High SAAR Control and High SAAR (n=20 for each diet). Mice had ad libitum access to their food and were on their diets for a duration of 1, 3, 7, 14, 21, and 35 days (n=3/timepoint/diet, except for day 7 which had n=5 animals/diet). At the beginning of the experimental diets, mice were intraperitoneally injected with a bolus of 99% deuterium oxide (^2H_2O) in 0.9% saline relative to 60% of their body weight. Drinking water was subsequently switched to contain 8% ^2H_2O. ^2H_2O is used to simultaneously monitor rates of protein and DNA synthesis by measuring incorporation of deuterium into sites with non-labile hydrogens of alanine and deoxyribose, respectively. This study design permits assessment of the rates of protein synthesis (k) for both shorter and longer-lived proteins. Animals were fasted for four hours and euthanized by decapitation. Brains, bone marrow, and the plasma fraction of blood were collected and frozen in liquid nitrogen. Tissue was stored at -80°C until analyses.

DIFFERENTIAL CENTRIFUGATION

Frontal cortex was sectioned from whole-brain under chilled conditions. 50-60 mg of frontal cortex was pulverized under liquid nitrogen using a mortar and pestle. 40 mg of pulverized frontal cortex was aliquoted for assessment of alanine deuterium enrichment, and 15 mg for deoxyribose enrichment. Differential centrifugation was used to isolate tissue fractions enriched in cytosolic, mitochondrial, and mixed proteins. Using a Bullet Blender® (NextAdvance, Troy, NY, USA), tissue was bead homogenized at 1:10 in mitochondrial isolation buffer and (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl2, 1 mM EDTA, 1 mM

ATP, pH 7.5) with HALT protease inhibitors (HALT, Thermo Scientific, Rockford IL) for two three-minute rounds at level three. After homogenization, tissue was centrifuged at 800g for 10 minutes at 4°C to obtain the mixed pellet. To obtain the mitochondrial pellet, supernatant from the mixed fraction was centrifuged at 9000g for 10 minutes at 4°C. To obtain the cytosolic fraction, supernatant from the mitochondrial fraction was collected and mixed an equal volume of 14% sulfosalicylic acid and incubated on ice for one hour. After the one-hour incubation completed, the mixture was centrifuged at 16,000g for 10 minutes at 4°C to obtain the cytosolic pellet.

Mixed, mitochondrial, and cytosolic pellets were rinsed with 500 µl of 100% ethanol and centrifuged at 1000g for 1 minute at 4°C. The pellets were then rinsed with 500 µl of 100% water and centrifuged at 1000g for 1 minute at 4°C. Both wash steps were repeated. Afterwards, 250uL 1M NaOH was added to the mixed and cytosolic pellets and shaken in 50°C heat for 15 minutes at 900 rpm. Next, 3 mL of 6M HCl was added to the cytosolic tube, 6 mL to the mixed tube, and 1.5 mL to the mitochondrial tube. All tubes were then hydrolyzed at 120°C for 24 hours.

PREPARATION OF ALANINE FOR ANALYSIS VIA GAS CHROMATOGRAPHY-MASS SPECTROMETRY

After hydrolysis, samples were ion-exchanged and dried down at low-temperature for 24 hours using a speedvac. Dried samples were reconstituted with 1 mL of MilliQ water. 500 µL of sample was mixed with 500 µL of acetonitrile, 50 µL of 1M K2HPO4, and 20 µL of pentafluorobenzyl bromide. The mixture was tightly sealed, vortexed thoroughly, and incubated at 100°C for 1 hour. After the incubation, 600 µL of ethyl acetate was added and

vortexed. The organic layer was removed and placed in glass vials and dried under nitrogen. Samples were reconstituted in 500 µL of ethyl acetate and sealed for gas chromatography/mass spectrometry (GC/MS) analysis.

GC/MS ANALYSIS OF ALANINE

Samples were analyzed for alanine on DB225 gas chromatograph column. Starting temperature was 100°C and increased by 10°C every minute until it reached a final temperature of 220°C. Helium was the carrier gas and methane was the reagent gas. The mass-to-charge ratios of 448, 449, and 450 were pentafluorobenzyl-N,N-di(pentafluorobenzyl)alaninate derivatives were recorded, and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Deuterium enrichment into alanine was calculated as the mass of alanine plus an addition of deuterium (M+1), divided by the sum of M+0 and M+0 (Hellerstein and Neese 1999). The newly synthesized fraction of brain proteins was calculated by dividing the enrichment of deuterium from body water in plasma. Body water enrichment of deuterium was measured in 125 µL of plasma that was placed into inverted plastic microcentrifuge tubes with a rubber O-ring and placed on a heat block at 80 °C overnight. During the incubation, evaporated water from the plasma was captured at the top of the tube. The water was collected and underwent proton exchange with 10 M NaOH and acetone overnight. The proton exchanged samples were derived in hexanes and into anhydrous sodium sulfate before they went GC/MS analysis. Using mass isotopomer distribution analysis (MIDA), the fraction of newly synthesized proteins was normalized to plasma $^{2}H_2O$ enrichment by individual animal as described previously (Miller, Robinson et al. 2012, Drake, Bruns et al. 2015).

DNA ISOLATION AND DERIVATION

15 mg of powdered brain frontal cortex was used for DNA isolation using the DNA mini kit protocols (Qiagen, Valencia, CA, USA). After DNA was isolated, 50 µ of nuclease S1 and potato acid phosphatase was added to the sample tubes and incubated at 37°C for 24 hours. After incubation, samples were transferred to a 13 x 800 glass tube. 150 µL of glacial acetic acid and 200 uL of pentafluorobenzylhydroxylamine was added to the sample and incubated at 100°C for 30 minutes. After the incubation, samples were cooled-down at room-temperature for 10 minutes. Using a pipette repeater, 2 mL of acetic anhydride was added. 200 µL of n-methylimidasole was added to the sample, vortexed, and quickly capped to avoid spillover from the reaction. The reaction was allowed to proceed for 15 minutes and afterwards, 3 mL of MilliQ water was added and vortexed. 1.25 mL of methylene chloride was added to each sample and properly vortexed. Samples were then spun at 150 g. The bottom liquid phase was carefully removed using a Pasteur pipette and transferred to another 13x800 glass tube containing granular anhydrous sodium sulfate. Addition of acetic anhydride and proceeding steps were repeated once. The mixture from the 13x800 glass tube was carefully transferred to a GC vial. Samples were dried down overnight using a speedvac at low temperature setting. Dried down samples were reconstituted in 80 µL of ethyl acetate and analyzed using a DB225 gas chromatograph column. The fractional molar isotope at m/z 435 and 436 of deoxyribose was measured and quantified using ChemStation Software (Agilent Technologies, Santa Clara, CA, USA).

TISSUE HOMOGENIZATION AND WESTERN BLOTTING

15 mg of brain frontal cortex was suspended in 112.5 mL of ice cold RIPA buffer (150 mM sodium chloride, Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0 with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL)) and sonicated for three rounds of 10 seconds on ice. After sonication, samples were centrifuged at 16,000g for 20 minutes. The supernatant was removed and placed into a new Eppendorf tube. Protein content was quantified using a bicinchoninic acid assay (BCA) according to the manufacturer's protocol (Thermo Fisher, Rockford, IL). Timepoints chosen for western blotting were day 1 and day 35 to identify differences in protein content in response to both acute and long-term SAAR treatment.

Each sample was diluted to a concentration of 2 μg/μL using 2X Laemmeli buffer. 50 μg of protein was loaded to 10-well and 26-well 10-20% Tris-HCl gels and separated by SDS-PAGE at 120V for 2 hours. After separation, proteins were transferred onto a polyvinylidenedifluoride (PVDF) membrane at 4°C for 45 minutes in chilled transfer buffer (20% methanol, 0.02% SDS, 192 mM glycine, 25mM Tris, pH 8.3). Ponceau staining was used to confirm a successful transfer of proteins to the PVDF membrane. Membranes were blocked in 5% BSA diluted in Tris Buffered Saline with Tween (TBST) at room temperature for one hour. The following primary antibodies (Cell Signaling) were used and diluted in 5% BSA in TBST: phospho-AMPKα[Thr172] #2531S (1:500 dilution), AMPKα #2532S (1:1000 dilution), RpS6 phospho-Ser[240/244] #4858S (1:1000 dilution), Phospho-eIF2α (Ser51) (D9G8) XP #3398T (1:1000 dilution), eIF2α (D7D3) XP #5324T (1:1000 dilution) and RpS6 #2217S (1:1000 dilution). Membranes were incubated with primary antibodies at 4°C overnight. After incubation, membranes were rinsed with TBST three

times for 10 minutes. Anti-rabbit horseradish peroxidase secondary antibody diluted 1:10,000 in 5% BSA in TBST was applied to the membranes and incubated for 1 hour at room temperature while rocking. Membranes were then rinsed with TBST three times for 10 minutes. Proteins of interest were visualized using enhanced chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, Rockford, IL) and imaged using a FluorChemE imager (bio-techne/ProteinSimple, San Jose, CA). Immunoblotting for phosphorylated proteins was carried out first. Membranes were then stripped using stripping buffer (20 mL SDS 10% ,12.5 mL Tris HCl, 67.5 mL distilled water, 0.8 mL ß-mercaptoethanol, pH 6.8) for 45 minutes at 50°C. After confirming removal of phospho-antibodies, membranes were re-probed for total protein.

STATISTICS

Statistical analysis was performed using PRISM GraphPad 9 Software (Graphpad Software, La Jolla, CA). Samples from SAAR mice were compared to those from the appropriate control group. Nonlinear regression of fraction new protein and DNA was calculated by using the mean value for the n= 3 animals of each timepoint for each dietary group. From the nonlinear regression, the slope of the curve (k) was calculated. For western blot analysis, average densitometry values were quantified using AlphaView SA (ProteinSimple, San Jose, CA) and groups were compared using an unpaired t-test. Statistical significance was set at $p<0.05$ or less. P values between 0.05 - 0.10 were reported as trends.

CHAPTER IV: RESULTS

MITOCHONDRIAL PROTEIN SYNTHESIS RATES ARE GREATER WITH LOW SAAR DIET

Rates of protein synthesis were measured in mixed, cytosolic, and mitochondrial fractions of brain frontal cortex with two levels of dietary SAAR. In animals on the Low SAAR diet, mitochondrial protein synthesis rates were significantly ($p<0.0001$) higher than the control diet (Figure 2A), however, no significant differences were observed in the cytosolic and mixed fractions. Unlike the Low SAAR diet, no significant differences were observed in rates of protein synthesis in animals on the High SAAR diet for the cytosolic, mixed, and mitochondrial fractions (Fig 2B).

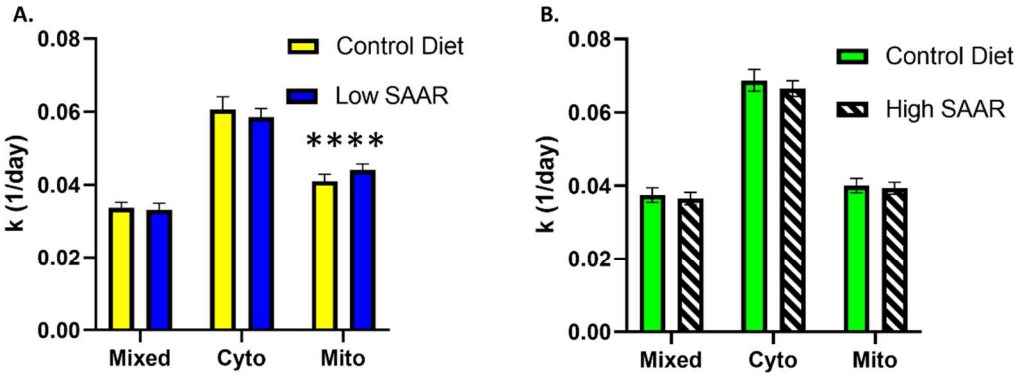

Figure 2. Protein synthesis rates (k) in subcellular fractions of brain frontal cortex of male C57Bl/6J mice that were fed a **(A)** SAA sufficient control diet (0.88% methionine) or a Low SAAR diet (0.18% methionine, 0% cysteine), or a **(B)** SAA sufficient control diet (0.85% methionine) or a High SAAR diet (0.12% methionine, 0% cysteine). Before starting their diets, mice were injected with a bolus of 99% ^2H_2O and had ad libitum access to drinking water enriched to 8% ^2H_2O. On days 1, 3, 7, 14, 21, and 35 of treatment, n=3-5 mice were sacrificed. A 1-phase association was used to calculate the rate of the rise of fraction new protein over time to determine the rate of synthesis (k, 1/d). Mitochondrial protein synthesis rates were significantly greater in the Low SAAR diet compared to its control diet. Data are presented as means +/- SD (n=18-20/diet group). Cyto, cytoplasmic; Mito, mitochondrial; SAA, sulfur amino acid; SAAR, sulfur amino acid restriction; ****p<0.0001, versus control diet.

SAAR DOES NOT ACTIVATE ENERGY SENSING PROTEINS

In liver, dietary SAAR activates key energy sensors that are involved in regulating protein synthesis (Pettit, Jonsson et al. 2017, Nichenametla, Mattocks et al. 2018, Jonsson, Margolies et al. 2021). To measure acute and long-term activation of AMPK, RPS6, and eIF2, we compared phosphorylated protein relative total protein at day 1 and day 35 for both levels of dietary SAAR (Figures 3 & 4). At day 1, p/t AMPK (Fig 3A) and p/t RPS6 (Fig 3C) were not significantly different in animals on the Low SAAR diet compared to control. However, p/t eIF2 (Fig 3E) tended ($p= 0.0922$) to be greater in Low SAAR compared to control. At day 35, p/t-AMPK (Fig 3B), p/t RPS6 (Fig 3D), and p/t eIF2 (Fig 3F) were not different in the Low SAAR diet compared to control. For the High SAAR diet, day 1 p/t AMPK (Fig 4A), and p/t RPS6 (Fig 4C) did not differ from the control. Day 1 p/t eIF2 was not included due to technical difficulties during western blotting procedure. At day 35, the High SAAR diet did not have any significant differences for p/t AMPK (Fig 4B), p/t RPS6 (Fig 4D), and p/t eIF2 (Fig 4F).

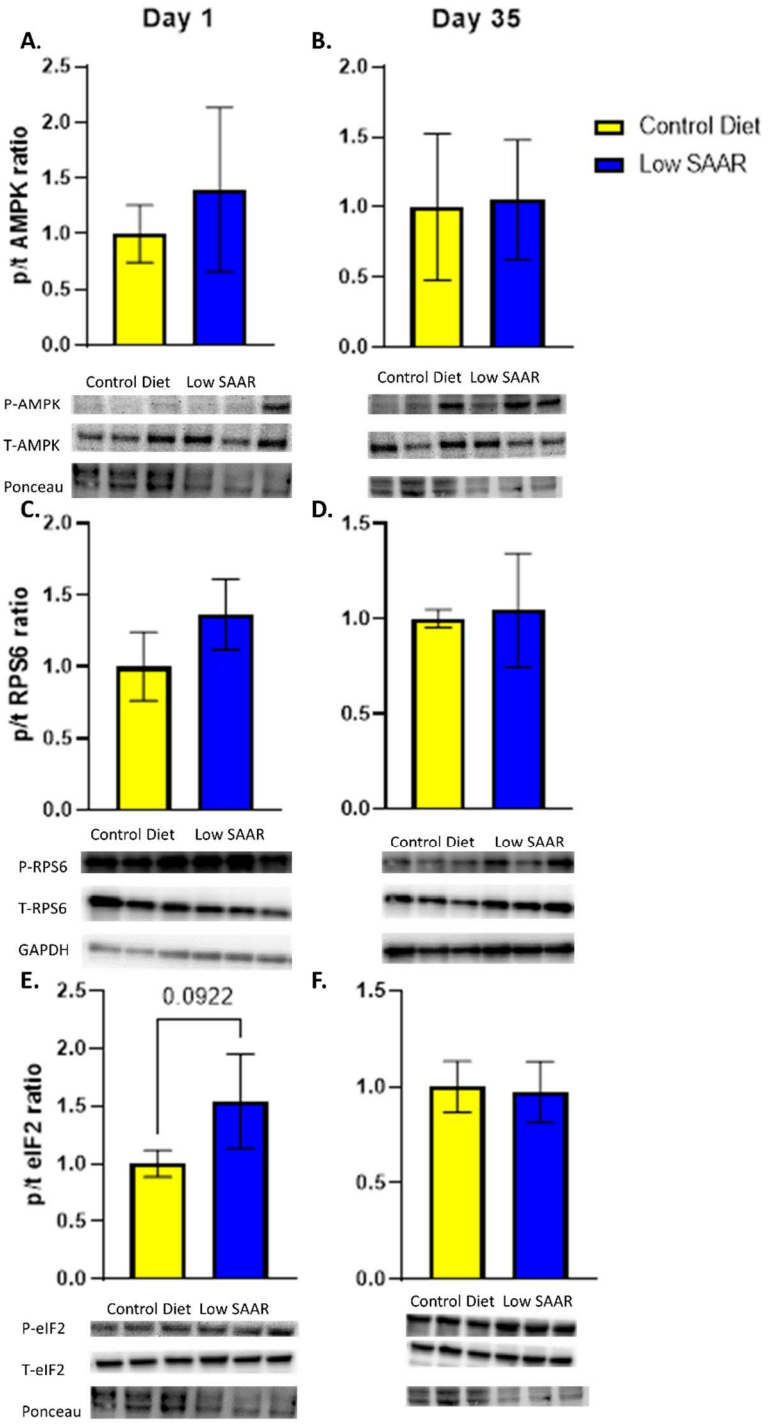

Figure 3. Western blotting for AMPK, RPS6, and eIF2 at day 1 and day 35 of the Low SAAR and control diets. Phosphorylated (p) and (t) total proteins were detected and expressed as p/t ratios to measure activation of proteins. GAPDH and Ponceau S were used to verify equal loading of protein. No significant differences in p/t ratios were observed between the low SAAR diet and its control diet for AMPK, RPS6, and eIF2 at day 1 and day 35. However, at day 1, p/t eIF2 tended (p=0.0922) to be greater in the Low SAAR diet compared to its control. Data are presented as mean +/- SD (n= 3/group). AMPK, AMP-activated protein kinase; RPS6, ribosomal protein S6; eIF2, eukaryotic initiation factor 2, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

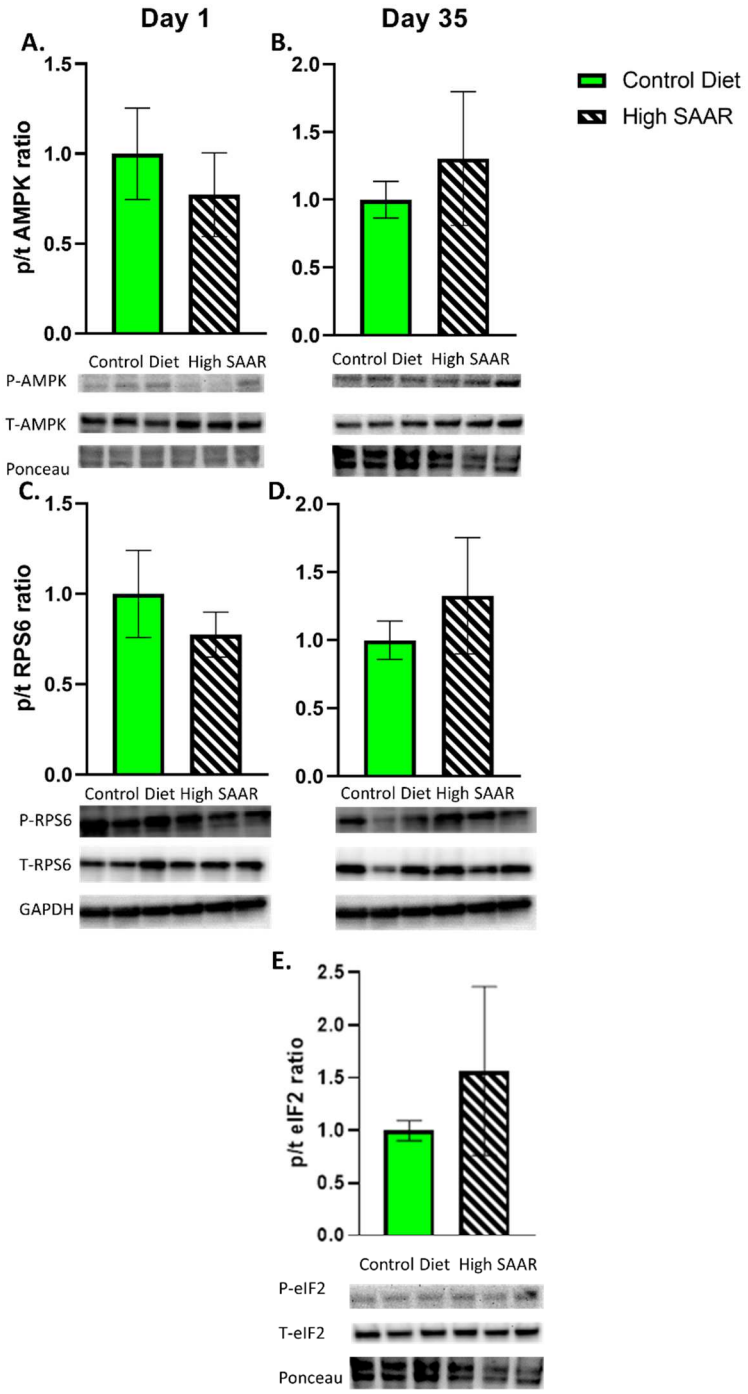

Figure 4. Western blotting for p/t AMPK, p/t RPS6, and p/t eIF2 at day 1 and day 35 of the High SAAR and control diets. At day 1, no significant differences were observed for p/t AMPK **(A),** and p/t RPS6 **(C)**. At day 35, no significant differences were observed for p/t AMPK **(B),** p/t RPS6 **(D),** p/t eIF2 **(E).** Data are presented as mean +/- SD (n= 3/group except for Day 35 p/t eIF2 High SAAR which included n=2). Data was not obtained for day 1 p/t eIF2 due to technical error during western blotting procedure. GAPDH and ponceau were used to verify equal loading of protein. AMPK, AMP-activated protein kinase; RPS6, ribosomal protein S6; eIF2, eukaryotic initiation factor 2.

CELL PROLIFERATION TENDS TO BE SLOWER IN THE HIGH SAAR DIET

To assess the influence of SAAR on brain cell proliferation, we measured rates of DNA synthesis. Low SAAR did not change DNA synthesis rates compared to control (Fig 5A). DNA synthesis rates tended to be slower (Fig 5B) in the High SAAR diet compared to control (p = 0.0868).

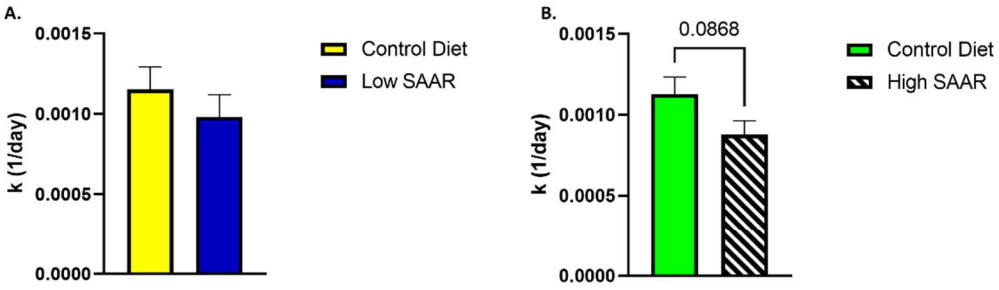

Figure 5. Rates of DNA synthesis were simultaneously measured with rates of protein synthesis. Rates of DNA synthesis were not significantly different in the Low SAAR diet **(A)**. Rates of DNA synthesis tended to be lower in the High SAAR diet **(B)** compared to control (p=0.086). Data are presented as means +/- SD.

NEWLY SYNTHESIZED PROTEINS ARE ALLOCATED TOWARDS PROTEOME MAINTENANCE UNDER HIGH SAAR

To assess the allocation of newly synthesized proteins for proteome maintenance versus for cellular proliferation, protein synthesis rates were expressed relative to DNA synthesis rates (PRO:DNA) for each subcellular fraction at each time point (Figure 6). At day 21 and 35, there were no significant differences between the Low SAAR diet and its control diet for the PRO:DNA ratios in the cytosolic, mixed, and mitochondrial fractions (Figure 7). PRO:DNA ratio in the mitochondrial fraction was significantly greater in the High SAAR diet at day 21 (Fig 6A) and day 35 (Fig 6B) (p= 0.0482 and p= 0.0377) compared to control. The High SAAR diet had significantly greater (p=0.0192 and p=0.0366) PRO:DNA ratios in the mixed fraction at day 21 (Fig 6C) and

day 35 (Fig 6D). The cytosolic PRO:DNA ratio did not differ at day 21 (Fig 6E) in the High SAAR diet compared to control. Cytosolic PRO:DNA ratio was significantly greater (p=0.026) at day 35 (Fig 6F) in the High SAAR diet compared to control at day 35.

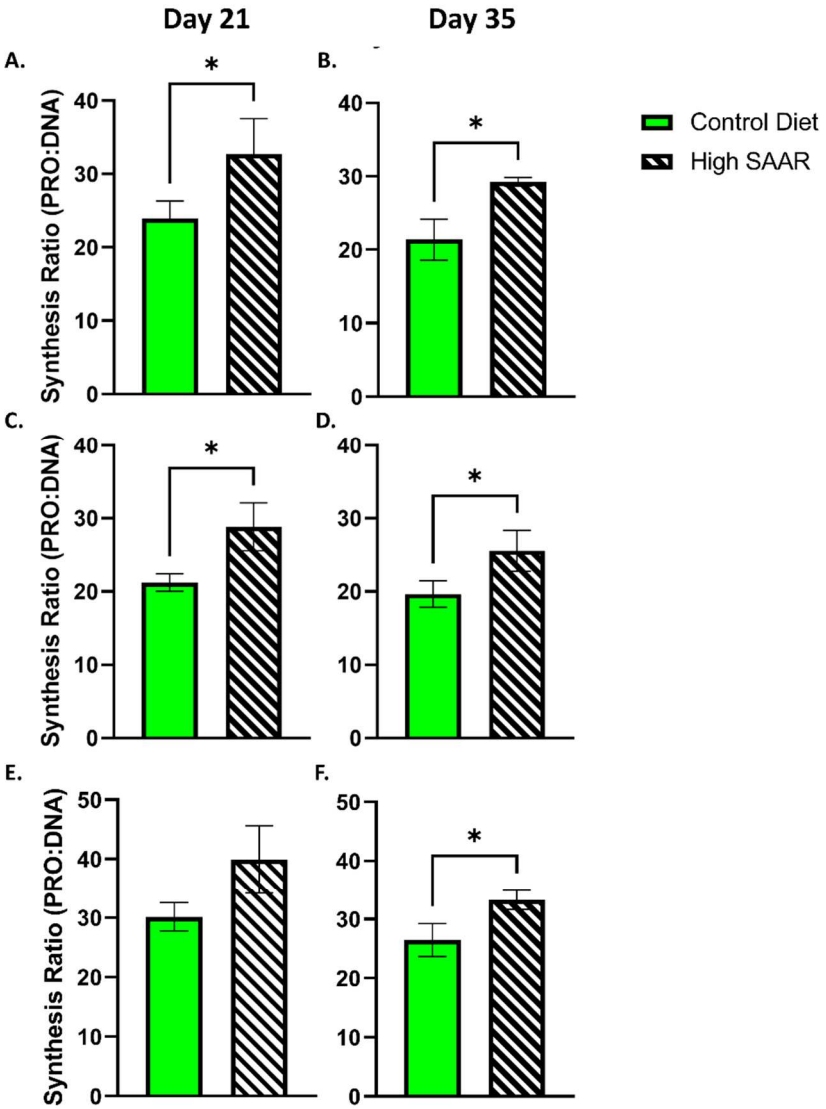

Figure 6: To assess allocation of newly synthesized proteins, protein synthesis was made relative to DNA synthesis (PRO:DNA) in the mitochondrial, mixed, and cytosolic fractions of brain frontal cortex at day 21 and day 35. The High SAAR diet had a greater PRO:DNA at day 21 in the mitochondrial **(A)** and mixed fraction **(C)** compared to control, however, the cytosolic fraction **(E)** was not significantly different. At day 35, PRO:DNA was significantly greater in the mitochondrial, **(B),** mixed **(D)**, and cytosolic fractions **(F)** compared to control. Data are presented as means +/- SD. PRO:DNA ratios for the Low SAAR are included in Supplemental Figure 1. *p<0.05, versus control diet.

CHAPTER V: DISCUSSION

SUMMARY OF FINDINGS

SAAR is a dietary intervention that extends longevity and improves health in experimental animal models (Orentreich, Matias et al. 1993, Miller, Buehner et al. 2005). Proper regulation of proteostasis is essential for maintaining cell function, and loss of proteostasis is a key driver of age-related ND (Yerbury, Ooi et al. 2016, Thibaudeau, Anderson et al. 2018, Kurtishi, Rosen et al. 2019). We have previously shown that dietary SAAR activates mechanisms of mitochondrial proteome maintenance in hepatic tissue (Pettit, Jonsson et al. 2017, Jonsson, Margolies et al. 2021). However, it was previously unknown how dietary SAAR impacts proteostasis in murine brain. Here, we examined the rates of protein synthesis in three tissue fractions of frontal cortex from mice treated with two levels of dietary SAAR. We found that rates of protein synthesis in the cytosolic and mixed fractions were maintained during both levels of dietary SAAR compared to their sulfur-amino acid sufficient control diets. In fact, in the Low SAAR diet, rates of mitochondrial protein synthesis were greater than control, but did not see a difference in mitochondrial protein synthesis rates the High SAAR diet. To complement these protein synthesis results, we used western blotting to assess activation of key nutrient sensing proteins that are involved in regulating protein synthesis. We found that compared to control, activation of AMPK, RPS6, and eIF2 were not altered in either SAAR diet at an acute (1 day) or longer term (35 days) time point. Lastly, to gain insight into the allocation of newly synthesized proteins, we assessed the rates of protein synthesis relative to the rates of DNA

synthesis (PRO:DNA). In the High SAAR diet, there was a greater allocation of newly synthesized proteins for maintenance of the existing proteome, rather than to cellular proliferation.

BRAIN ENERGY SENSING PATHWAYS ARE NOT ACTIVATED BY SAAR

AMPK is a sensor of cellular energy status and master regulator of metabolism that is activated under conditions of nutrient stress to maintain energetic homeostasis (Steinberg and Carling 2019). Intriguingly, hyperactivation of AMPK has been reported in AD (Mairet-Coello, Courchet et al. 2013, Ma, Chen et al. 2014). However, many reports have argued that activating AMPK is beneficial for improving brain health, depicting a clear gap in knowledge of understanding when activation of AMPK is beneficial or harmful. For example, pharmacological activation of AMPK improves cognition in young mice (Kobilo, Guerrieri et al. 2014) and could protect against AD-associated cognitive decline (Zhang, Zhang et al. 2015). Pharmacological inhibition of electron transport chain complex I has also been shown to activate AMPK (Brunmair, Staniek et al. 2004, Jenkins, Sun et al. 2013). Interestingly, a seven-week 0.17% SAAR diet decreased mitochondrial content and activity of electron transport chain (ETC) complex I from total rat brain homogenate (Naudi, Caro et al. 2007). Therefore, we hypothesized that dietary SAAR would activate AMPK in brain frontal cortex. Contrary to our hypothesis, we did not detect any changes in AMPK activation in either of the SAAR diets at day 1 or day 35 (Figure 3A, 3B, 4A and 4B). Very few studies have measured AMPK activation during dietary SAAR. In one study, dietary SAAR activated AMPK and delayed kidney senescence in 22 month old C57BL/6 mice that were fed a 0.15% SAAR diet (Wang, Wang et al. 2019). In liver tissue from progeroid mice, a 0.12% SAAR diet induced AMPK activation (Bárcena, Quirós et al. 2018). Furthermore, a 0.15% SAAR diet activated AMPK in primary mouse endothelial cells

(Longchamp, Mirabella et al. 2018). In another study, no changes in AMPK activation were observed in adipose tissue of F344 rats that underwent 0.17% dietary SAAR (Perrone, Mattocks et al. 2008). Therefore, SAAR-induced AMPK activation could be dependent on tissue type, however, this is the first study investigating AMPK activation in the brain in response to SAAR treatment.

Cell growth is modulated by another key energy sensor, mTOR (Johnson, Rabinovitch et al. 2013). Inhibition or reduced activity of mTOR is a shared characteristic of lifespan-extending interventions and long-lived animals (Miller, Harrison et al. 2011, Johnson, Rabinovitch et al. 2013). Amino acid availability, including methionine (Zhou, Ren et al. 2016, Meng, Yang et al. 2020), regulates mTOR activity. RPS6 is a downstream effector of mTOR and activation of RPS6 promotes protein synthesis (Chauvin, Koka et al. 2014). Given that methionine activates mTOR and the downstream target RPS6, we hypothesized that SAAR would inhibit mTOR with subsequent decreases in RPS6 activation. Contrary to our hypothesis, we did not observe any significant differences in activated RPS6. These results are interesting because methionine and its byproduct, SAM, have been established as potent activators of mTOR in HEK-293 cells, therefore, dietary SAAR should inhibit mTOR (Gu, Orozco et al. 2017). In one study, RNAseq analysis shows a decrease in expression of mRNA for proteins regulating global protein translation within 3 and 6 hours of dietary SAAR in liver tissue. Additionally, the authors mentioned they did not detect any changes in RPS6 activation in liver extracts at 1, 3, or 6 hours after initiating dietary SAAR (Stone, Ghosh et al. 2021).

The beneficial effects of SAAR have been attributed to the activation of the integrated stress response (ISR) (Kilberg, Shan et al. 2009). Induction of the ISR through eIF2 activation

reduces general protein translation, but also directs preferential expression of stress responsive mRNAs, primarily through ATF4 activation (Kilberg, Shan et al. 2009). Amino acid deprivation results in phosphorylation of eIF2 via general control nondepressible 2 (GCN2) to maintain the amino acid availability (Zou, Ouyang et al. 2017). We have previously published that five weeks of dietary SAAR increased activation of hepatic eIF2 in male wild-type mice (Pettit, Jonsson et al. 2017, Jonsson, Margolies et al. 2021), a finding also reported by others (Nichenametla, Mattocks et al. 2018). Therefore, we explored eIF2 activation in brain following SAAR. Unlike hepatic tissue, we did not observe any significant change in eIF2 activation in brain, however, at day 1 of the Low SAAR diet, eIF2 activation tended to be greater ($p= 0.0922$) than the control diet, but at day 35 there was no significant difference. At day 35, the High SAAR diet did not have a significant difference in eIF2 activation compared to control. These results are surprising because amino acid deficiencies are detected in the piriform cortex and lead to GCN2 activation and subsequent activation of eIF2 (Hao, Ross-Inta et al. 2010). This observation leads to the question of whether eIF2 activation is brain region dependent or if changes in sulfur amino acid content were detected during a 0.12% or 0.18% SAAR diet.

MECHANISMS FAVORING PROTEOSTASIS ARE MAINTAINED DURING DIETARY SAAR

To our knowledge, this is the first study to simultaneously assess rates of protein synthesis and cell proliferation in the frontal cortex during dietary SAAR. Different levels of dietary SAAR produce different metabolic phenotypes. For example, in another study, mice on a 0.25% SAAR diet weigh more than mice on 0.17% SAAR diet, which is the most commonly used diet throughout studies. Additionally, mice on a 0.12% SAAR diet have less adiposity than mice on a 0.17% diet. Furthermore, a SAAR diet below 0.12% causes rapid loss of body weight

and death (Forney, Wanders et al. 2017). Therefore, we used two levels of dietary restriction, either 0.18% SAAR or 0.12% SAAR, to evaluate differences in rates of protein synthesis in different subcellular fractions of frontal cortex compared to their sulfur amino acid sufficient control diets. We chose the frontal cortex due to its decline in volume as aging progresses and the loss of proteostasis being a contributing factor (Pabba, Scifo et al. 2017, Sabath, Levy-Adam et al. 2020). Therefore, this study provides insight into the effect of two levels of dietary SAAR on proteostasis in this important brain region.

We have previously shown that mice undergoing the two levels of dietary SAAR used in this study have slower rates of hepatic protein synthesis in the cytosolic and mixed fractions, while the mitochondrial fraction maintains rates of protein synthesis compared to mice in the control diet (Jonsson, Margolies et al. 2021). Given our previous knowledge of protein synthesis and dietary SAAR, we hypothesized to see the same results as liver in frontal cortex.

Contrary to our hypothesis, in animals on the Low SAAR diet, rates of protein synthesis were maintained in the cytosolic and mixed fraction. Additionally, we observed that, the mitochondrial protein synthesis rate was significantly greater than the control diet (Fig 2A). In the High SAAR diet, rates of protein synthesis were maintained in all subcellular fractions (Fig 2B). These results are consistent with our observations that SAAR did not activate AMPK, RPS6, and eIF2, however, these findings in brain are in contrast to observations in other tissues. Another study found that protein synthesis was decreased in mixed protein fraction of liver, however, rates of protein synthesis in hippocampus were unaffected after 12 weeks of 0.17% SAAR (Nichenametla, Mattocks et al. 2018). In our previous studies we reported that rates of cytosolic, mixed, and mitochondrial protein synthesis in whole brain did not differ between a

rapamycin treated group, in which mTOR was inhibited, and controls (Reid, Linden et al. 2020), re-affirming our observation that brain could be spared from the global suppression of protein synthesis observed in peripheral tissues. However, the lack of difference in rates of protein synthesis might occur from the dietary intervention itself. Glucose is the brain's main source of energy, and under conditions of low or impaired glucose uptake, neurons can switch their fuel sources to ketone bodies, lactate, or amino acids (Mächler, Matthias et al. 2016). Carbohydrate content is not lowered under dietary SAAR, and instead, glucose metabolism is improved by dietary SAAR (Hasek, Stewart et al. 2010, Luo, Yang et al. 2019). Potentially, this might improve glucose metabolism within the brain, leading to the lack of use of amino acids as energy sources. A shift in neuronal substrate utilization from glucose to ketones occurs during caloric restriction in response to low glucose levels (Guo, Bakshi et al. 2015), however, it is unknown if dietary SAAR induces this same shift in substrate utilization. Therefore, brain metabolism under dietary SAAR should be studied to close that knowledge gap.

Maintenance of the mitochondrial proteome is a shared characteristic in animal models that exhibit slowed aging and of life-/healthspan interventions (Hamilton and Miller 2017). Mitochondria have important roles within the cell that include ATP production, energy sensing, and stress-adaptation signaling (Rose, Santoro et al. 2017). A loss of mitochondrial function is considered to be a driver of brain aging (Mattson and Arumugam 2018). Our previous studies have shown that mTOR inhibition through chronic rapamycin feeding for 12 weeks maintains rates of mitochondrial protein synthesis in skeletal muscle of UM-HET3 mice (Drake, Peelor et al. 2013). Rates of mitochondrial protein synthesis are maintained in liver, heart, and skeletal tissue of B6DF21 mice undergoing caloric restriction for 6 weeks at a young, middle, and old

age (Miller, Robinson et al. 2012). As previously mentioned, hepatic mitochondrial protein synthesis rates are maintained under dietary SAAR, despite a decrease in cytosolic and mixed protein synthesis (Jonsson, Margolies et al. 2021). A unique finding from our study in brain was that rather than observing a maintenance of mitochondrial protein synthesis, as seen with our previous studies, rates of protein synthesis were greater in the Low SAAR diet compared to its control diet (Fig 2A). This unique finding in brain suggests that mitochondrial biogenesis is increased during a Low SAAR diet, potentially providing a mitohormetic effect to strengthen mitochondrial function, though, more research is needed to draw conclusions.

Furthermore, it is important to consider protein synthesis in the context of cellular proliferation, which we accomplished by simultaneously measuring protein and DNA synthesis rates. The ratio of protein synthesis to DNA synthesis rates provides valuable insight into the allocation of newly synthesized proteins for proteome maintenance versus for newly proliferating cells, in response to dietary SAAR. In our study, rates of cellular proliferation tended ($p=0.0868$) to be lower (Fig 5B) in the High SAAR diet compared to control, while there was no significant difference in the Low SAAR compared to control (Fig 5A). Despite the slower rate of cell proliferation in the High SAAR diet, rates of protein synthesis were maintained in all fractions, indicating that more newly synthesized proteins were directed towards somatic maintenance. Allocation of newly synthesized proteins towards somatic maintenance of liver and skeletal muscle has been observed in long-lived Snell Dwarf Mice (Drake, Bruns et al. 2015) and a long-lived crowded litter model, which imposes nutrient stress on the mice by increasing litter size (Drake, Bruns et al. 2014). Collectively, our studies show a preference for somatic

maintenance, especially for mitochondria, under life-/healthspan interventions and long-lived animal models.

COMPARISONS TO OTHER LIFESPAN-EXTENDING INTERVENTIONS

In this study, we show that during dietary SAAR, rates of protein synthesis are maintained in the brain, despite slower rates of cellular proliferation, suggesting somatic maintenance. Along with dietary SAAR, somatic maintenance is observed in response to other lifespan-extending interventions. For instance, caloric restriction maintains short and long-term rates of protein synthesis in heart, liver, and skeletal muscle, despite slower rates of cellular proliferation (Miller, Robinson et al. 2013). In the long-lived Snell Dwarf mice, which is a model of growth restriction, rates of protein synthesis are lower than control mice in skeletal muscle and heart, however, rates of cellular proliferation are also lower, indicating that newly synthesized proteins are being allocated towards somatic maintenance (Drake, Bruns et al. 2015). Chronic administration of the lifespan extending mTOR inhibitor, rapamycin, maintains skeletal muscle mitochondrial protein synthesis despite a reduction in cytosolic and mixed fractions and slower rates of cellular proliferation (Drake, Peelor et al. 2013)

It has been hypothesized that lifespan-extending interventions, including SAAR, may delay age-related cognitive decline. In one study, three months of dietary SAAR attenuated memory decline in 15 and 18 month old mice. The improvements in cognition are suggested to be mediated by an increase in hippocampus FGF21 concentration which increases expression of genes related to brain mitochondrial biogenesis in response to dietary SAAR (Ren, Wang et al. 2021). Accordingly, we observed elevated rates of mitochondrial protein synthesis, a true

measure of mitochondrial biogenesis, in the Low SAAR diet compared to control (Fig 2A). A modest improvement in cognition and increased mitochondrial biogenesis related-genes have also been observed in mice that underwent caloric restriction and a low-protein high carbohydrate (LPHC) diet (Wahl, Solon-Biet et al. 2018). Long-lived Snell-Dwarf mice have better performance in cognitive tests at 3, 12, and 24 months of age compared to wild-type mice (Sharma, Haselton et al. 2010). Additionally, adult Snell-dwarf mice have greater generation of newborn neurons in the dentate gyrus compared to control mice (Sun, Evans et al. 2005). Cognitive performance is improved in 18 month old mice treated with a lifespan-extending dose of rapamycin beginning at two months of age compared to control treated mice (Majumder, Caccamo et al. 2012). In contrast, a study in lemurs showed that life-long 30% calorie restriction extends life-/healthspan and preserves brain white matter, but accelerates the loss of grey matter with no differences in cognitive performance (Pifferi, Terrien et al. 2018). These studies show that life-/healthspan extending interventions have the potential to be powerful tools to delay age-related declines in cognition, however, there a major gaps in knowledge in understanding the mechanisms driving those improvements.

The activation of AMPK and inhibition of mTOR, to promote stress-resistance and/or somatic maintenance, is a common characteristic of lifespan extending interventions and of long-lived animal models. Dietary SAAR shares those same characteristics in various peripheral tissues (Pettit, Jonsson et al. 2017, Bárcena, Quirós et al. 2018, Wang, Wang et al. 2019), however, the effects of dietary SAAR in the brain are mostly unknown.

LIMITATIONS

This study was specifically designed to measure rates of synthesis of both short-lived and long-lived proteins, with timepoints ranging from 1 to 35 days of dietary intervention (Miller, Wolff et al. 2015). The experimental design is not powered to detect differences in protein content at any single timepoint (n=3-5/timepoint). Therefore, the lack of statistical differences in activation of AMPK, RPS6, and eIF2, measured on day 1 and day 35 of treatment, may be influenced by the experimental design. Rates of protein synthesis were analyzed from frontal cortex which was fractionated into different subcellular compartments, but does not take into account specific brain cell types. Finally, this study only used male mice and, therefore, does not consider any potential sex-differences in SAAR-induced effects on nutrient sensing or proteostasis in the brain

CONCLUSIONS AND FUTURE DIRECTIONS

This is the first study to simultaneously assess rates of protein synthesis and cellular proliferation in brain frontal cortex in response to two levels of dietary SAAR in mice. In our previous study in liver, we found that rates of protein synthesis were lower the cytosolic and mixed fractions, while the mitochondrial fraction was maintained. We hypothesized that we would see the same results in brain as liver. Contrary to our hypothesis and to our surprise, we found that in the Low SAAR diet, there were greater rates of mitochondrial protein synthesis in the brain, suggesting that mechanisms promoting mitochondrial biogenesis and maintaining proteostasis were activated. Furthermore, a High SAAR diet promoted the allocation of more newly synthesized proteins towards somatic maintenance compared to control. To our surprise,

dietary SAAR did not activate the key energy sensing proteins, AMPK, RPS6 as a measure of mTOR activity, and eIF2 at day 1 and day 35. These results suggest that protein synthesis and energy sensing in brain may be handled differently under dietary SAAR compared to liver. The lack of differences between the SAAR diets and their control diets may be due to a lack of change in brain substrate metabolism, however, this unknown. To address the lack of difference in activation of energy sensing pathways, we recommend assessing how substrate metabolism in brain is affected by dietary SAAR. Furthermore, the observation that a Low SAAR diet with associated with greater rates of mitochondrial protein synthesis may implicate the idea that mitochondria are becoming more resilient. In the context of brain aging and NDs, more resilient mitochondria could potentially reduce ROS-induced damage to proteins and ameliorate signals to activate neuroinflammatory processes. Therefore, future studies should consider using dietary SAAR as an intervention to improve the brain aging process or age-related ND pathologies.

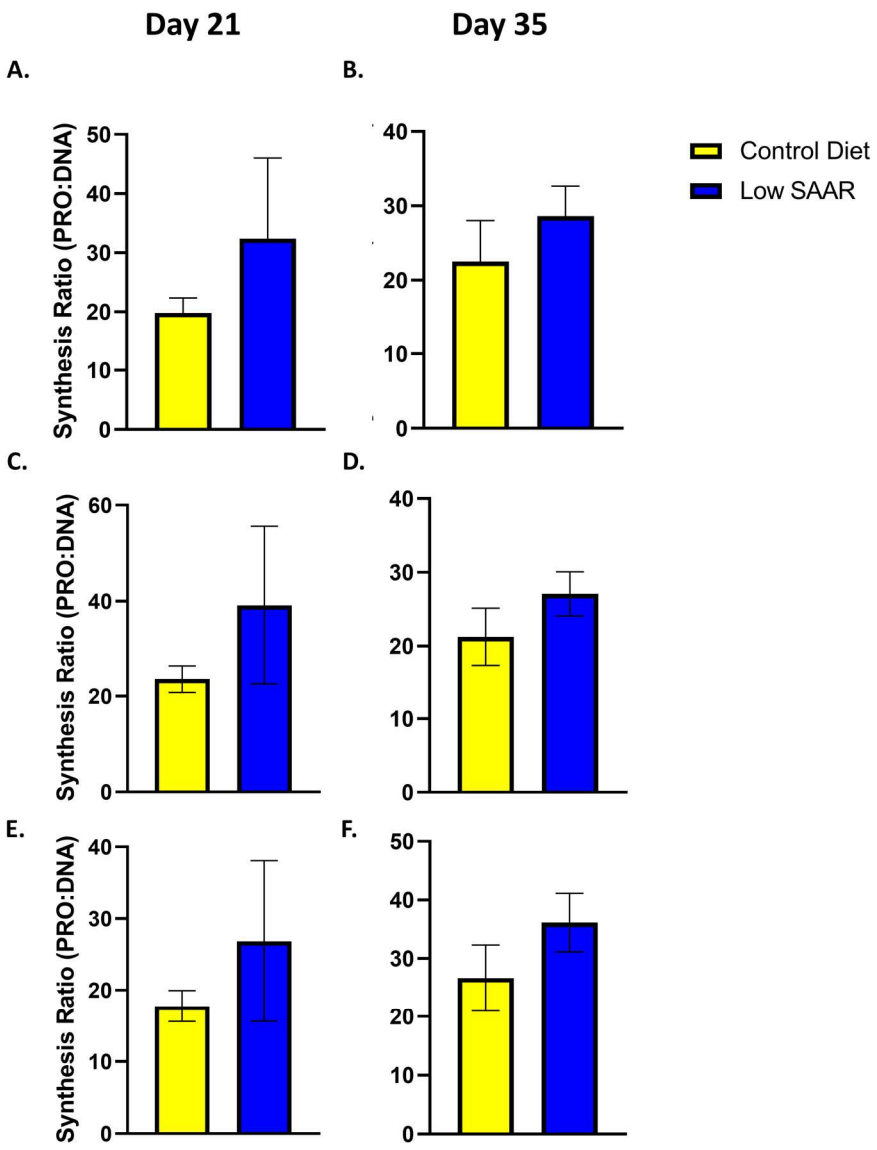

Figure 7. PRO:DNA ratios in the mitochondrial, mixed, and cytosolic fractions of brain frontal cortex at day 21 and day 35 of the Low SAAR diet. At day 21, there were no significant differences between the Low SAAR diet and control in the mitochondrial **(A)**, mixed **(C)**, and cytosolic **(E)** fractions. At day 35, there were no significant differences between the Low SAAR diet and control in the **(B)** mitochondrial, **(D)** mixed, and cytosolic **(F)** fractions. Data are presented as means +/- SD.

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