THESIS

NOVEL INSIGHTS INTO PROTEIN SYNTHESIS RATES IN THE BRAIN FOLLOWING TWO LIFESPAN-EXTENDING TREATMENTS

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ABSTRACT

NOVEL INSIGHTS INTO PROTEIN SYNTHESIS RATES IN THE BRAIN FOLLOWING TWO LIFESPAN-EXTENDING TREATMENTS

The number of individuals 65 years or older is rapidly increasing. Aging is the predominant risk factor for chronic disease and disability. Dramatic increases in the number of individuals living with chronic disease or disability will present unique societal challenges. Accordingly, much research has focused on treatments that slow the aging process to prevent many chronic diseases simultaneously. Treatments using two pharmaceuticals, rapamycin and rapamycin plus metformin, have been shown to extend lifespan and improve health in model organisms. Neurodegenerative diseases represent an important subset of debilitating chronic diseases, for which treatment is currently limited. The use of slowed aging treatments in research of neurological function may provide insight into causes of neurodegenerative disease. Protein homeostasis (proteostasis) is crucial for cell and organismal health. Loss of proteostasis is characteristic of aging and chronic disease, and slowed aging treatments improve proteostasis-related outcomes. Protein synthesis is a necessary component of proteostasis. The effect of rapamycin and rapamycin plus metformin on protein synthesis rates *in vivo* is unexplored. Investigation into the effect of both slowed aging treatments on protein synthesis rates in the brain could inform effects on neuronal health, which may have implications for neurodegeneration. The purpose of the current study is to establish the use of deuterium oxide as a stable isotopic tracer for brain

protein synthesis rates *in vivo*, and to determine the effect of two slowed aging treatments on brain protein synthesis rates. Supportive measurements related to proteostasis were also made. Deuterium oxide labeling allowed for measurement of subcellular brain protein synthesis rates with ample sensitivity to detect sex differences and responses to treatment. The results demonstrated a strong influence of sex in response to both rapamycin and rapamycin plus metformin. Both slowed aging treatments had differing effects on protein synthesis as well as other markers of proteostasis. This study is the first to demonstrate the use of deuterium oxide for protein synthesis rates in the brain, which represents a novel methodology for evaluating proteostasis in neuronal tissue. Further, this is the first study to explore and reveal the effects of rapamycin and rapamycin with metformin on protein synthesis rates in the brain. Future studies using these methods and slowed aging interventions in models of neurodegenerative disease may prove insightful in determining causes, pathologies, and treatments of age-related neurological disorders.

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CHAPTER I: INTRODUCTION

The median age of the human population is rapidly increasing worldwide. In the United States alone, conservative estimates predict that by 2030 the number of individuals older than 65 years will exceed 70 million (Olshansky, Goldman, Zheng, & Rowe, 2009). This shift in part reflects increased life expectancy due to advancements in healthcare and higher standards of living (Harper, 2014). However, this sudden change will present unique societal issues given that aging is the predominant risk factor for many chronic diseases (Anton et al., 2015; Rowe & Kahn, 1998), including an important subset of debilitating chronic diseases that affect the central nervous system, known as neurological disorders (World Health Organization, 2006).

The fact that aging is the leading risk factor for most chronic diseases suggests that aging is a target for preventing many chronic diseases simultaneously (Seals, Justice, & LaRocca, 2016). Despite the intricacies of aging, slowing the aging process may be an effective alternative to treating the primary etiology or individual symptoms of individual chronic diseases. For that reason, it is important to identify and understand the mechanistic causes of aging so that they may be targeted by slowed-aging treatments (Kennedy et al., 2014).

Slowed aging treatments are currently the focus of much research (López-Otín, Galluzzi, Freije, Madeo, & Kroemer, 2016). Two pharmaceutical compounds, rapamycin and metformin, extend lifespan and improve health outcomes (Anisimov, 2013; Harrison et al., 2009). Both appear to exert their benefits through coordination of overlapping cellular signaling cascades. Metformin and rapamycin are tools for increasing scientific knowledge of the aging process. Alterations in the mammalian target of rapamycin

(mTOR) signaling pathway are thought to underlie some of the beneficial effects of rapamycin and metformin treatment.

The maintenance of protein function and concentration is of particular importance at both the cellular and organismal level. Protein homeostasis (proteostasis) is the result of the collective processes of protein folding, degradation, and synthesis (Balch, Morimoto, Dillin, & Kelly, 2008). Loss of proteostasis is associated with aging and many chronic diseases (Labbadia & Morimoto, 2015a; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Loss of proteostasis appears to be particularly important in the context of neurodegenerative disease (Eftekharzadeh, Hyman, & Wegmann, 2016; Smith & Mallucci, 2016).

Important regulators of proteostasis are under the control of mTOR (Laplante & Sabatini, 2009), and thus may be affected by rapamycin and metformin. In this way, treatment with rapamycin and metformin may be used to better understand the effects of slowed aging treatments on proteostatic maintenance in the brain. The information gained from experiments on slowed aging treatments in brain could inform mechanisms that underlie age-related neurodegeneration and the onset of neurodegenerative disorders, as well as how each can be prevented.

Study purpose

The purpose of this study was to evaluate the effect of the slowed aging treatments, rapamycin and metformin, on proteostatic maintenance in the brains of male and female mice in both young and old cohorts.

Statement of Problem

Proteostatic Maintenance is a complex and dynamic cellular process that is necessary for proper cell function and organismal health. The capacity for proteostatic maintenance decreases with age and in disease. The central nervous system is particularly sensitive to loss of proteostasis, which is a characteristic of many neurodegenerative diseases. The effect of the health-promoting and lifespan extending treatments on proteostatic maintenance in the brain has yet to be evaluated.

Hypotheses

We hypothesize that the slowed aging treatments, rapamycin and rapamycin plus metformin in combination, will improve the outcomes of various markers of proteostatic maintenance in the brains of young and old mice when compared to mice that receive no treatment.

CHAPTER II: LITERATURE REVIEW

An Aging Global Population

The median age of the human population is rapidly increasing. In the United States, the number of individuals aged 65 years or older is expected to double between 2000 and 2030 (Olshansky et al., 2009). By the year 2050, the aging demographic will constitute 25-40% of the total population of most developed nations (Petsko, 2008). Global life expectancy is roughly seventy years, the highest it has ever been (H. Wang et al., 2016). Increased life expectancy shifts the age composition of a population by increasing the proportion of older individuals in a given population. Simultaneously, the absolute number of older individuals is increased. Thus, the primary outcome of increases in life expectancy is a greater total and relative number of older persons.

Current life expectancy is in stark contrast with the predicted lifespan of early Paleolithic human ancestors, who lived a mere 15-20 years in comparison (Gurven & Kaplan, 2007). Early increases in life expectancy followed the advent of early agriculture (Galor & Moav, 2007), while more recent advances can be attributed to high standards of living and access to advanced medical care in many areas of the world (Harper, 2014). Life expectancy has increased at a remarkably steady rate since the mid-19th century, and humans have consistently lived longer than their ancestors regardless of social, economic, and environmental crises (Oeppen & Vaupel, 2002). Further, human life expectancy is predicted to increase over the next four decades (Hackett, Cooperman, & Ritchey, 2015). Given the current global life expectancy, and the fact that it is likely to continue to increase, it is wise to anticipate that the elderly demographic will only continue to grow.

Increased global life expectancy is the culmination of advances made in healthcare and medical science. However, a rapid increase in the size of the elderly population is likely to present unique societal challenges. Many healthcare-related fields responsible for the well-being of older individuals may be strained as the demand for care and treatment increases proportionally with the elderly population.

Aging, Chronic Disease, and Disability

Despite advances made in extending total years lived (lifespan), aging remains the predominant risk factor for most chronic diseases, and is associated with general physiological decline (Seals et al., 2016). A person's risk for disability and chronic disease increases with age (Anton et al., 2015; Rowe & Kahn, 1997; Seals et al., 2016). Specifically, aging is the main risk factor for cancer, heart disease, stroke, diabetes, kidney disease, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Niccoli & Partridge, 2012; Seals et al., 2016). Peripheral complications associated with aging, such as obesity and chronic pain, are themselves risk factors for chronic disease (Anton et al., 2015).

Because aging is so strongly associated with chronic disease and disability, some predict an impending healthcare crisis due to such a rapid increase in the size of the older population (Olshansky et al., 2009; Petsko, 2008). As the number of older individuals in the world increases, the number of people living with chronic disease and disability will increase proportionally (Olshansky et al., 2009). Even now, roughly one half of all adults in the US are afflicted by at least one chronic disease (Ward, Schiller, & Goodman, 2014), and chronic disease is the leading cause of disability (Roper et al., 2009). Regardless of where a certain element of aging falls on the spectrum of general

physiological decline and full-blown disease, the current ailments associated with aging decrease quality of life, increases likelihood of injury, and increase the prospect that a person will be dependent on the care of others.

Aging and Neurological Disorders

Neurological disorders (ND) are an important subset of chronic diseases that impact the nervous system, and are often categorized by their effect on memory, cognitive function, and motor function. Similar to other chronic diseases, the risk for developing ND is extremely sensitive to increases in age (World Health Organization, 2006). One prominent example is Alzheimer's disease (AD), a devastating disorder that progressively worsens with time and primarily affects communication, judgement, and behavior (Alzheimer's Association, 2017). In the United States, 10% of individuals 65 years or older has AD, and the likelihood of having diagnosable AD increases to over 30% once a person is 85 years old (Hebert, Weuve, Scherr, & Evans, 2013).

As the number of elderly individuals increases, so too will the number of people living with ND. It is expected that by 2050 there will be nearly 1 million new cases of Alzheimer's each year (Alzheimer's Association, 2017). AD is a significant source of morbidity and mortality among the elderly. Those diagnosed with AD frequently need assistance with daily living. Eventually, AD patients become entirely dependent (Alzheimer's Association, 2017), and survival rates following AD diagnosis are very low (Larson et al., 2004). Similarly, the number of people living with Parkinson's disease, another prevalent and debilitating, age-associated ND, will have doubled between 2005 and 2030 (Dorsey et al., 2007).

ND will also cause economic strain, illustrating the type of issues facing a world with such a large elderly population. It's estimated that by 2050 the United States will spend 1 trillion dollars annually on ND (a large increase from current spending of 300 billion) (Petsko, 2008). Given the human welfare and economic impact of the current and rising incidence of ND, it is imperative to develop preventative medicine and treatments that may delay the onset or minimize the severity of ND.

Healthspan

Aging has been identified as the primary risk factor for chronic disease, and represents a viable target for minimizing the incidence and impact of several chronic diseases simultaneously (Blagosklonny, 2012c; Kennedy et al., 2014; Seals et al., 2016). In other words, an intervention that slows the aging process may prevent many chronic diseases at once. Slowing aging has been a long-time goal of humanity. When current human life expectancy is compared to historic human life expectancy (Wilmoth, 2000) it may appear that this goal has been accomplished. In some sense this is true, as most humans can expect to live considerably longer than their ancestors. However, most humans that do not die of accidents or acute causes experience some combination of physiological decline, disability, and chronic disease at the end of life (Bauer, Briss, Goodman, & Bowman, 2014). Thus, there is frequently a discrepancy between the total number of years a person lives (lifespan) and the number of years a person lives free from the burden of chronic disease and disability (healthspan).

The concept of healthspan has recently become a prominent feature of aging research (Kaeberlein, Rabinovitch, & Martin, 2015; Seals et al., 2016), and is the focus of a new field of aging research known as geroscience (Kennedy et al., 2014). In

general, the duration of healthspan is shorter than that of lifespan, and the end of healthspan is marked by the onset of disability and chronic disease. It has been proposed that the primary goal of aging research should be to increase the duration of healthspan, or to minimize the amount of time a person spends in physiological decline (Anton et al., 2015; Kennedy et al., 2014; Seals et al., 2016). Aging research should be conducted with the primary goal of improving health through minimizing or slowing the aging process, rather than simply extending the total number of years lived regardless of health status.

Conventionally, chronic disease research has focused on either the prevention of specific diseases, or treatment of individual symptoms once disease is established. Take, for example, the recommendation of sunscreen use to prevent cancer (Green, Williams, Logan, & Strutton, 2011), or the prescription of insulin to treat type 2 diabetes (Charbonnel, Penfornis, Varroud-Vial, Kusnik-Joinville, & Detournay, 2012). In these cases and others, this approach is relatively effective. However, the continually high prevalence of disability and chronic disease, particularly among the elderly, illustrates the shortcomings of utilizing such a narrow approach. If aging is the primary risk factor for many chronic diseases and disability, then the biology of aging is a suitable target for addressing many chronic diseases simultaneously. Thus, targeting aging is preferable to a single disease focus for chronic disease prevention. Increasing healthspan by slowing the aging process will prevent general age-associated physiological decline, and subsequently chronic disease.

Theoretical Causes of Aging

For some time, aging was thought to be the consequence of accumulated and irreversible molecular and macromolecular damage, caused primarily by reactive oxygen species (ROS) (Anson & Bohr, 2000; Beckman & Ames, 1998; Harman, 1956). Recently, it has become apparent that damage accumulation likely represents only part of the aging process, (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Doonan et al., 2008; Speakman & Selman, 2011) as treatments that reduced oxidative damage failed to extend lifespan in model organisms (Bjelakovic et al., 2007; Doonan et al., 2008; Pérez et al., 2009). Additional studies revealed that long-lived organisms do not necessarily possess increased protection against destructive ROS (Andziak, O'Connor, & Buffenstein, 2005; Bjelakovic et al., 2007; Voituron, de Fraipont, Issartel, Guillaume, & Clobert, 2011), and that impairing antioxidant defense systems does not shorten lifespan (Huang, Carlson, Gillespie, Shi, & Epstein, 2000; Pérez et al., 2009; Van Raamsdonk & Hekimi, 2009; W. Yang, Li, & Hekimi, 2007). It is true that markers of oxidative damage increase with aging and molecular damage has a role in age-associated physiological decline (Forster et al., 1996; Sohal, Agarwal, Dubey, & Orr, 1993). However, it appears that either molecular damage is merely a component of aging (Kennedy et al., 2014; Salmon, Richardson, & Pérez, 2010), or that a more sophisticated understanding of redox biology is required to fully appreciate its causal role in the aging process (Halliwell, 2012; Murphy et al., 2011).

At the same time, it has been demonstrated that the activity of highly conserved cellular signaling pathways also contributes to aging (Blagosklonny, 2008; M. Kaeberlein et al., 2005; Kapahi et al., 2010). Manipulation (both directly and indirectly)

of various processes involved in growth and responses to nutrient intake consistently extend lifespan and improve health outcomes in a diverse range of species, ranging from single-celled organisms to non-human primates (Blagosklonny, 2006). The existence of specific and conserved signaling cascades that directly regulate aging suggests that aging is a coordinated and programmed process, similar to those that regulate development and organismal function. However, considered in the context of evolution this theory also loses credibility. A synchronized process that serves only to promote physiological decline and increase susceptibility to disease would not be maintained through natural selection (Blagosklonny, 2007; Kirkwood, 2005; Williams, 1957). Additionally, humans surviving into what is currently considered “old age” is a very recent phenomenon on the timescale of human evolution (Gurven & Kaplan, 2007; Oeppen & Vaupel, 2002). The likelihood of natural selection to have the potential to select for genes that act only in “old age” is therefore extremely low (Kirkwood, 2005; Williams, 1957).

Evolutionary theory then eliminates the possibility of a direct, programmed cause of aging. This conclusion is problematic, however, as it is in conflict with the repeated observation that identifiable genes and molecular signaling pathways regulate longevity. As mentioned, the cellular networks that regulate aging are also necessary for developmental growth, as well as responses to nutrient intake (Gangloff et al., 2004; Kapahi et al., 2010). Commonalities between these two processes indicate that organismal growth and aging share a common mechanism. Based on this idea, it has been hypothesized that aging is not necessarily programmed, but is simply the inappropriate and unnecessary continuation of growth and development when they are

no longer needed (Blagosklonny, 2006, 2010; Gems & de la Guardia, 2013). Thus, aging may be “quasi-programmed” in that it is the needless activation of cellular programs that otherwise serve necessary purposes.

In the quasi-programmed, or “hyperfunction”, model of aging organismal senescence is caused by processes that promote growth during development but do not cease when growth is no longer necessary (Blagosklonny, 2007, 2010; Gems & de la Guardia, 2013). Continuation of these processes leads to cellular and organismal hyperfunction, which is a detriment to the cell and eventually the organism (Blagosklonny, 2012b, 2013a). This model is appealing because it appears to be well supported experimentally. Treatments that slow growth or decrease the activity of cellular mechanisms that contribute to growth improve health outcomes and extend lifespan with remarkable consistency (Kaeberlein et al., 2005; Kapahi et al., 2004a; Selman et al., 2009). Additionally, unlike theories of programmed aging, the “quasi-programmed” theory of aging is feasible in the context of evolution. Until very recently, humans did not live into old age due to acute causes of death. In a scenario where humans do survive into old age, there is no selective pressure to inhibit processes that beneficially promote growth early in life, but eventually cause aging late in life (Blagosklonny, 2007).

That hyperfunction precedes age-related physiological decline can be observed in many age-related chronic diseases (Tsang, Qi, Liu, & Zheng, 2007). For example, hyperactivity of cells that secrete cytokines causes inflammation, a hallmark of aging (Kennedy et al., 2014) and ubiquitous characteristic of chronic disease (Hunter, 2012). Cancer also provides an example of hyperfunction resulting in dysfunction which

contributes to chronic disease. It is true that cancer can arise from genetic mutations caused by damage (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). However, DNA damage in itself is not sufficient for cancer, and cancer does not occur unless genetic damage somehow causes dysregulation of cell growth and proliferation (Guertin & Sabatini, 2005; Vogelstein & Kinzler, 2004).

Although the hyperfunction model of aging addresses inconsistencies that arise in some theories of aging, it is not without its own limitations. For example, many age-associated disease, such as sarcopenia (Brooks & Faulkner, 1994) and Alzheimer's disease (de la Monte, 1989; Swaab, Hofman, Lucassen, Salehi, & Uylings, 1994), are associated with cell atrophy, which is in conflict with the idea that it is cell growth and proliferation that underlies aging. Proponents of the hyperfunction model of aging may contest that some form of hyperactivity precedes such atrophy (Blagosklonny, 2012a). It has been argued that if hypertrophy and hyperactivity are vaguely defined, nearly physiological characteristic can be attributed to them, and that the generation of damage inducing-ROS is in fact due to cellular hyperactivity (Zimniak, 2012).

The disposable soma theory of aging posits that energetic resources are allocated in a way that either promotes growth and reproduction, or favors conditions of maintenance and repair (Kirkwood & Holliday, 1979; Kirkwood, 2005). The basis of the disposable soma theory is that in the wild, the soma need be maintained only as long as an organism has a realistic chance of surviving and reproducing (Kirkwood & Austad, 2000). Under favorable energetic conditions, processes that promote reproduction will be favored over those which would slow aging through cellular and tissue maintenance and repair (termed "somatic maintenance"). From an evolutionary standpoint,

maintenance and repair (which contribute longevity of the individual) is of less importance than the passing of genetic material to future generations, especially when the probability of extrinsic mortality is high (Kirkwood & Rose, 1991). In the wild, the benefit of investing in maintenance and repair is outweighed by the benefit of investing in short-term survival and reproduction. The disposable soma theory is supported by studies showing that reproduction is inversely related to survival in humans (Tabatabaie et al., 2011) and that the elimination of the germ line extends lifespan in some (Berman & Kenyon, 2006; Hsin & Kenyon, 1999), but not all (Barnes, Boone, Jacobson, Partridge, & Chapman, 2006), model organisms tested.

One possible reconciliation of the discrepancies in each of the theories of aging is that no theory is mutually exclusive from another. One characteristic that is constant in each of the aforementioned theories of aging is that biological imperfections accumulate with time and impair cell and organismal function. It has been proposed that aging arises from a combination of *both* random and intrinsic processes. These imperfections constitute the “deleteriome”, which encompasses damage (in its classical sense) and all detrimental processes initiated by genetics and cell signaling (Gladyshev, 2016). Over time, the deleteriome grows in both its size and effect on the organism, which gradually results in aging. The deleteriome model of aging offers a potential compromise between molecular damage and cell signaling pathway-based theories of aging.

Clearly, aging is complex and much remains to be learned about its underlying causes. Although there is no consensus on any single factor that is the proximal cause of aging, long-lived model organisms and treatments that extend lifespan can be used

to study the impact of various biological processes and factors that may contribute to the aging process. Experimental models of slowed aging can be used in laboratory settings to help better understand why we age and how the rate of aging can be altered.

Models of Slowed Aging

A variety of experimental interventions known as slowed aging treatments act on growth-inducing and nutrient-sensing pathways to extend lifespan and improve health. The link between growth and aging is readily observed in the case of caloric restriction (CR), or decreased caloric intake without causing malnutrition. CR is the most well-established (Heilbronn & Ravussin, 2003; Masoro, 2003, Masoro 2005) and long-known (Osborne, Mendel, & Ferry, 1917) longevity-promoting experimental intervention. CR improves survival and health outcomes in a wide range of species, from single-celled budding yeast to non-human primates (Bodkin, Alexander, Ortmeyer, Johnson, & Hansen, 2003; R. J. Colman et al., 2009; Ricki J. Colman et al., 2014; Mattison et al., 2012). Even upon its discovery a century ago, the lifespan extending effects of CR were correctly (if indirectly) associated with its effect on growth (Osborne et al., 1917).

Recently, slowed aging treatments that utilize genetic manipulation have also proven to be indispensable for investigating the mechanisms that underlie aging and how they can be targeted (Blagosklonny, 2009). Similar to CR, many of these manipulations involve pathways related to nutrient sensing and growth. Ames and Snell dwarf mice lack growth hormone signaling, and are both long-lived (Bartke et al., 2001; Bartke, 2006; Brown-Borg, Borg, Meliska, & Bartke, 1996; Miller, 1999). A wide range of animals genetically engineered to be deficient in various components of the insulin/insulin-like growth factors signaling axis (IIS) are long-lived (Kenyon, 2010; van

Heemst, 2010), including *Drosophila melanogaster* (Tatar et al., 2001)*, Caenorhbaditis. Elegans* (Friedman & Johnson, 1988)*,* and *Mus Musculus* (Selman et al., 2007). Reductions in IIS have also been proposed to play a role in longevity in humans (Kenyon, 2010).

In addition, certain pharmaceutical compounds have become prominent players in aging research as potent slowed aging treatments. The immunosuppressant, rapamycin (also known as siromilus) is one such compound (Ehninger, Neff, & Xie, 2014; Harrison et al., 2009). Currently, the anti-diabetic drug Metformin is the topic of extensive study in aging research (Anisimov, 2010, 2013; Anisimov et al., 2008b). As will be discussed further, both have been shown to extend lifespan and improve health in a diverse array of species including humans.

Each slowed aging intervention provides unique insight to the aging process. Identification, understanding, and refinement of these treatments is essential to understanding aging so that eventually it may be slowed. It should be a high priority to identify common underlying mechanisms that link each of these treatments together.

Molecular Mechanisms of Aging

Many experimental treatments that extend lifespan mimic or induce growth restriction. This shared characteristic opens the possibility that multiple slowed aging treatments, although unique in nature, converge on a single physiological element. Much research suggests that this is may be the case. At the nexus of each the aforementioned lifespan extension treatments is the conserved serine/threonine kinase, mechanistic target of rapamycin (mTOR) (Kapahi et al., 2010). mTOR lies downstream to a variety of growth-promoting pathways, such as the insulin/insulin-like growth factor

signaling axis (IIS) and the growth hormone signaling axis (Laplante & Sabatini, 2009). Additionally, mTOR is sensitive to cellular energy status and responds to changes in cellular AMP/ATP levels through inhibition by 5' adenosine monophosphate-activated protein kinase (AMPK) (Inoki, Zhu, & Guan, 2003). mTOR also acts as a direct nutrient sensor and is stimulated by amino acids (Nobukuni et al., 2005).

In a general sense, mTOR is a ubiquitous anabolic promoter, inducing cell growth and proliferation in response to nutrients and growth factor binding (Kim, Buel, & Blenis, 2013). One of the primary roles of mTOR is as a key regulator of protein synthesis, primarily through translation initiation, elongation, and ribosomal biogenesis (Kapahi et al., 2010). mTOR also regulates lipid biosynthesis to promote cell division, inhibits catabolic processes (mainly autophagy), and promotes progression through the cell cycle (Fingar et al., 2004; Laplante & Sabatini, 2009). Under the proper circumstances, cell growth and division induced by mTOR are necessary components of healthy cell and organismal function and development. The importance of mTOR during development is demonstrated by studies which show that mTOR knockouts are embryonic lethal (Guertin et al., 2006; Murakami et al., 2004). That reducing the sources of input to mTOR results in lifespan extension, coupled with the fact that mTOR is a crucial regulator of growth and development, implicates mTOR as a possible mechanistic link between nutrient intake/status, growth, and aging (Johnson, Rabinovitch, & Kaeberlein, 2013).

The notion that mTOR connects nutrient intake, growth, and aging is supported by a growing body of research that shows inhibition of mTOR, directly or indirectly, extends lifespan. In *Saccharomyces cerevisiae*, deletion of genes involved in the mTOR

pathway extends lifespan (Kaeberlein et al., 2005; Powers, Kaeberlein, Caldwell, Kennedy, & Fields, 2006). The use of RNA interference in *C. elegans* has demonstrated that deficiency in mTOR signaling is sufficient for lifespan extension (Vellai et al., 2003), and mTOR is involved in IIS-regulated lifespan extension (K. Jia, Chen, & Riddle, 2004). In *D. melanogaster,* decreased activity of both upstream and downstream components of mTOR by genetic manipulation results in increased lifespan (Kapahi et al., 2004b). Overexpression of tuberous sclerosis complex, a primary negative regulator of mTOR, improves a number of health outcomes and extends lifespan in mice (Zhang, Diaz, Walsh, & Zhang, 2017). In the long-lived Ames dwarf mice, mTOR signaling is significantly reduced (Sharp & Bartke, 2005). Additionally, deletion of ribosomal protein s6 kinase (S6K), one of two primary effectors of mTOR that promotes translation, is sufficient for lifespan extension in mice (Selman et al., 2009).

Although mTOR activity appears to have an important role in longevity, it cannot explain all models of slowed aging. However, not all models of slowed aging demonstrate decreased mTOR signaling, and modification of mTOR signaling is not always required for lifespan extension. Caloric restriction during suckling by increasing litter size in (crowded litter, CL) increases lifespan (Sadagurski et al., 2014). In CL mice mTOR signaling is unaffected at 4 months and increased at 7 months of age (Drake et al., 2014). Additionally, restriction of dietary methionine promotes longevity without altering mTOR signaling (Sun, Sadighi Akha, Miller, & Harper, 2009), and branched-chain amino acid supplementation extends lifespan despite increases in mTOR substrate activation (D'Antona et al., 2010).

Clearly, mTOR signaling is a key piece of the aging puzzle. Repeatedly, modulations in mTOR signaling extend lifespan in model organisms, and decreased activity through the mTOR pathway is a common feature of models of slowed aging. Despite that mTOR inhibition is not necessary in all models of slowed aging, the growing body of literature on mTOR-mediated lifespan extension warrants further investigation.

Regulation of Aging by mTOR

Decreased activity through the mTOR signaling pathway, either directly or indirectly, improves health and increases lifespan in many model organisms. Signaling through the mTOR pathway is required for development and mTOR knockouts are not viable (Guertin et al., 2006; Murakami et al., 2004). However, reductions in mTOR activity underlie lifespan extension in various models of slowed aging, including impaired IIS (K. Jia et al., 2004) and caloric restriction (Kaeberlein et al., 2005). Additionally, genetic inhibition of the mTOR signaling pathway is sufficient for lifespan extension (Selman et al., 2009). That mTOR activity is required for development, and its inhibition promotes longevity seems paradoxical. This raises the question of how mTOR regulates lifespan extension. The general role of mTOR is to induce cell growth and proliferation in response to nutrients, growth factors, and high energy status (Fingar et al., 2004; Laplante & Sabatini, 2009). In regard to aging, it appears that mTOR regulates the aging process primarily through protein synthesis, protein catabolism (autophagy), responses to stress, and alterations in metabolism (Kapahi et al., 2010).

In some cases, inhibition of translation is sufficient for lifespan extension. mTOR stimulates protein synthesis by phosphorylation of its downstream effectors, eukaryotic

initiation factor eIF4E-binding protein (4E-BP) and S6K (Kapahi et al., 2010). Reducing translational rates by decreasing 4E-BP, S6K, or ribosomal proteins extends lifespan in *C. elegans* (Hansen et al., 2007), as does direct inhibition of translation (Pan et al., 2007). This finding has been repeated in other model organisms (M. Kaeberlein et al., 2005; Kapahi et al., 2004b; Selman et al., 2009; Steffen et al., 2008). The translational repressor, 4E-BP, is required for CR induced lifespan extension (Zid et al., 2009). Constitutively active 4E-BP extends lifespan as well, which is not furthered by CR (Zid et al., 2009). Additionally, protein synthesis negatively corresponds with lifespan (Katewa & Kapahi, 2011). Although reduced rates of protein synthesis may be important for mTOR-mediated lifespan extension, none of the aforementioned studies measured the effects of cell proliferation, which may be the primary, or an additional, mechanism by with mTOR inhibition extends lifespan.

Autophagy is one of two primary means of protein degradation in the cell and acts as a clearance mechanism of cellular damage. mTOR inhibits autophagy (Jung, Ro, Cao, Otto, & Kim, 2010), and increased autophagic activity has been strongly implicated in longevity (Madeo, Tavernarakis, & Kroemer, 2010; Petrovski & Das, 2010). In *C. elegans*, autophagy is necessary for CR to increase lifespan (Hansen et al., 2008), and inhibition of translation of autophagic proteins shortens lifespan (Hars et al., 2007).

In addition to its effect on protein synthesis and degradation, mTOR is also involved in cellular stress resistance. Pharmacological inhibition of mTOR in both *C. elegans* and *M. muluscus* activates endogenous xenobiotic defense systems, regulated by SKN-1 and NRF2, respectively (Robida-Stubbs et al., 2012; Steinbaugh, Sun, Bartke, & Miller, 2012). Despite large-scale decreases in protein translation following

mTOR inhibition (X. M. Ma & Blenis, 2009), certain nuclear-encoded mitochondrial proteins maintain translational rates (Zid et al., 2009), and the function of these mitochondrial proteins is required for CR-induced lifespan extension. Mitochondrial protein synthesis may reflect increased or maintained oxidative capacity during CR, which could ensure adequate ATP stores for energetically costly but beneficial processes (Zid et al., 2009), such as protein turnover. Additionally, CR-induced mitochondrial biogenesis is associated with increased mitochondrial efficiency and lower ROS production (López-Lluch et al., 2006), adding a further layer of stress resistance attributable to mTOR inhibition.

Slowed Aging Tools - Rapamycin and Metformin

The pharmaceuticals, rapamycin and metformin, are among the growing list of slowed aging treatments garnering much attention in the field of geroscience. Each serves as a valuable tool for investigating the mechanisms of aging and effects of slowed aging treatments. Originally isolated as a macrolide antibiotic from the bacteria, *Streptomyces hygroscopicus*, rapamycin was discovered in soil obtained from the island of Rapa Nui (Vézina, Kudelski, & Sehgal, 1975). Rapamycin has conventionally been prescribed to prevent organ rejection following transplantation (Groth et al., 1999), and to treat various forms of cancer (Seto, 2012). In model organisms, rapamycin extends lifespan and improves a multitude of health outcomes.

In 2009, rapamycin became the first compound to extend lifespan in both sexes of a mammalian species (Harrison et al., 2009). A multi-site study conducted through the National Institute on Aging's Intervention Testing Program (ITP) showed that consumption of rapamycin significantly extended median and maximal lifespan in both

male and female genetically heterogeneous mice (Harrison et al., 2009). Importantly, lifespan extension was observed when rapamycin was administered late in adult life (600 day-old mice, roughly the equivalent of 60 year-old humans), showing that lifespan extending treatments may be effective even when initiated in old age.

Additionally, the relative contributions of age-related causes of death were not different between rapamycin-treated and control mice, suggesting that the increase in lifespan indicates slowed aging, rather than the prevention of any single disease (Harrison et al., 2009). That rapamycin improves certain health outcomes, in addition to extending lifespan, has been shown repeatedly. In mice, rapamycin prevents age-associated macular degeneration (Kolosova et al., 2012), increases in obesity (S.-B. Yang et al., 2012), decreased cognitive function (Halloran et al., 2012), liver degeneration (Wilkinson et al., 2012), declines in physical activity and strength (Wilkinson et al., 2012; Xue et al., 2016), arterial stiffness (Lesniewski et al., 2017), and tumorigenesis in mice genetically predisposed to cancer (Komarova et al., 2012). The results of a comprehensive study on the effect of rapamycin on healthspan and aging appeared to suggest that the effect of rapamycin-mediated lifespan extension was primarily due to the prevention of cancer (Neff et al., 2013). However, this conclusion has been contested as a misinterpretation (Blagosklonny, 2013b; Johnson, Martin, Rabinovitch, & Kaeberlein, 2013). Further, rapamycin extends lifespan in organisms that do not develop cancer (Bjedov et al., 2010; Powers et al., 2006; Robida-Stubbs et al., 2012)

Metformin is widely prescribed to improve insulin sensitivity for treatment of type 2 diabetes (T2D). Metformin has also been suggested as a feasible slowed aging

treatment for use in humans (Barzilai, Crandall, Kritchevsky, & Espeland, 2016). The use of metformin to slow aging in the general population is the primary focus of the recently proposed, large-scale clinical trial, “TAME” (Targeting Aging with MEtformin) (Barzilai et al., 2016), which would represent the first clinical trial of a drug intended to slowing aging and increase the duration of healthspan.

In both model organisms and humans, metformin appears to induce a variety of protective effects. In nematode worms, metformin mimics the effects of CR and increases healthspan (Onken & Driscoll, 2010). In mice, metformin decreases body weight and extends both median and average lifespan (Anisimov et al., 2008; Martin-Montalvo et al., 2013), although the direct effect of metformin on lifespan in rodents is somewhat inconsistent (Strong et al., 2016). Metformin improves measurements of cognitive function in older mice, in addition to increasing lean body mass and protecting against fat accumulation in the face of high fat diet (Allard et al., 2016). In individuals diagnosed with T2D, metformin may decrease risk for atherosclerosis (Goldberg et al., 2017) and cardiovascular disease (UK Prospective Diabetes Study Group, 1998). Results from the United Kingdom Prospective Diabetes Study demonstrated that metformin decreased diabetes-related mortality by 42%, and all cause-mortality was decreased by 36% in those taking metformin (UK Prospective Diabetes Study Group, 1998). Epidemiological data suggests that not only do type 2 diabetics who are prescribed metformin live longer than those prescribed other common antidiabetic drugs, they may even surpass their nondiabetic counterparts in survival (Bannister et al., 2014).

The apparent efficacy and relative safety of both rapamycin and metformin as slowed aging treatments begs the question of why each or both is not currently used for widespread disease prevention in humans. In large part, this is due to a lack of clinical trials for either as slowed aging tools. The absence of a clear, clinical definition of aging contributes to inconsistencies in opinion of how aging should be targeted, and limits progress in developing anti-aging clinical trials (Barzilai et al., 2016). Additionally, although rapamycin extends lifespan and is beneficial to many aspects of health, chronic rapamycin treatment has negative side effects, including testicular degeneration and cataracts in mice (Wilkinson et al., 2012). Similarly, chronic rapamycin treatment impairs glucose homeostasis by decreasing hepatic insulin sensitivity (Lamming et al., 2012). Based on this, intermittent treatment of rapamycin has been proposed to potentially negate these effects, and in limited testing of intermittent treatment has shown be effective (Anisimov et al., 2011; Arriola Apelo, Pumper, Baar, Cummings, & Lamming, 2016). Finally, co-treatment with metformin may offset the impaired glucose tolerance associated with rapamycin (Aliper et al., 2017; Blagosklonny, 2017), and this approach may demonstrate additive effects of both drugs on healthspan.

Modification of Signaling Pathways by Rapamycin and Metformin

mTOR was discovered during exploration of the anti-growth properties of rapamycin, for which the protein kinase is named (Heitman, Movva, & Hall, 1991). Upon testing it was revealed that rapamycin-induced growth inhibition is mediated by a protein (or several proteins, in yeast) deemed target of rapamycin (and subsequently, mechanistic target of rapamycin) (Heitman et al., 1991). Rapamycin is a direct and potent inhibitor of mTOR. The primary mechanism by which rapamycin inhibits mTOR is

through allosteric inhibition. Rapamycin binds 12-kDa FK506-binding protein (FKBP12), which inhibits the assembly of mTOR complex 1 (mTORC1) (Laplante & Sabatini, 2009). The main action of mTORC1 is to regulate cell growth and division (Fingar et al., 2004). mTOR forms a second complex, (mTORC2) that regulates cytoskeletal organization and dynamics and is rapamycin-insensitive (Jacinto et al., 2004). However, chronic administration of rapamycin affects both mTOR complexes, and inhibition of mTORC2 contributes to rapamycin-induced hepatic insulin resistance (Lamming et al., 2012).

Although the exact molecular mechanism of metformin remains unclear (Rena, Hardie, & Pearson, 2017), it is currently thought that the beneficial effects of metformin are stimulated by activation of AMPK (Zhou et al., 2001). A widely accepted explanation of this effect is that metformin inhibits complex I of the electron transport chain, increasing the ratio of cellular AMP:ATP, thereby activating AMPK (El-Mir et al., 2000; Owen, Doran, & Halestrap, 2000). However, AMPK activation may not be required for the insulin sensitizing effects of metformin (Foretz et al., 2010; Guigas et al., 2006). Activated AMPK inhibits mTOR (Xu, Ji, & Yan, 2012). Specifically, AMPK targets tuberous sclerosis complex 2, which forms a complex with tuberous sclerosis complex 1 to inhibit the formation of mTORC1 (Howell et al., 2017a; Inoki et al., 2003). Additionally, AMPK phosphorylates the mTOR binding protein, raptor, further preventing the formation of mTORC1 (Gwinn et al., 2008). In this way, both rapamycin and metformin inhibit the growth and proliferative effects of mTOR, potentially explaining their effect on lifespan and health.

Rapamycin and Metformin in the Brain

The rise to prominence of metformin and rapamycin in aging research has sparked investigation into the effect of each on the central nervous system. Although most research has focused on the effect of rapamycin and metformin in peripheral tissues, each crosses the blood brain barrier (Halloran et al., 2012; Łabuzek et al., 2010; Meikle et al., 2008) and may affect the central nervous system. In mice, rapamycin reduces age-associated decreases in physical activity (Miller et al., 2011; Neff et al., 2013; Wilkinson et al., 2012) and improves performance in tasks involving learning and memory (Neff et al., 2013). Near lifelong treatment of rapamycin improves cognitive function in older mice and may be related to decreases in brain inflammation (Majumder et al., 2012). One comprehensive study replicated findings that rapamycin treatment improves spatial learning and memory in both young and old mice (Halloran et al., 2012). It was also revealed that rapamycin increased the levels of the neurotransmitters; norepinephrine, dopamine, and 5-hydroxytryptamine, in conjunction with apparent decreased anxiety and depressive-like behavior (Halloran et al., 2012b).

Recently, rapamycin has been proposed as a treatment for multiple forms of ND (Bové, Martínez-Vicente, & Vila, 2011). Rapamycin is thought to be neuroprotective, in part, through induction of autophagic processes (Cai & Yan, 2013; Jahrling & Laberge, 2015). In transgenic mouse models of AD, rapamycin improves cognitive function, promotes autophagy, and reduces the levels of amyloid-B (AB) (Spilman et al., 2010). Similarly, there is strong evidence for the role of mTOR in tau hyperphosphorylation, a potential contributor to AD (Gong & Iqbal, 2008). Rapamycin prevents this process and restores normal cognitive function (Caccamo et al., 2013). Neuroprotection has been

observed using rapamycin and a rapamycin ester in *Drosophila* and mouse models of Huntington's disease, respectively (Ravikumar et al., 2004). Additionally, rapamycin prevents loss of cognitive function in a mouse model of Parkinson's disease by restoring mitochondrial homeostasis via induction of mitophagy (Siddiqui et al., 2015).

Although much research is still needed, metformin represents another potential treatment in chronic neurodegenerative disorders (Markowicz-Piasecka et al., 2017). Metformin appears to elicit similar effects in the brain as in peripheral tissue, such as AMPK activation and subsequent increases in autophagy (Jiang et al., 2014). Metformin extends lifespan of male mice by 20% in a transgenic model of Huntington's disease in a dose-dependent manner (Ma et al., 2007). Type 2 diabetics are at increased risk for AD, and T2D can speed the rate of cognitive decline (Ascher-Svanum et al., 2015; Luchsinger, Tang, Shea, & Mayeux, 2004). The effect of T2D on cognitive function appears to be reduced by metformin treatment (Cheng et al., 2014). Interestingly, the apparent neuroprotection offered by metformin in those with T2D is dependent on the duration of treatment, and not observed following treatment of all types of antidiabetic medication. Mice subjected to AB show decreased long-term potentiation, a neural adaptation process that is the basis of synaptic plasticity and memory. Metformin attenuates this decrease, suggesting neuroprotective effects and implicating metformin as a potential treatment for AD (Asadbegi, Yaghmaei, Salehi, Ebrahim-Habibi, & Komaki, 2016). Additionally, metformin increases neuronal insulin sensitivity, a process which reverses AD-like characteristics of hyperglycemia-exposed neuronal cells (Gupta, Bisht, & Dey, 2011). However, one study found that treatment with metformin increases AB production *in vitro* (Chen et al., 2009). Co-treatment with insulin reversed this effect

and lowered AB biogenesis overall (Chen et al., 2009), suggesting that a combination of metformin and insulin may protect against AD. This is congruent with epidemiological research that has demonstrated protection against AD in type 2 diabetics when insulin and metformin are taken together (Beeri et al., 2008).

Neurodegenerative diseases are commonly associated with protein aggregation (Eftekharzadeh et al., 2016). Although some research is conflicting, it appears that both rapamycin and metformin offer neuroprotection under certain conditions. Given the effect of rapamycin and metformin on both protein synthesis and breakdown, it is possible that the primary means by which both rapamycin and metformin maintain or promote neuronal function is through preservation of the proteome.

Proteostasis

Protein homeostasis, or proteostasis, refers to the collective processes that maintain protein concentration, location, and conformation (Balch et al., 2008). Maintenance of the proteome (proteostatic maintenance) is accomplished by an extraordinarily complex and coordinated system known as the “proteostasis network” (PN), which can be divided into three broad branches; protein translation (synthesis), degradation, and folding (Klaips, Jayaraj, & Hartl, 2018). The cyclical process of protein synthesis and degradation is often referred to as protein turnover (Hinkson & Elias, 2011). Protein turnover is a crucial cell, tissue, and organismal function. Loss of proteostasis is characteristic of many chronic diseases as well as aging (Cohen & Kelly, 2003; Labbadia & Morimoto, 2015b).

Protein Synthesis

As discussed, protein synthesis is largely under the control of mTORC1, and is regulated by a wide variety of factors including energy status, growth factor signaling, mechanostransduction, hypoxia, and nutrients (Laplante & Sabatini, 2009; X. Wang & Proud, 2006). Protein synthesis is a critical component of cell function and proteostatic maintenance, determining many important characteristics of the proteome (Arnsburg & Kirstein-Miles, 2014). Protein synthesis is required for the cell growth and division (Polymenis & Aramayo, 2015), as well as adaptations to stress and hormesis (Holcik & Sonenberg, 2005; Radak, Chung, Koltai, Taylor, & Goto, 2008; Rogers et al., 2011), a concept that has implications for health and aging. For example, in response to energetic stress (such as in CR and exercise), low cellular ATP levels stimulate the synthesis of mitochondrial proteins (Hood, 2009; Miller, Robinson, Bruss, Hellerstein, & Hamilton, 2012; Zid et al., 2009), an outcome that better equips the cell and organism to handle energetic stress in the future.

Protein Folding

In order to properly function, proteins must contain the correct sequence of amino acids. However, the appropriate amino acid sequence alone is not sufficient for protein function, and proteins must be folded into highly specific 3-dimensional structures known as conformations. Following translation, proteins spontaneously adapt a partially folded state due to the loss of free energy associated with intramolecular bonding within a polypeptide (Onuchic & Wolynes, 2004). Mechanisms that aid in protein folding are necessary, as spontaneous folding is error prone (Schubert et al.,

2000), and proteins must be refolded following oxidative damage (Wickner, Maurizi, & Gottesman, 1999).

Because of the importance of protein folding, cellular machinery exists solely for the purpose of ensuring adequate protein folding. The heat shock protein family (HSP) and chaperonins are molecular protein folding aids (Klaips et al., 2018). Heat shock proteins recognize hydrophobic amino acid sequences that become exposed upon misfolding (Rüdiger, Buchberger, & Bukau, 1997), and promote the correct conformation utilizing ATP hydrolysis (Clerico, Tilitsky, Meng, & Gierasch, 2015). If the HSP system is not successful in folding a protein, it may be transferred to a number of chaperonins (Lopez, Dalton, & Frydman, 2015), adding another layer of regulation for protein folding.

Protein Degradation

If protein folding mechanisms fail, misfolded and damaged proteins must be degraded to avoid their accumulation. This process is essential, as the accumulation of damaged proteins and protein aggregates is toxic to cells (Morimoto, 2008). Additionally, protein degradation is essential for cell and tissue remodeling (Mizushima & Komatsu, 2011). Breakdown of proteins generally occurs through one of two mechanisms; autophagy and proteosomal degradation (Mizushima & Klionsky, 2007; Mortimore & Poso, 1987; Poppek & Grune, 2006). Proteosomal degradation tends to target individual proteins, whereas autophagic processes are better equipped for large protein aggregates and whole organelles. Protein degradative processes are essential, as the enzymatic capacity for protein repair is low (Mary et al., 2004). Thus, the primary

means of protein “repair” is through degradation and re-synthesis (Mary et al., 2004; Mortimore & Poso, 1987).

Maintenance of the proteome is accomplished by a wide range of complex and highly coordinated processes. Given the necessity of appropriate protein concentration and function, it is difficult to overstate the importance of proteostatic maintenance in health and disease, as well as slowed aging.

Proteostasis and Slowed Aging

Recently, proteostasis was identified as one of the seven pillars of aging (Kennedy et al., 2014). Loss of proteostasis is thought to be a major contributor to both aging and age-related disease (Kaushik & Cuervo, 2015; Labbadia & Morimoto, 2015b; López-Otín et al., 2013). Thus, proteostasis is a target for slowed aging treatments and the prevention of chronic disease (Balch et al., 2008). As discussed, a simplified view of the PN is that it is comprised of cellular machinery responsible for protein synthesis, folding and refolding, as well as degradation. Importantly, each branch of the PN is compromised with aging.

Protein translation decreases with age (Rattan, 1996), and in age-related chronic disease (Proctor, Balagopal, & Nair, 1998; K. L. Smith & Tisdale, 1993). This is somewhat paradoxical as treatments that inhibit translation prolong lifespan (Hansen et al., 2007). Some have speculated that reduced translation may lessen the burden of the other components of the PN, thus promoting proteostatic maintenance (Taylor & Dillin, 2011; Walter & Ron, 2011). Additionally, decreased protein synthesis is associated with decreased rates of cell proliferation, which may indicate preferential synthesis of proteins for proteostatic maintenance (Miller et al., 2012). Indeed, in some settings

reduced translation promotes resistance to external stressors (Hansen et al., 2007). Additionally, treatments that reduce translation may act to promote the synthesis of proteins involved in other components of the PN that contribute to proteostatic maintenance (Hansen et al., 2007; McColl et al., 2010; Rogers et al., 2011) . Based on this, it has been hypothesized that decreased rates of translation and cell proliferation shift cells to a state that favors the allocation of resources to maintenance and repair (Drake et al., 2015; Hansen et al., 2007), consistent with the disposable soma theory of aging. This shift would also decrease rates of growth, and is therefore a potential link between inhibition of mTOR and lifespan extension.

The capacity for protein folding also decreases with age, a phenomenon that contributes to an accumulation of damaged proteins (Stadtman, 1992). In model organisms, the ability of protein chaperons to maintain proteostasis decreases with age (Ben-Zvi, Miller, & Morimoto, 2009). The capacity of protein folding mechanisms is implicated in lifespan extension. Function of the HSP family of chaperones is required for CR lifespan extension in nematodes (Morley & Morimoto, 2003), and long-lived IIS mutants show increased stress resistance through induction of the HSP, *hsp-16A* (Walker & Lithgow, 2003). Additionally, overexpression of *hsp22* is sufficient for lifespan extension in *Drosophila* (Morrow, Samson, Michaud, & Tanguay, 2004).

As discussed, proteins that cannot properly be refolded are targeted for degradation by the ubiquitin-proteasome system, or the autophagy-lysosomal system. Damaged proteins must be degraded and then resynthesized in order for proteostasis to be maintained. Autophagic clearance of damaged proteins decreases with age (Cuervo, 2008; Simonsen et al., 2008a; Vittorini et al., 1999). Several studies have

demonstrated the necessity of autophagy for lifespan extension in CR (Hansen et al., 2008; Kailiang Jia & Levine, n.d.; Tóth et al., 2008) and IIS mutants (Melendez et al., 2003; Tóth et al., 2008). Additionally, induction of autophagy alone is sufficient for lifespan extension in some cases (Simonsen et al., 2008b), and suppression of autophagy decreases lifespan (Tóth et al., 2008).

Neuronal Proteostasis & Slowed Aging

Proteostatic maintenance in the brain is a crucial area of aging research. Although neurodegenerative disease states are influenced by different factors, it appears that a general decrease of protein turnover and repair plays an important role (Radak, Zhao, Goto, & Koltai, 2011). Loss of proteostasis is a characteristic of many age-related neurodegenerative disorders (Eftekharzadeh et al., 2016; Smith & Mallucci, 2016).

Mitochondrial dysfunction has been strongly implicated in ND, and some have proposed that neurodegeneration is caused by a loss of mitochondrial proteostasis (Chen & Chan, 2009). Parkinson's disease, which is characterized by tremors and impaired motor control, is thought to be related to PTEN-induced kinase 1 (Pink1) and Parkin (Pickrell & Youle, 2015). Both Pink1 and Parkin are required for autophagic clearance of dysfunctional mitochondria (Narendra et al., 2010; Vives-Bauza et al., 2010). As a result, Pink1 mutant *Drosophila* have abnormally large and dysfunctional neuronal mitochondria (Park et al., 2006). Similarly, a transgenic mouse model of PD showed morphologically abnormal mitochondria (Stichel et al., 2007). Mitochondrial protein synthesis may also play a role in ND, as decreased expression of transcriptional

regulators of mitochondrial biogenesis has been observed in AD patients (Sheng et al., 2012).

In addition to impaired mitochondrial proteostatic maintenance, reduced clearance of cytosolic proteins also contributes to neurodegeneration. In the case of AD, dementia arises and progressively worsens as synaptic regions in areas of the brain responsible for memory and cognition are disrupted by the deposition of self-assembling protein aggregates known as beta-amyloid plaques (AB) (Reddy & Beal, 2008; Selkoe, 1994). It has been proposed that even small reductions in the rate of AB clearance can considerably worsen pathologies (Saido, 1998).

The influence of proteostasis on neuronal function identifies mTOR as a potential mediator of central nervous system health. Complete loss of mTOR activity induces neuronal cell death (Choi, Kim, Ha, Kim, & Son, 2010). Alternatively, mouse models of AD show inappropriate activation of mTOR (Caccamo, Majumder, Richardson, Strong, & Oddo, 2010), and research has suggested that mTOR signaling contributes to tau hyperphosphorylation (Khurana et al., 2006; Meske, Albert, & Ohm, 2008). Further, mTOR inhibition decreases AB formation in mice, which is associated with increased autophagy (Spilman et al., 2010).

Due to the effect of rapamycin on mTOR and autophagy, rapamycin has been proposed as a potential Alzheimer's treatment (Bové et al., 2011). Indeed, rapamycin, through modulation of proteostatic maintenance and autophagy, has been demonstrated as protective in a variety of neurodegenerative disorders, including in models of AD (Dehay et al., 2010). Hyperactivation of mTOR in mouse models of AD is reversed by rapamycin, which reduces AB levels by induction of autophagy, prevents

tau phosphorylation, and restores cognitive function (Caccamo et al., 2010). Granted these results represent only one study using a transgenic mouse model, it appears as though rapamycin completely reversed the symptoms of AD. That rapamycin reduces AB levels and restores cognitive function has been repeated in separate models of AD (Spilman et al., 2010). Interestingly, rapamycin has been shown to effectively prevent cognitive decline in mouse models of AD when administered before, but not after, disease onset, suggesting an anti-aging effect of rapamycin in neuronal tissue (Majumder, Richardson, Strong, & Oddo, 2011). It should be noted that the use of rapamycin as either a treatment or prevention of ND is complicated by the fact that mTOR-mediated protein synthesis is required for long-term potentiation and synaptic plasticity (Tang et al., 2002). Additionally, one study showed that rapamycin may promote AB formation (Zhang et al., 2010).

In some models of ND it is the repression of translation by rapamycin, rather than induction of autophagy, that alleviates symptoms. In *Drosophila* models of PD, rapamycin prevents the progressive degeneration of dopaminergic neurons specifically through translation inhibition (Tain et al., 2009). The finding that reduced protein synthesis, mediated by rapamycin, is beneficial in neurons has been repeated in other models of PD (King et al., 2008), as well as in models of Huntington's disease (Wyttenbach, Hands, King, Lipkow, & Tolkovsky, 2008). Inhibition of mTOR shifts protein synthesis to predominantly 5' cap-independent translation, which promotes translation of proteins involved in responses to cellular stress (Holcik & Sonenberg, 2005), such as protein chaperones (Meyer et al., 2015), and may additionally contribute to rapamycin-induced neuroprotection.

Although recent progress has been made in understanding the effects of rapamycin on proteostasis in neuronal tissue, less is known about the effect of metformin. As discussed, one of the primary actions of metformin is to activate AMPK, which then inhibits mTOR. Therefore, in neurons the effect of metformin may be similar to rapamycin. It has been shown that increased AMPK signaling reduces AB production in mouse models of AD through inhibition of mTOR and induction of autophagy (Vingtdeux et al., 2010). In db/db obese mice, metformin improves memory and decreases AB transport across the blood brain barrier by decreasing the expression of receptor for advanced glycation end products (Chen et al., 2016). Metformin may also provide neuroprotection independently of AMPK. Metformin promotes dephosphorylation of tau by activation of protein phosphatase 2A, which does not require AMPK activation (Kickstein et al., 2010). In peripheral tissue metformin inhibits mTOR and thus protein synthesis through both AMPK dependent (Howell et al., 2017) and independent (Kalender et al., 2010) mechanisms. Whether metformin inhibits protein translation and thus promotes neuronal health in a similar manner remains to be seen. Additionally, in cultured cells and mice, metformin has been shown to increase the expression of beta-secratase 1, the enzyme thought to be primarily responsible for cleavage of amyloid precursor protein and the subsequent formation of AB (Chen et al., 2009).

Currently, it appears that both rapamycin and metformin, in some cases, provide neuroprotection through alterations in proteostatic maintenance. However, much is still unknown about these alterations in proteostatic mechanisms and how rapamycin and metformin may be refined or best utilized in the context of ND. Thus, accurate

measurements of the different components of proteostasis in the brain following treatment with rapamycin and metformin are needed.

Protein Synthesis in the Context of Cell Proliferation

The importance of proteostasis in health and disease has made measurements of protein synthesis a fundamental part of physiological research. The uses of protein synthesis measurements are decidedly varied. For example, protein synthetic rates can reflect proteostatic maintenance and provide insight into how cells maintain function during stress, as well as the wide array of adaptations and recovery processes that follow exposure to stress (Arnsburg & Kirstein-Miles, 2014; Miller, Drake, Naylor, Price, & Hamilton, 2014). Alternatively, protein synthesis occurs for the purpose of cell division (Grebien, Dolznig, Beug, & Mullner, 2005). These two distinct purposes of protein synthesis complicate interpretations of protein synthesis measurements. However, by simultaneously measuring the rate of both protein synthesis and cell proliferation, the relative proportion of protein synthesis for maintenance, repair, and adaptation, can be distinguished from protein synthesis for cell proliferation.

Rates of cellular proliferation can be estimated by measuring DNA synthesis rates (Neese et al., 2001). Prior to dividing, a cell must double its genome so that each daughter cell has a complete set of genetic material (Stoeber et al., 2001). Similarly, the cell must double its proteome to ensure each cell possesses a full complement of functional proteins (Grebien et al., 2005). In this scenario, there is a proportional synthesis of protein and DNA. If this is considered as a ratio of the rate of protein synthesis to the rate of DNA synthesis (PRO:DNA), this ratio is unchanged by the fact that a cell is dividing.

Alternatively, if an existing cell synthesizes proteins for adaptations or repair, there would be synthesis of proteins without a concurrent synthesis of DNA. In this case, if protein synthesis is considered in its relationship to DNA synthesis, the ratio of the rate of protein to DNA synthesis would increase. Thus, measuring the rate of DNA synthesis simultaneously with protein synthesis allows for more accurate and insightful interpretations to be made about protein synthesis rates, either for proteostatic maintenance or cell division.

Ratio of PRO:DNA Synthesis In Models of Slowed Aging

Consistently, an increased ratio of PRO:DNA synthesis has been observed in various body tissues from models of slowed aging (Miller, Drake, Naylor, Price, & Hamilton, 2014). The importance of considering cell proliferation in the context of protein synthesis is clearly illustrated in studies using CR mice. Measuring long-term rates of protein synthesis using stable isotopic tracers, it was found that in multiple subcellular fractions, both long and short-term protein synthesis rates in skeletal muscle, heart, and liver of CR mice were not different from mice allowed to feed *ad libitum* (with the exception that short-term, mitochondrial protein synthesis was decreased by CR in liver) (Miller et al., 2013). This finding is counterintuitive, given the expected effect of caloric restriction on mTOR, and subsequently, protein synthesis. However, when the rates of protein synthesis were expressed as a ratio compared to DNA synthesis, clear differences emerged. For example, in calorically restricted mice, the ratio of PRO:DNA synthesis was increased compared to control in skeletal muscle in all subcellular fractions measured (Miller, Drake, Naylor, Price, & Hamilton, 2014). This difference can be interpreted as an increase in the rate of protein synthesis for the

purpose of maintenance, repair, and adaptation of existing cells, as opposed to growth and proliferation. Given the importance of proteostatic maintenance in aging it might be reasonable to expect that this characteristic is shared among other models of slowed aging, and this indeed the case.

Long-lived crowded-litter mice (which are calorically restricted during postnatal development) show increased levels of PRO:DNA in all of skeletal muscle, heart, and liver (Drake et al., 2014). Rapamycin treated mice also show increased PRO:DNA synthesis rates (Miller et al., 2014). Finally, Snell Dwarf mice, which have decreased growth hormone, thyroid stimulating hormone, and prolactin due to underdeveloped anterior pituitary grand, show both increased lifespan and increased proteostatic maintenance, evidenced by an increased PRO:DNA in skeletal muscle and heart (Drake et al., 2015).

Measurements of protein and DNA synthesis have provided valuable insight into proteostatic maintenance as it relates to slowed-aging. Differences observed across tissues and between models highlights the importance of considering treatment effects in different portions of the body separately. Heart, skeletal muscle, and livermay respond differently to a given treatment. Whether these effects are consistent in nervous system tissue in models of slowed aging has yet to be evaluated.

Knowledge Gaps

Measurements of protein synthesis and proteostatic maintenance have revealed important mechanisms underlying improved health and extended lifespan various models of slowed aging. Frequently, treatment effects are tissue specific. Whether or

not slowed aging treatments alter protein synthesis rates or proteostatic maintenance in the brain of models of slowed aging is an unexplored area of research.

Study purpose

The purpose of this study was to determine the impact of rapamycin and rapamycin plus metformin in combination on various measurements of proteostatic maintenance in the brains of old and young mice.

CHAPTER III: METHODS

Animal Care

Genetically heterogeneous (UM-HET3) mice were crossbred from two different F1 strains: (BALB/cByJ x C57BL/6J) F1 females (JAX stock 10009) and (C3H/HeJ x DBA/2J) F1 males (JAX stock 10004). Animals were housed at the University of Michigan on a 12-hour light/dark cycle. All procedures met or exceeded the standards for facilities housing animals described in the Animals Welfare Act regulations, the Guide for the Care and Use of Laboratory Animals, and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Animal Diet Composition and Study Design

Male and female mice were divided into two groups that received treatment diets at either 4 (YOUNG) or 17 (OLD) months of age. Mice from OLD and YOUNG cohorts were randomized into one of three treatment groups that received either a standard control diet (Purina 5LG6) (CON), a standard diet supplemented with rapamycin (Purina 5LG6 with 14 parts per million rapamycin) (RAP), or a standard diet supplemented with rapamycin and metformin (Purina 5LG6 with 100 parts per million metformin and 14 parts per million rapamycin) (RAP+MET). Each sex was equally represented among groups. Mice received treatment diet for 8 weeks prior to sacrifice, making YOUNG and OLD 6 and 19 months of age at sacrifice, respectively. Additionally, each treatment group was separated into five groups for different D_2O labeling periods, which allowed for estimation of long-term protein synthesis rates in a time-course style experiment (n=3 per group/sex/timepoint). An overview of the study design is illustrated in Figure 1.

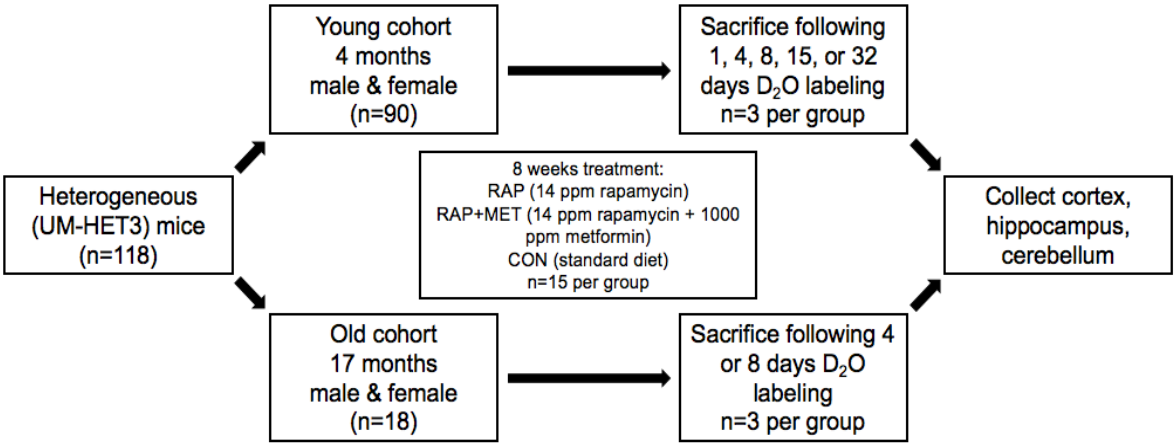

Figure 1. Schematic Representation of treatment groups and diet.

Stable Isotope Labeling Technique

For isotopic tracer enrichment measurements, mice received intraperitoneal injections of deuterium oxide (D_2O) (Sigma Aldrich, St. Louis, MO, USA) relative to 60% of body weight as previously described (Miller et al., 2013). Subsequently, drinking water was supplemented with 8% D_2O. In the young cohort, each of the five groups within dietary treatment by sex received D_2O for either 1, 4, 8 15, or 32 days and was sacrificed upon completion of D_2O treatment (Figure 2). In the old cohort mice received D_2O for 4 (male) and 8 (female) days before sacrifice (Figure 1). Mice were fasted for 12 hours prior to sacrifice and anesthetized by carbon dioxide. Upon sacrifice, cortex, hippocampus, and cerebellum were collected and immediately frozen on liquid nitrogen. Blood was obtained by cardiac venipuncture and bone marrow was extracted from tibia

using phosphate-buffered saline, and each tissue was frozen on liquid nitrogen and stored at -80 C° for later analysis.

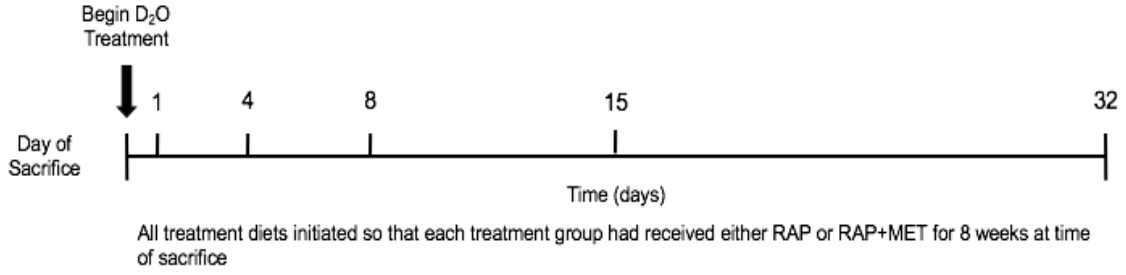

Figure 2. Overview of deuterium oxide (D_2O) labeling scheme for the young mice cohort.

Alanine Enrichment Analysis

Differential Centrifugation

Whole brain tissue compartments were combined and pulverized under liquid nitrogen. 40-50 milligrams of tissue was aliquoted for analysis of alanine isotopic enrichment. Tissue was processed by differential centrifugation as previously described (Miller et al., 2013). In brief, tissue was homogenized by Bullet Blender® (Next Advance, Troy, NY, USA) in 1:10 mitochondrial isolation buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.5) supplemented with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL, USA). Following homogenization, tissue was centrifuged for 10 minutes at 800g to obtain the mixed subcellular protein fraction (MIX) pellet. Supernatant from the MIX pellet was centrifuged at 9000g for 10 minutes to separate a mitochondria-enriched pellet (MITO). Supernatant from the MITO pellet was incubated on ice with equal volume 14% sulfosalicylic acid for one hour to precipitate cytosolic proteins before a 10 minute 16,000g spin to obtain a cytosolic protein pellet (CYTO).

After the MIX and CYTO fractions were pelleted, each was washed with 500 microliters (μl) of 100% ethanol (EtOH), and was centrifuged for 1 minute at 1000g before the removal of EtOH. This wash was followed by a similar wash with 500 μl water, and both washes steps repeated. Both pellets were then solubilized in 250 μl 1M NaOH and heated for 15 minutes at 50° C while shaking at 900 rotations per minute. Afterwards, the NaOH solutions containing the solubilized CYTO and MIX pellets were added to 3 and 6 milliliters (ml) 6M HCl, respectively. The MITO pellet was washed with 200 μl mitochondrial isolation buffer #2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris Base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4) and centrifuged for 10 minutes at 8000g. The first addition of buffer #2 was removed, and a second addition of 100 μl was added followed by a 10 minute 6000g spin. The MITO pellet was solubilized in 250 μl of 1M NaOH and heated to 50° C while shaking at 900 RPM for 15 minutes. The solubilized MITO pellet was then added to 1.5 mL 6M HCl. Each centrifugation was performed at 4° C and all samples were kept on ice throughout the procedure. All HCl solutions were incubated for 24 hours at 120° C for protein hydrolysis.

Alanine Derivation and Analysis

Protein hydrolysates were cation exchanged and dried as previously described (Miller et al., 2012). Dried samples were reconstituted in 1 mL of Milli-Q (Millipore Sigma, Darmstadt, Germany) ultrapure filtered water. One-half of reconstituted sample (500 μl) was combined with 500 μl acetonitrile, 50 μl 1M K_2HPO_4, and 20 μl pentafluorobenzyl bromide. Samples plus reagents were vortexed and incubated for 1 hour at 100° C. Following incubation, 600 μl ethyl acetate was added and samples were

vortexed vigorously. The organic layer was extracted, dried under nitrogen gas, and reconstituted in 700 μl of ethyl acetate for analysis by gas chromatography/mass spectrometry (GC-MS) (Agilent Technologies, GC 5975C/MS7890A). Using a DB225 gas chromatograph column, temperature was increased from 100 C° to 225 C° at a rate of 10 C° per minute. The mass-to-charge ratios (m/z) of 448, 449, and 450 were measured for the pentafluorobenzyl-*N,*N-di(pentafluorobenzyl)alanine derivative and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Deuterium enrichment was calculated as the M+1 mass isotopomer (449) divided by the sum of the M+0 (448) and M+1 (449) mass isotopomers (Hellerstein & Neese, 1999). Deuterium enrichment of alanine was used to estimate the proportion of newly synthesized protein by dividing the deuterium enrichment of alanine by the precursor enrichment from body water. Body water enrichment was estimated using the deuterium content of extracted plasma. The fraction of newly synthesized tissue alanine was then adjusted for precursor enrichment using mass isotopomer distribution analysis (MIDA) (Hellerstein & Neese, 1999).

Body Water Enrichment Analysis

Body Water Derivation and Analysis

Body water enrichment of D_2O was measured from plasma as previously described (Drake et al., 2015). 125 μl of plasma was heated overnight at 80 C° for the extraction of water and D_2O. After cooling to room temperature, 2 μl of 10 mM NaOH and 20 μl of HPLC grade acetone was added to each sample. Samples were vortexed and incubated overnight at room temperature. Next, 200 μl of hexanes was added, and each sample vortexed. The organic layer was transferred to gas chromatography vials

using 200 μl pipette tips filled with sodium sulfate. The m/z ratios of 58 and 60 were measured and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Analysis was performed on GC-MS (Agilent Technologies GC 5975C/MS7890A) in tandem on EI mode with a DB-17MS column.

DNA Enrichment Analysis

DNA Isolation and Derivation

DNA was isolated with QIAamp DNA mini kit using 30 milligrams whole brain tissue and bone marrow according to manufacturer instructions (Qiagen, Valencia, CA, USA). Following extraction, samples were incubated overnight at 37° C with nuclease S1 and potato acid phosphatase. The next day, 80 μl glacial acetic acid and 100 μl pentafluorobenzyl hydroxylamine solution was added and samples were incubated for 30 minutes at 100° C. Samples were cooled to room temperature and 1 ml acetic and hydride and 100 μl n-methylimidazole were added. Samples were allowed to cool to room temperature and 2 ml molecular biology grade water and 800 μl methylene chloride were added. Each sample was vortexed and the organic layer extracted. A second addition of 800 μl methylene chloride was extracted, combined with the first, and transferred to glass tubes containing granular, anhydrous sodium sulfate. Samples were dried under vacuum and reconstituted in 70 μl ethyl acetate and analyzed by GC-MS (Agilent Technologies, GC 7890B/MS 7697) with a DB-17MS column using negative chemical ionization. Helium was used as a carrier gas and methane as the reagent. The pentafluorobenzyl triacetyl derivative of purine deoxyribose was measured for the fractional molar isotope abundances at m/z 435 and 436 and quantified using ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

Protein Quantification and Aggregation Assays

Tissue Homogenization and Lysis

40 milligrams of pulverized whole brain was homogenized in 300 μl lysis buffer (100 mM NaF, 50 mM HEPES, 12 mM Sodium Pyrophosphate, 10 mM EDTA, 1% 100X-Triton by volume) with phosphatase and protease inhibitors (HALT, Thermo Scientific, Rockford, IL). Samples were centrifuged at 16,000g for 20 minutes. Supernatant was removed and protein content was quantified by bicinchoninic acid assay (BCA) (Thermo Fisher, Rockford, IL).

Protein Quantification by Western Blot

Each sample of homogenized tissue was then diluted so that total protein concentration was equal to 2 μg/μl. Samples were then incubated with Lamelli buffer. 30 μg protein was loaded into a 26-well, 10-20% Tris-HCl gel (Criterion, Bio-Rad, Hercules, CA) and separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100V. Samples were transferred to a polyvinylidene difluoride (PVDF) membrane at 4° C for 45 minutes at 100V in transfer buffer (20%w/v methanol, 0.02% SDS, 192 mM glycine, 25mM Tris, pH8.3). Membranes were blocked in 5% milk in Tris Buffered Saline with Tween (TBST) (get from SOP) for 2 hours at room temperature. before primary antibodies were added. Antibodies were purchased from Cell Signaling Technologies (Boston, MA; RpS6 #2217S, RpS6 phospho-Ser[240/244] #4858S, AMPKα #2532S, phosphor-AMPKα[Thr172] #2531S). Blots were incubated overnight in primary antibodies at a concentration of 1:1000 in 5% BSA and TBST at 4° C. Membranes were rinsed in TBST and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody diluted to a concentration of

1:5000 in 5% milk in TBST. After a 1 hour room temperature incubation membranes were rinsed in TBST followed by chemiluminescence detection (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific, Rockford, IL). Images were captured and densitometry was analyzed using a UVP Bioimaging system (Upland, CA). Blots were first probed for phosphorylated proteins. Membranes were then stripped (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL) and re-probed for total protein.

Protein Aggregation Assay

Protein aggregation was measured in homogenized, lysed tissue samples using a PROTEOSTAT® protein aggregation assay (ENZ-51023, Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer instructions. A standard curve was generated using PROTEOSTAT® protein aggregation standards (ENZ-51039, Enzo Life Sciences, Inc., Farmingdale, NY, USA), also prepared according to manufacturer instructions. Samples and standards were prepared in duplicate on a 96-well plate. Mean optical density values were converted to percentage of aggregated protein based on values obtained from the standard curve.

Statistics

Statistical Analysis was performed using PRISM GraphPad 7 Software (Graphpad Software, La Jolla, CA). In the young cohort, nonlinear regressions of fraction new were calculated using mean values for each timepoints in all subcellular protein fractions for the three treatment groups in both sexes. Slope of the curve for each nonlinear regression (k) was calculated. Within sex and age comparisons of k values for each fraction, protein abundance measured by Western blot, and protein

aggregation content between groups were made using Tukey's multiple comparison test. Between sex and between age comparisons of k for each fraction and group, as well as individual protein content, and concentration of aggregated protein were made using Sidak's multiple comparisons test. Fraction new DNA as well as PRO:DNA synthesis ratios were compared in the 32 day timepoint of the young cohort using Tukey's multiple comparisons test. In the older cohort, fraction new was analyzed for individual timepoints in male (8 days D_2O labeling) and female (4 days labeling) using Tukey's multiple comparisons test. Critical value for significance was set *a priori* at $p < 0.05$.

CHAPTER IV: RESULTS

Rapamycin and Metformin Affect Long-Term, Subcellular Brain Protein Synthesis Rates in Young Female Mice

In young male mice, mitochondrial, cytosolic, and mixed protein synthesis rates were not different in any group (Figure 3). In young female mice mitochondrial protein synthesis value was lower in RAP+MET compared to CON and RAP ($p<0.05$) (Figures 4A and 4B). Additionally, cytosolic protein synthesis was lower in young female RAP+MET than in CON ($p<0.05$) (Figures 4C and 4D). In the mixed protein fraction, no differences in synthesis rates were observed in among treatment groups in female mice (Figures 4E and 4F).

Protein Synthesis Rates Differ Between Male and Female Mice in Each Treatment Group

When subcellular protein synthesis rates were compared between male and female mice for each group, it was found that there was a main effect of sex ($p<0.05$) in the CON group, with females showing greater protein synthesis rates ($p<0.05$) (Figure 5A). Additionally, Female CON mice had higher rates of mixed fraction protein synthesis compared to males ($p<0.05$) (Figure 5A). In RAP, no differences were observed between sexes (Figure 5B). In RAP+MET, there was a main effect of sex ($p<0.05$) and a sex by fraction interaction ($p<0.05$), with males demonstrating higher rates of protein synthesis, but no differences in synthesis rates for individual protein fractions (Figure 5C).

Rapamycin and Metformin Decrease Subcellular Protein Synthesis Rates in Older Mice

Due to limited tissue availability, only individual time points were analyzed for older mice (Figure 6A-6F). Analysis was made on male mice that underwent 8 days of D_2O labeling, and females following 4 days of D_2O treatment. In males, RAP showed lower mitochondrial protein fraction new compared to CON ($p<0.05$) (Figure 6A). Also in male RAP, cytosolic protein synthesis was decreased compared to CON ($P<0.05$) (Figure 6B), while decreased mixed fraction protein synthesis in male RAP mice approached statistical significance decreased synthesis rates compared to RAP+MET ($p=0.084$) (Figure 6C). In females, mixed protein synthesis was lower in RAP and RAP+MET compared to CON ($p<0.05$) (Figures 6D). In old female RAP mice cytosolic protein synthesis also approached statistical significance ($p=0.067$) (Figure 6F), and no differences were found between treatment groups for mitochondrial protein synthesis (Figure 6B).

DNA Synthesis is Affected Differently By Rapamycin and Metformin in Male and Female Mice

Brain has low rates of cell proliferation, and due to low DNA enrichment values only data for the final timepoint (day 32) for young mice were included in the analysis. In young male mice RAP, but not RAP+MET, decreased the rates of DNA synthesis compared to CON ($p<0.05$) (Figure 7A). In young female mice, both RAP and RAP+MET showed increased rates of DNA synthesis compared to CON ($P<0.05$) (Figure 7B).

Rapamycin Increases PRO:DNA in Young Male Mice

To evaluate the influence of RAP and RAP+MET on proteostatic maintenance, protein and DNA synthesis for the 32 day timepoint for young male and female mice was expressed as a ratio (PRO:DNA) in (Figure 8). In male mice, PRO:DNA synthesis was increased in all protein fractions in RAP compared to CON ($p<0.05$) (Figures 8A, 8C, and 8E). Conversely, RAP and RAP+MET showed decreased PRO:DNA synthesis rates in young female mice compared to CON (Figures 8B, 8D, and 8F).

Rapamycin Decreased Aggregated Protein Content in Young Male Mice

In young male mice RAP decreased the percentage of protein aggregates relative to total protein ($P<0.05$) (Figure 9A). However, no other differences were observed in any treatment group in either age group or sex (Figures 9B-9D). In addition, no differences in protein aggregation were observed when male and female mice were compared within young and old cohorts, or in comparisons between age in male and female mice (Figures 10A-10D).

Rapamycin and Rapamycin Plus Metformin Decrease Phosphorylated RPS6 in Older Mice

In the young cohort, the proportion of p-RpS6 compared to total RpS6 was not different in either male or female mice (Figures 11A and 11B). In older male mice, RAP decreased p-RpS6 ($P<0.05$) (Figure 11C). In female mice, both RAP and RAP+MET decreased p-RpS6 relative to total RpS6 compared to CON ($P<0.05$) (Figure 11D). No statistically significant differences in overall p-RpS6 content were observed between treatment groups in any age or sex (Figures 12A-12D). Total RpS6 was significantly

higher in RAP and RAP+MET in old female mice ($P<0.05$) (Figure 13D), and no other treatment differences were observed (Figures 13A-13C).

Neither Treatment Group Demonstrated Increased Levels of Activated AMPK in Any Age or Sex

When comparisons were made between groups and among age and sex, no differences in p-AMPK relative to total AMPK were detected (Figures 14A-14D). Both RAP and RAP+MET showed increased absolute levels of p-AMPK in young female mice ($P<0.05$) (Figure 15B), but not in other old mice or either age in males (Figures 15A-15D). No differences were observed in total AMPK content in any age or sex (Figures 16A-16D).

Figures

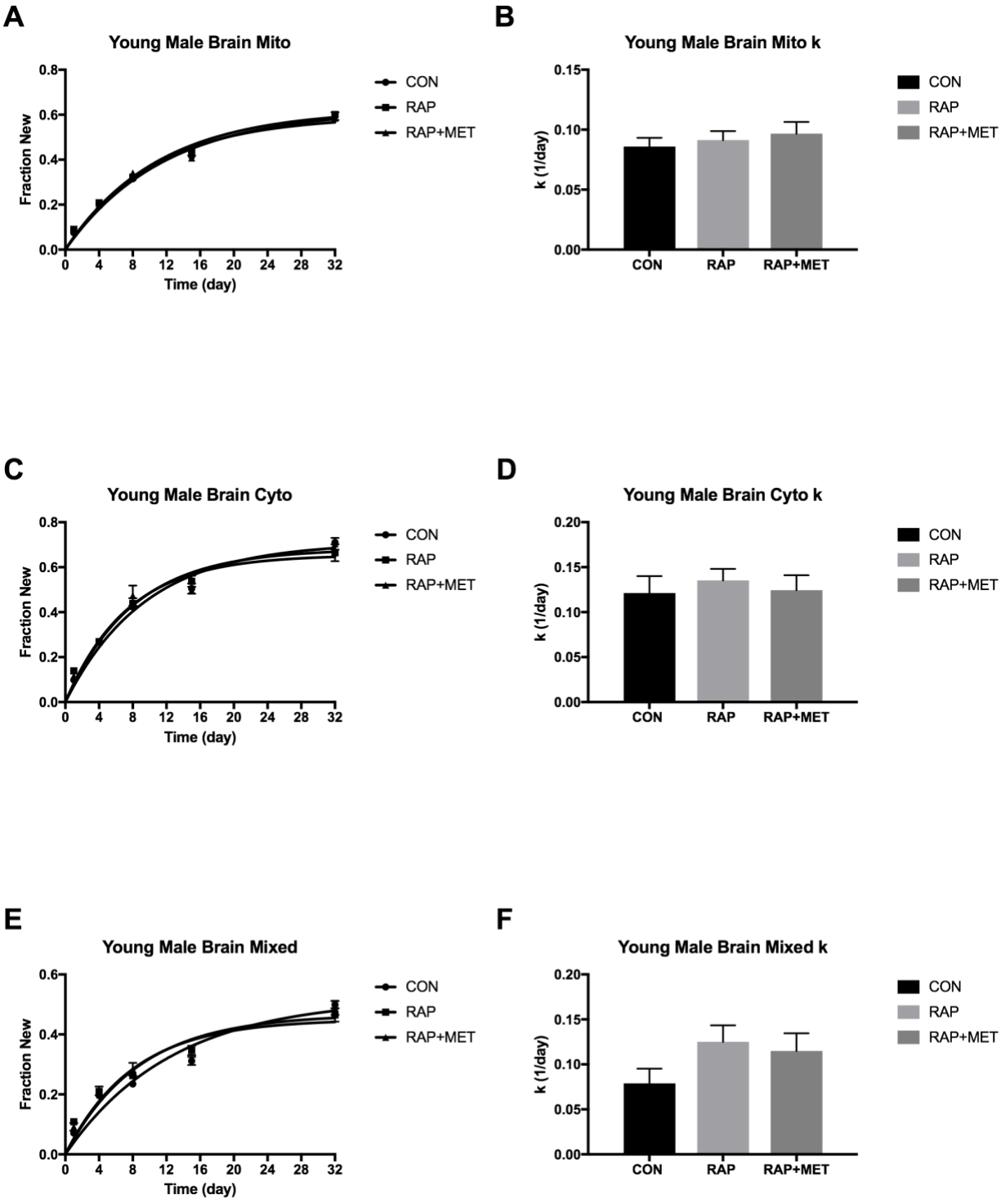

Figure 3. Nonlinear regressions and slope of the regression (k) of fraction new protein for mitochondrial (A and B), cytosolic (C and D), and mixed (E and F) fractions in young male mice. Data presented as means +/- SEM.

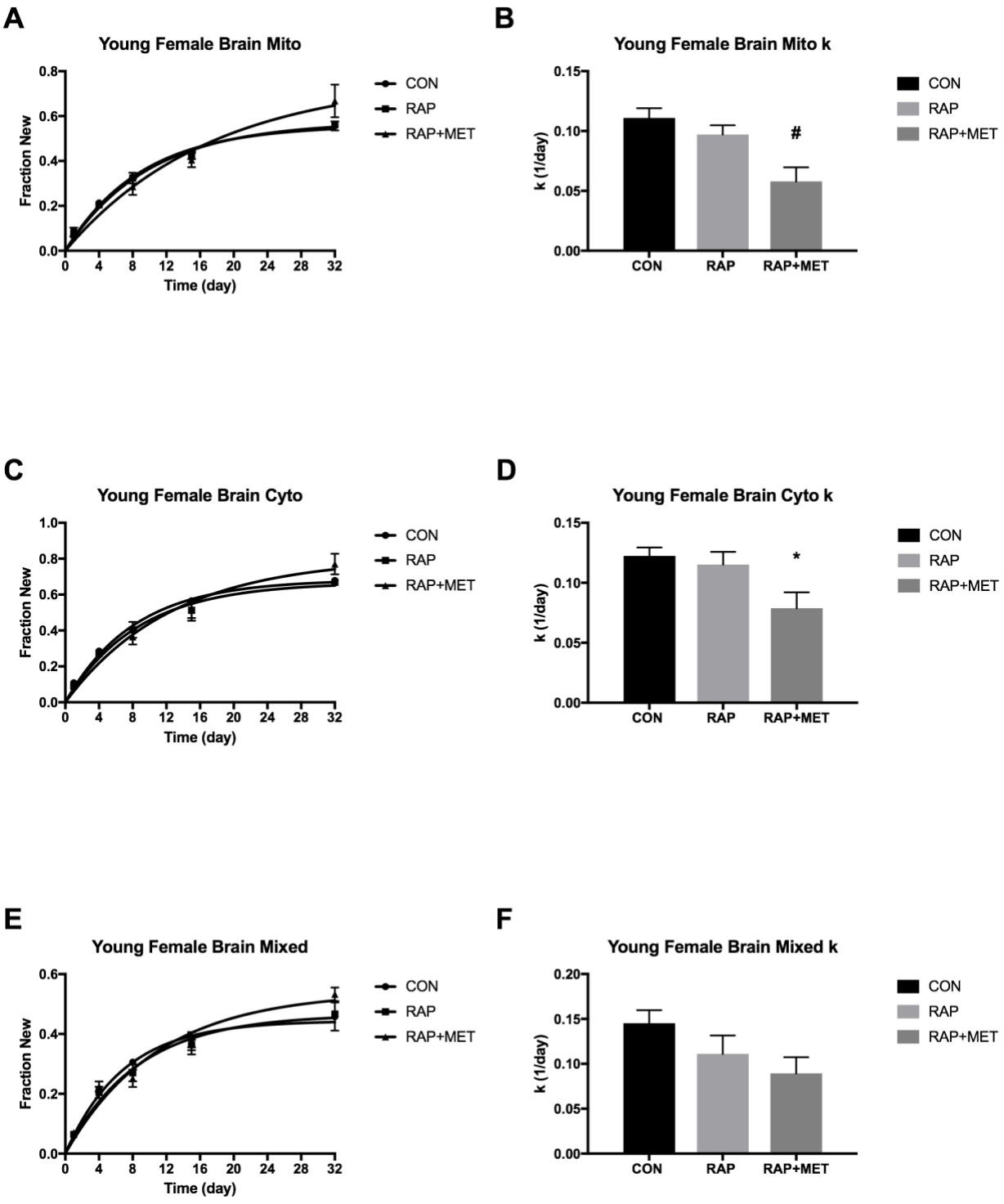

Figure 4. Nonlinear regressions and slope of the regression (k) for fraction new protein of mitochondrial (A and B), cytosolic (C and D), and mixed (E and F) fractions in young female mice. #Statistically different from CON and RAP ($p<0.05$). *Statically different from CON ($p<0.05$). Data presented as means +/- SEM

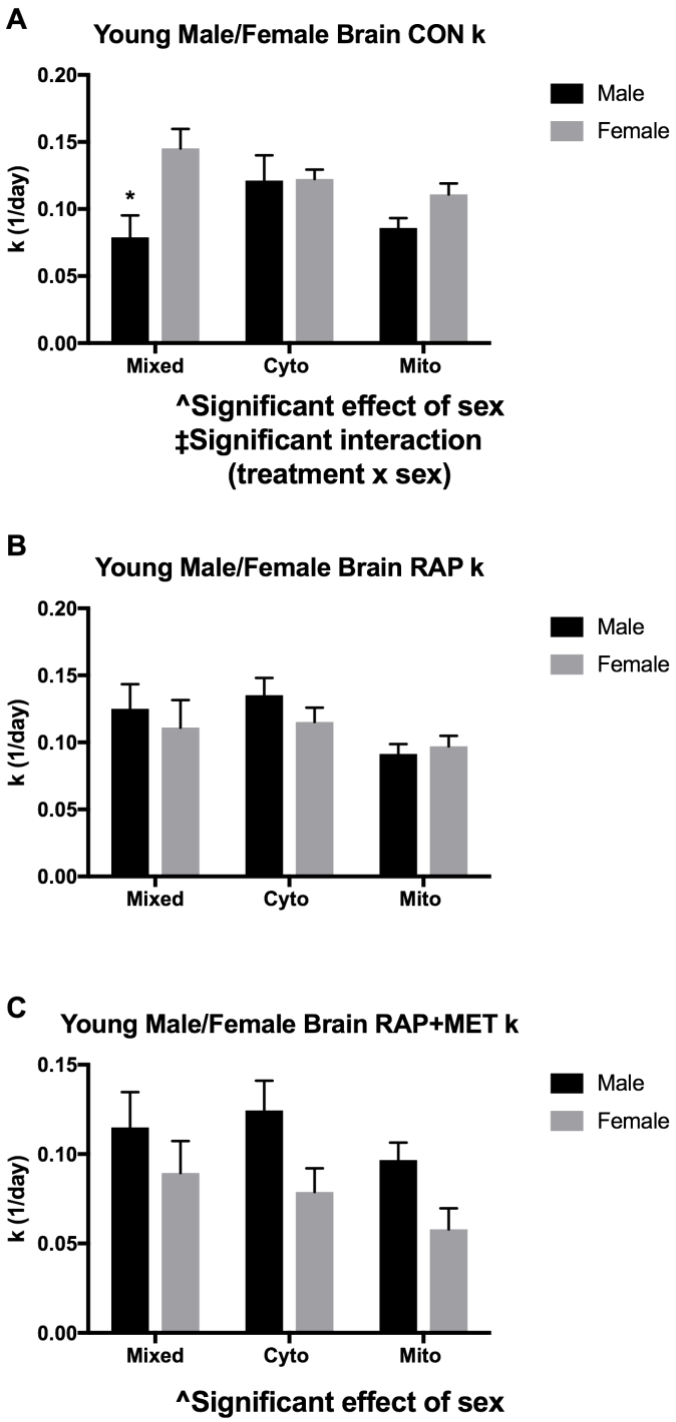

Figure 5. Comparisons between male and female k for each group in young mice. *Significantly different from female (p<0.05). ^Main effect of sex (P<0.05). ‡Significant sex by fraction interaction (p<0.05). Values presented as mean +/- SEM.

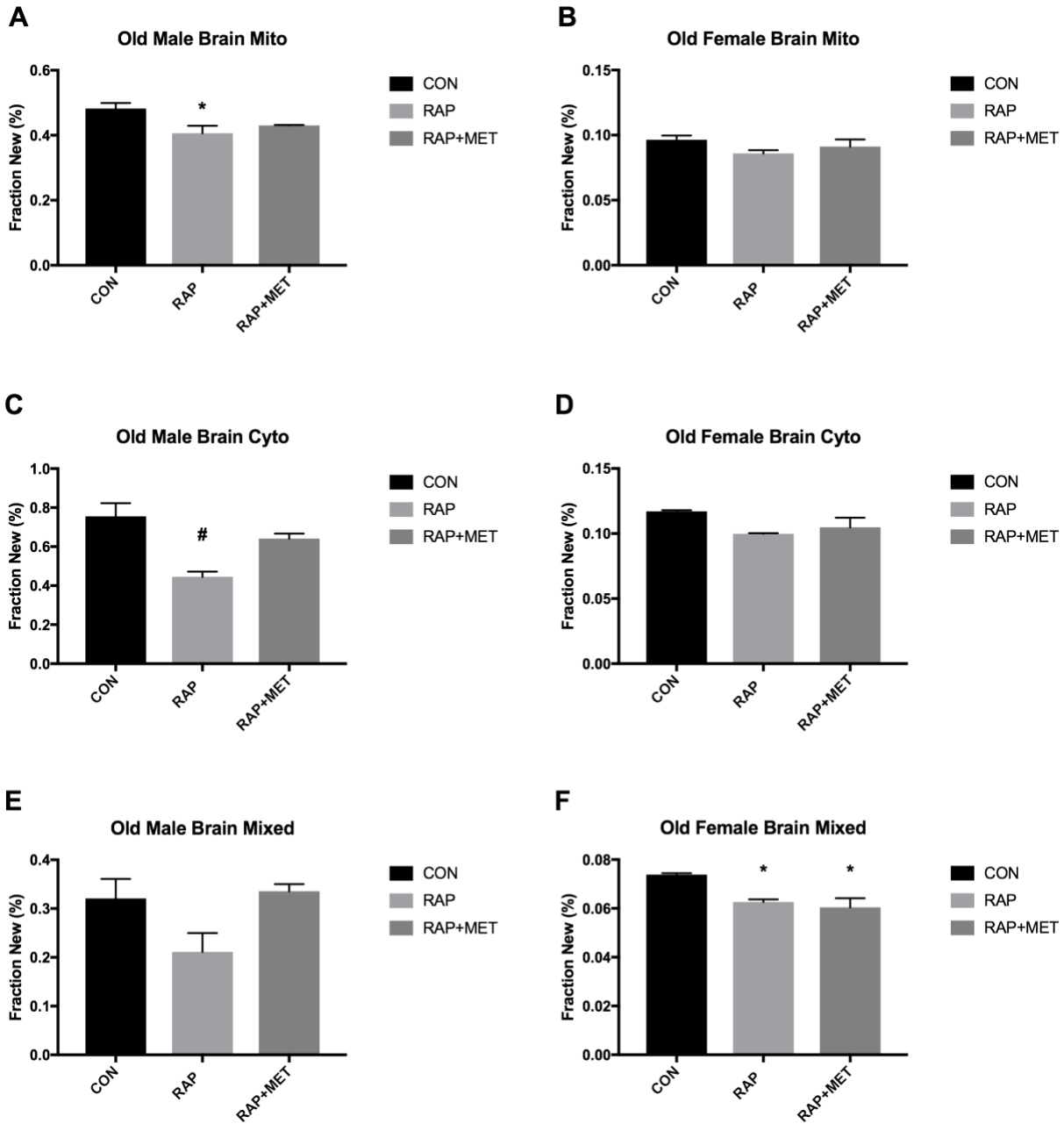

Figure 6. Fraction new at individual time points for mitochondrial, cytosolic, and mixed protein fractions in older mice. *Significantly different from CON ($p<0.05$). #Significantly different from CON and RAP+MET ($p<0.05$) Values expressed as mean +/- SEM.

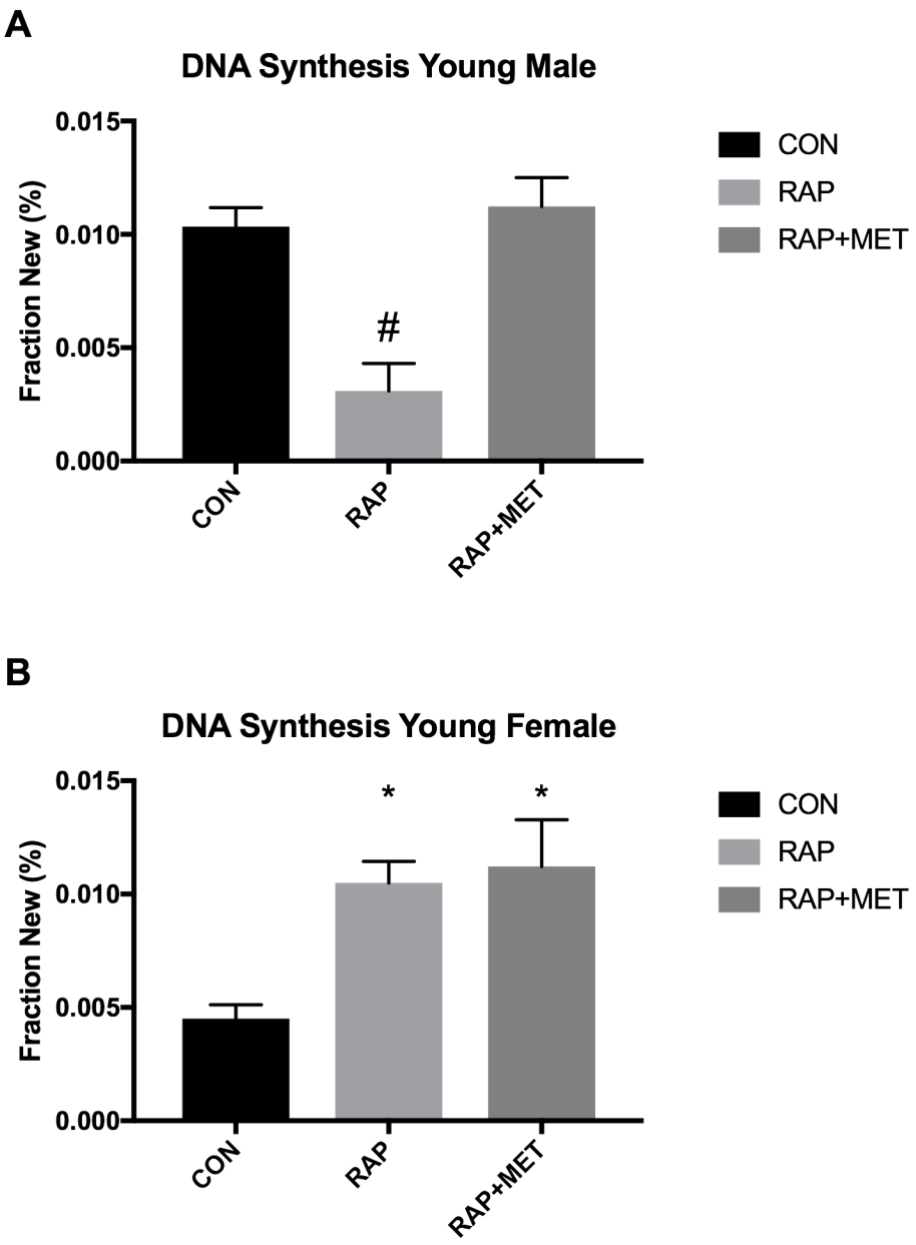

Figure 7. DNA fraction new for young mice at the 32 day timepoint. #Significantly different from CON and RAP+MET ($p<0.05$). *Significantly different from CON ($p<0.05$). Values expressed as mean +/- SEM.

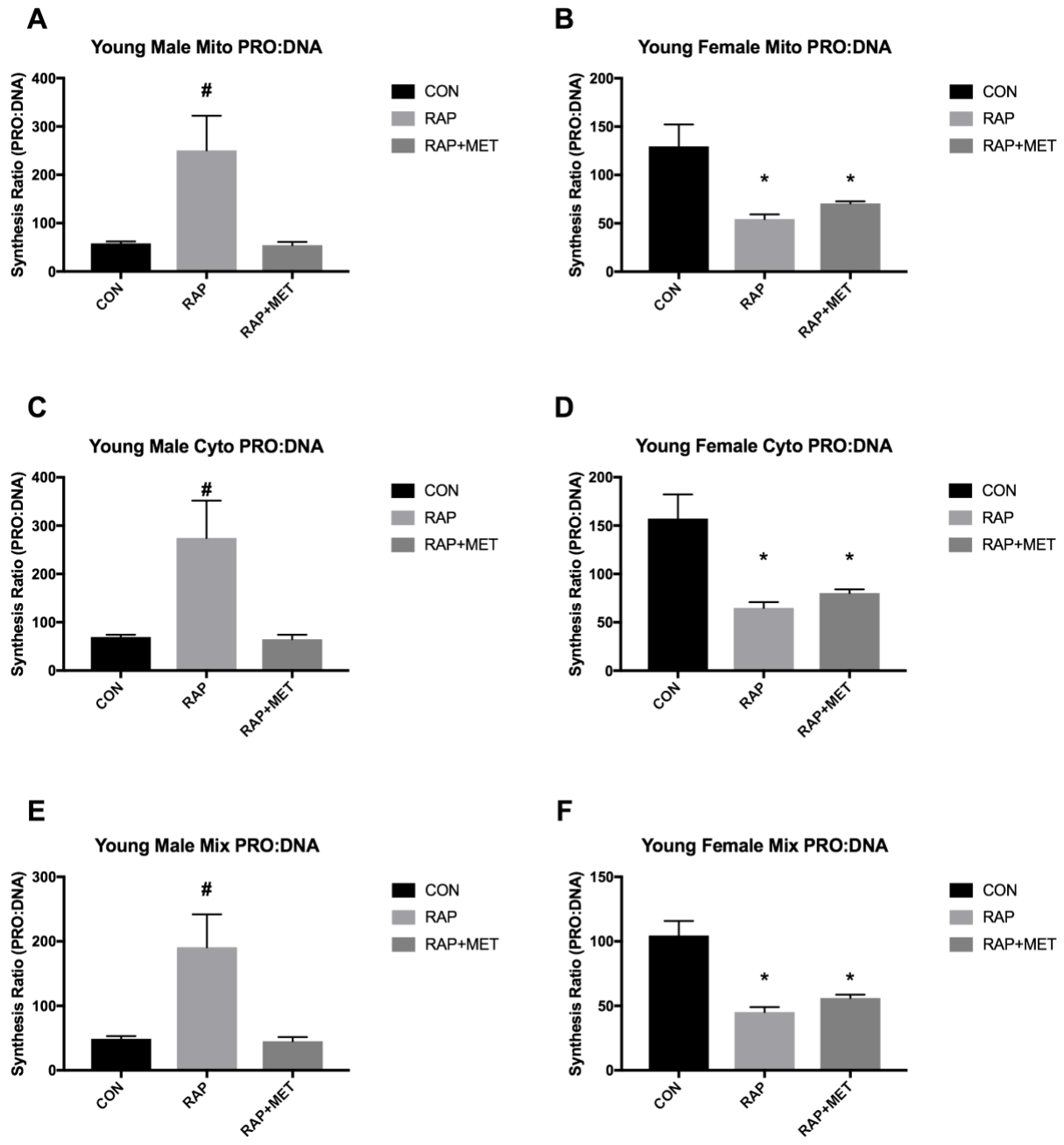

Figure 8. Ratio of PRO:DNA synthesis at the 32 day timepoint for young mice. #Significantly different from CON and RAP+MET ($p<0.05$). *Significantly different from CON ($p<0.05$). Values expressed as mean +/- SEM.

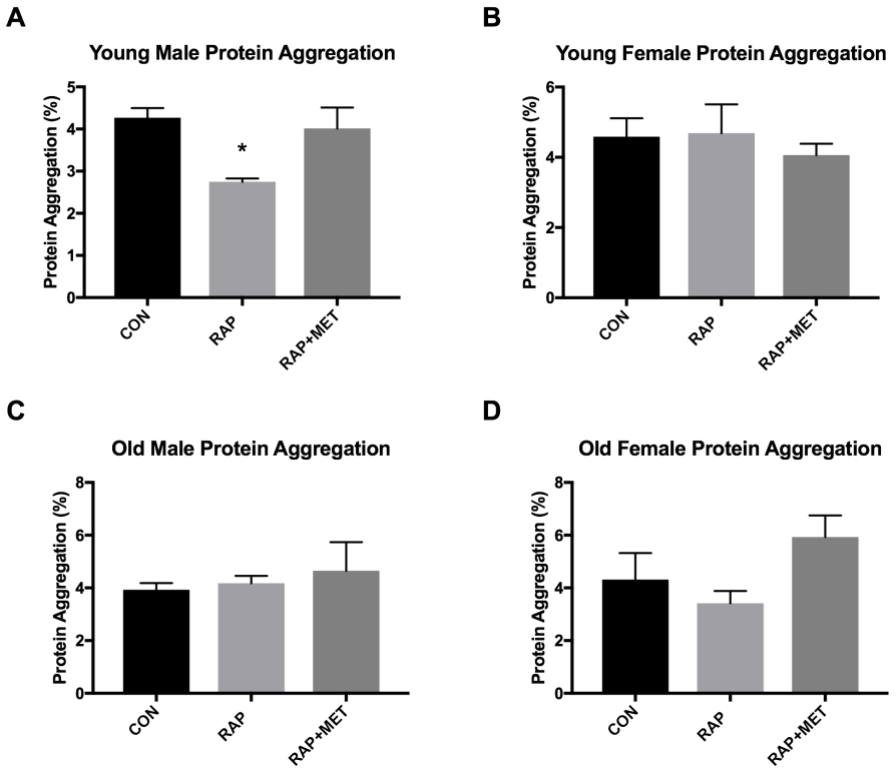

Figure 9. Protein aggregation as a percentage of total protein. *Significantly different from CON ($p<0.05$). Values expressed as mean +/- SEM.

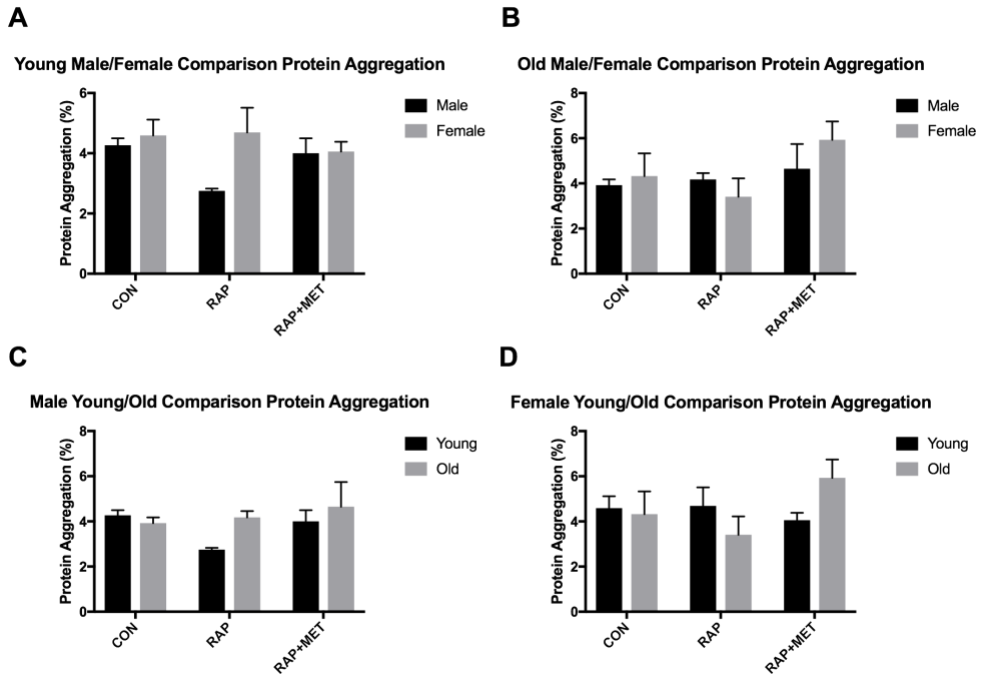

Figure 10. Comparisons of protein aggregation content between male and female and young and old mice. Values expressed as mean +/- SEM.

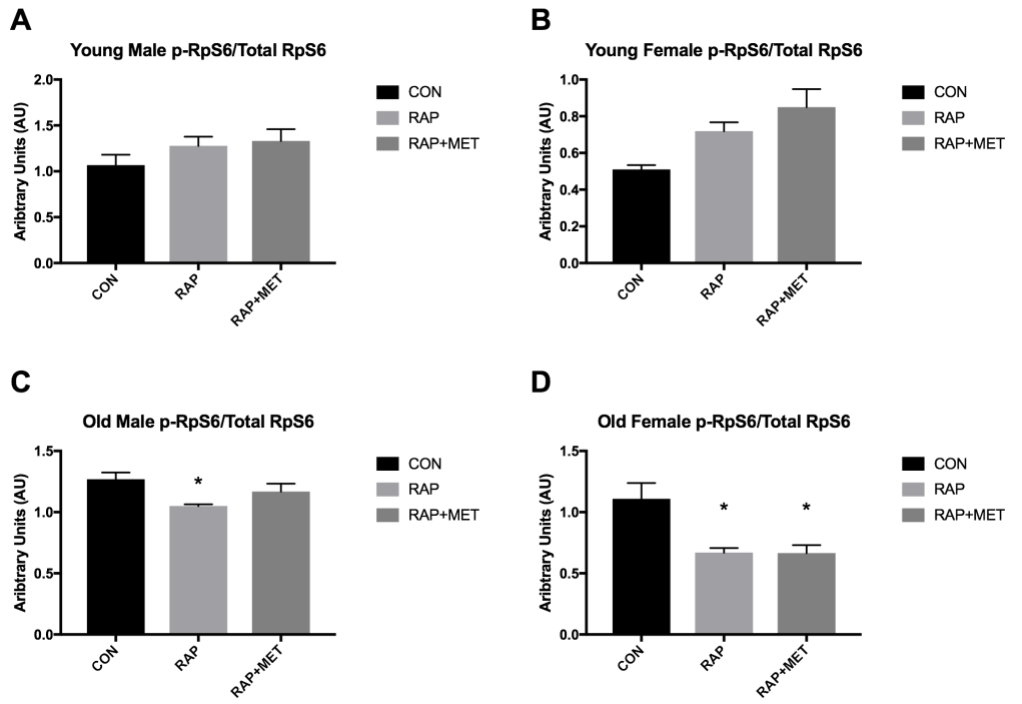

Figure 11. Phosphorylated RpS6 relative to total RpS6 in young and old mice. *Significantly different from CON ($p<0.05$). Values expressed as mean +/- SEM.

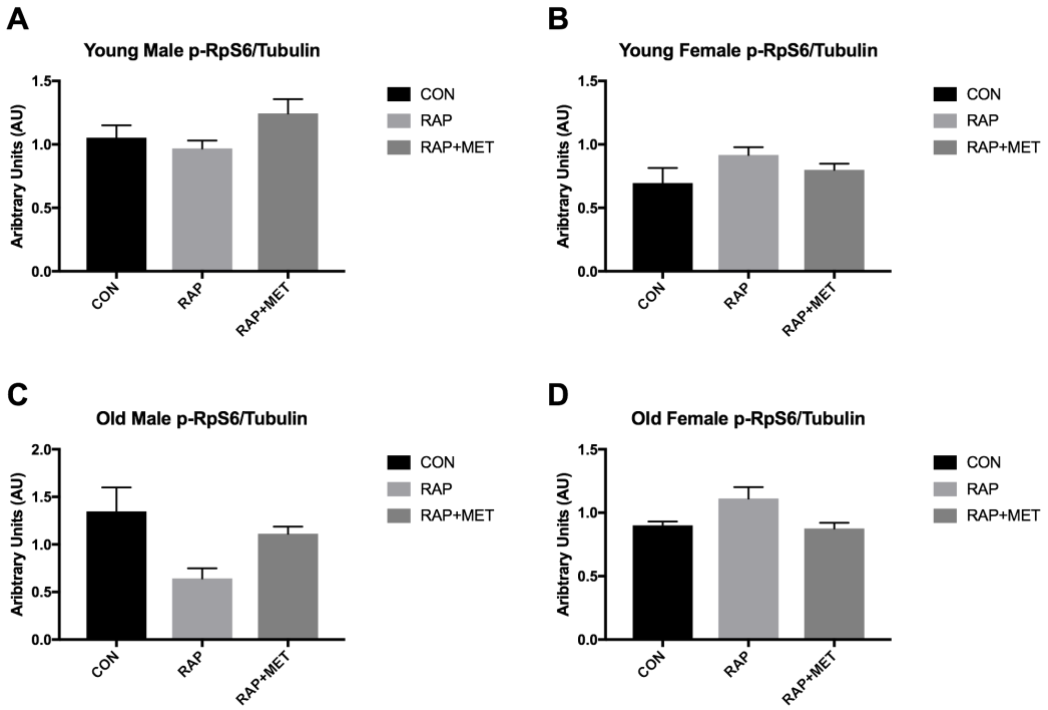

Figure 12. Phosphorylated RpS6 normalized to tubulin in young and old mice. Values expressed as mean +/- SEM.

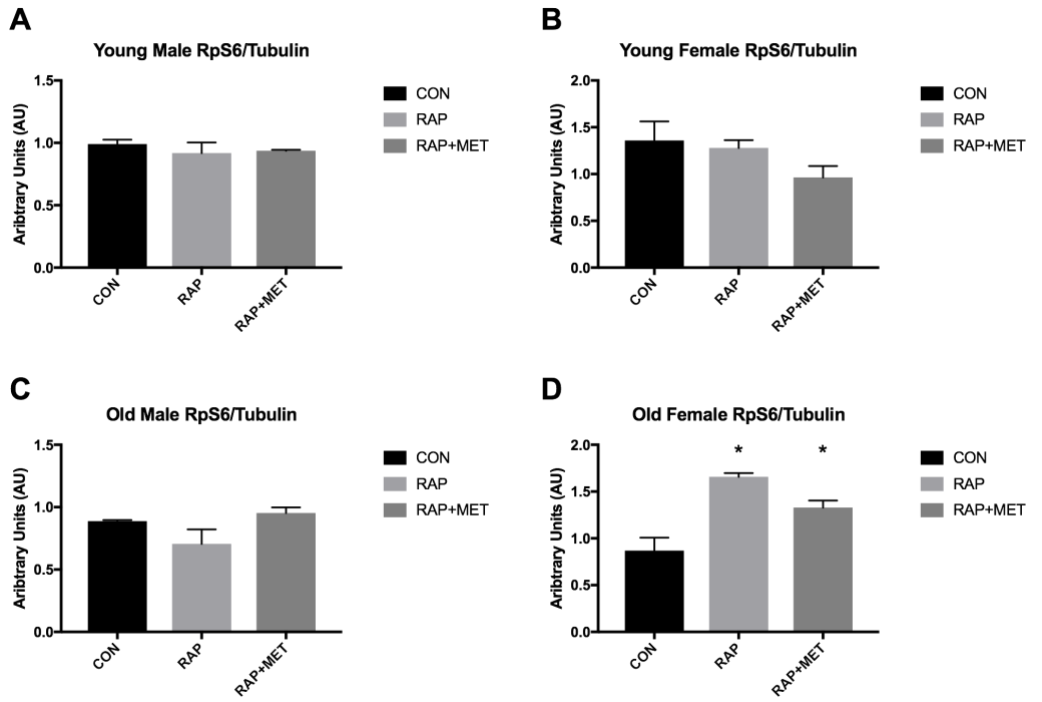

Figure 13. Total RpS6 normalized to tubulin in young and old mice. *Significantly different from CON ($p<0.05$). Values expressed as mean +/- SEM.

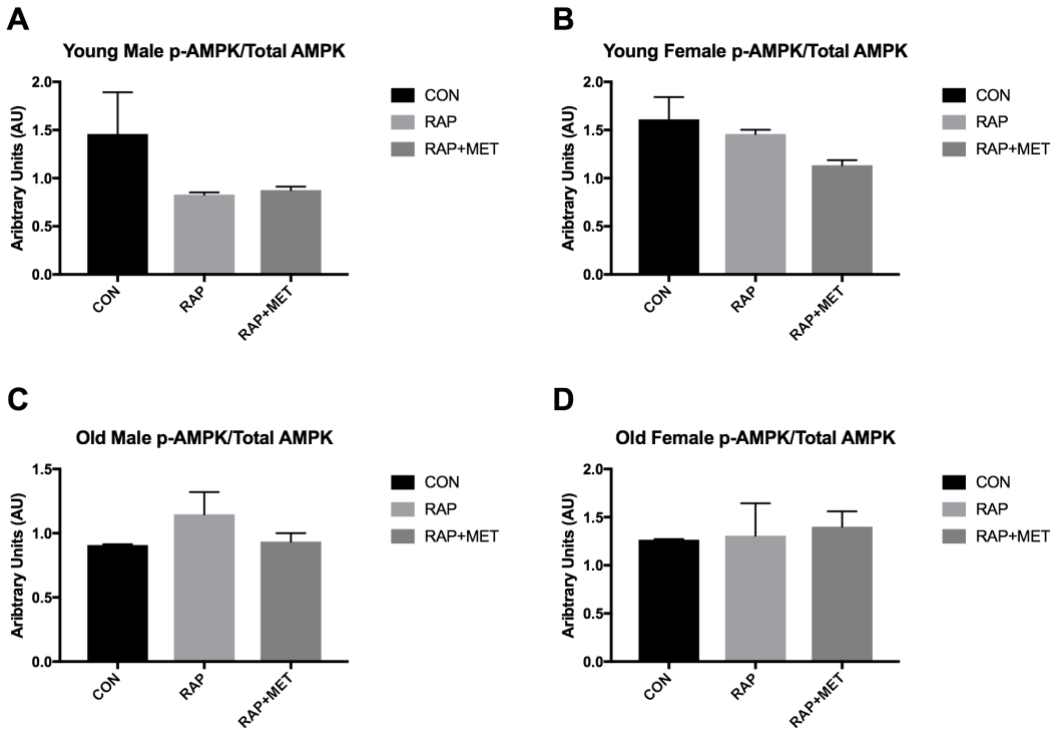

Figure 14. Phosphorylated AMPK relative to total AMPK in young and old mice. Values expressed as mean +/- SEM.

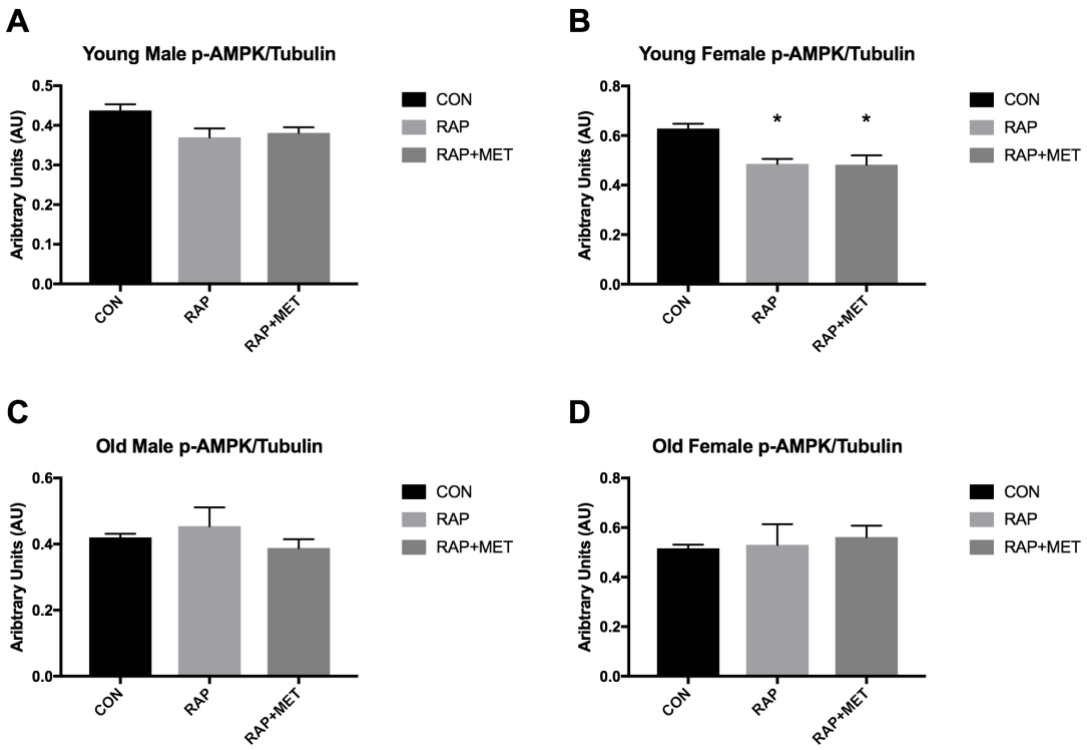

Figure 15. Phosphorylated AMPK normalized to tubulin in young and old mice. *Significantly different from CON (P<0.05). Values expressed as mean +/- SEM.

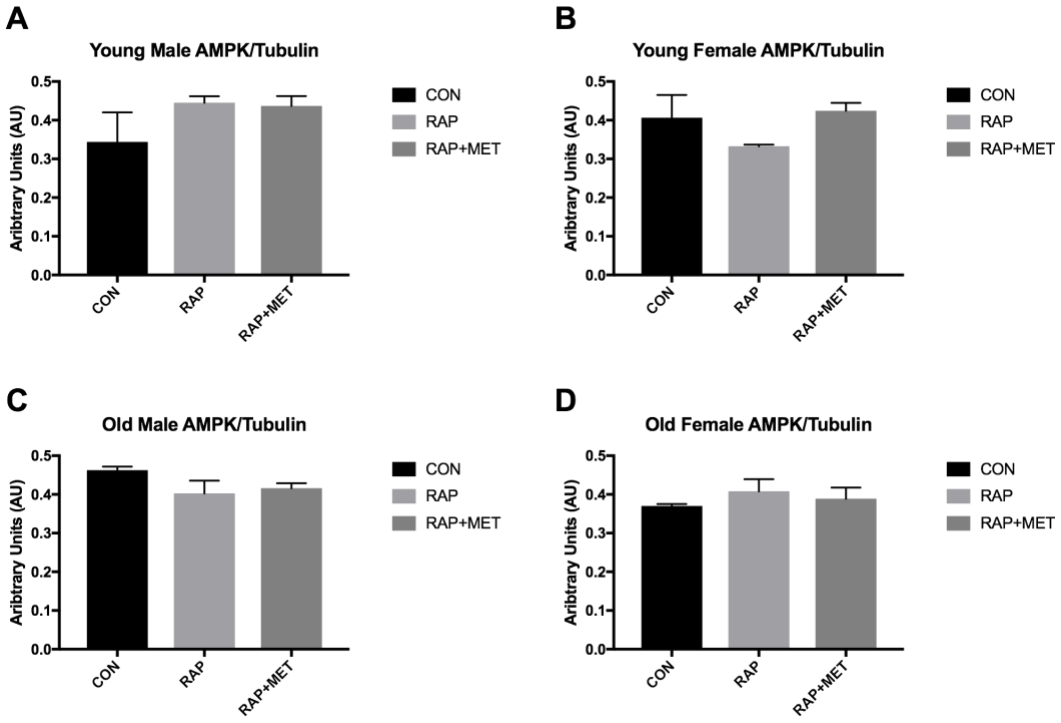

Figure 16. Total AMPK normalized to tubulin in young and old mice. Values expressed as mean +/- SEM.

CHAPTER V: DISCUSSION

Summary of Findings

This study is the first to report the use of deuterium oxide as a stable isotopic tracer for measurements of protein and DNA synthesis in the brain. The primary findings of this study are that in the brains of UM-HET3 mice, treatment with RAP and RAP+MET differently affects components of proteostatic maintenance, and that there are differences between sexes in brain turnover with and without lifespan extension treatments. These data are important because they provide evidence that protein turnover in the brain is malleable, and that sex should be considered when targeting treatments for brain aging and disease.

Novel Insights and Outcomes

The use of deuterium oxide as a stable isotopic tracer for measurements of biogenesis in cell culture (Miller, Wolff, Peelor, Shipman, & Hamilton, 2015) and *in vivo* is well established (Drake et al., 2013, 2015; Miller, Robinson, Bruss, Hellerstein, & Hamilton, 2012). However, this is the first study to demonstrate that deuterium oxide can be used to assess protein and DNA synthesis rates in whole brain. In addition, these results show that measurements of protein and DNA synthesis rates using D_2O in mouse brain are sensitive enough to detect sex differences, as well as differences in response to treatment. These differences were consistent even in the relatively shorter-term measurements used in analysis of protein synthesis in the older cohort. In addition to our proteostatic assessments in peripheral tissues, we now add D_2O approaches use for evaluation of proteostasis in the brain. This approach represents a new methodology

for studying protein and DNA synthesis rates in the central nervous system, a crucial area for research on aging and neurodegenerative disease.

The present study is the first study to examine the effects of two treatments known to extend lifespan on multiple characteristics of proteostatic maintenance in the brain. Here, we have demonstrated that treatment with RAP and RAP+MET differently affects subcellular protein synthesis rates, DNA synthesis rates, PRO:DNA synthesis ratios, protein aggregation, and activity in the mTOR signaling pathway in the brain. These findings support existing research that implicates the use of slowed aging treatments to study, prevent, or treat age-related cognitive decline and neurodegeneration (Dehay et al., 2010; Markowicz-Piasecka et al., 2017; Moll, Cohen, & El-Ami, 2014).

Sex Differences in Outcomes of Proteostatic Maintenance

Notably, the responses to RAP and RAP+MET were consistently different between sexes in young mice. Sex-dependent differences in either the presence or magnitude of response to a given dose of rapamycin have previously been reported (Fischer et al., 2015; Fok et al., 2014; Miller et al., 2014) and attributed to elevated levels of blood rapamycin in female mice compared to males . Although blood levels of rapamycin were not measured in the current study, a difference in bioavailable rapamycin is a plausible explanation for the different protein synthesis rates in female RAP+MET compared to males. It should be acknowledged that male and female comparisons were not made in the old cohort, as different timepoints were used for each sex.

In addition to discrepancies in the presence of an effect of treatment between sexes, we found that the direction of phenotypic change in response to RAP and RAP+MET was also distinct between male and female mice. In young male mice RAP decreased DNA synthesis, whereas both RAP and RAP+MET increased DNA synthesis in female mice. Differences in the direction of a phenotypic change in response to rapamycin treatment in mice has previously been reported (Fischer et al., 2015). The single study reporting such differences found that female mice had increased body mass and body fat (without increased food consumption), while males had lower body mass and body fat in response to rapamycin treatment (Fischer et al., 2015).

Brain Protein Synthesis Rates in Comparison to Other Body Tissues

Data that directly compares synthesis rates in brain to other body tissues is limited. It has been shown that brain has among the lowest protein synthesis rates of any tissue in mice (Shahbazian, Jacobs, & Lajtha, 1987; Wu, MacCoss, Howell, Matthews, & Yates, 2004). When brain protein synthesis data from the current study are compared to protein synthesis rates in other tissues from the same animals, the results are in agreement with previous findings (Sayegh & Lajtha, 1989; Shahbazian et al., 1987). Consistent with published literature, protein synthesis rates in each subcellular fraction in brain are, in general, much lower than those in liver. For example, in young male CON, the k value for cytosolic and mixed protein synthesis in brain was 0.1212 and 0.0789, respectively. In contrast, liver k values for were 0.2469 for cytosolic protein synthesis 0.3088 for mixed protein synthesis. However, protein synthesis rates in brain were considerably higher than in skeletal muscle (cytosolic=0.02246, mixed=0.01792), and comparable to those in heart (cytosolic=0.07296 and mixed=0.06147).

It also appears that, in general, mitochondrial protein synthesis rates may be higher in brain (k=0.0858 in young male CON) than in heart (k=0.0510) and skeletal muscle. (k=0.0216). Mitophagy is reduced in some types of ND (Chen & Chan, 2009), and it is possible that mitophagy is important in tissue that has high rates of mitochondrial protein synthesis, such as brain. If mitochondrial protein synthesis is not matched by mitophagic processes, dysfunctional mitochondria may accumulate, leading to the generation of ROS, protein damage, and potentially protein aggregation.

Do Rapamycin and Metformin Slow Aging?

The results in this thesis could be used to determine if the lifespan-extending effect of the slowed aging treatments, RAP and RAP+MET, also extend healthspan. Although the lifespan-extending effects of rapamycin (Harrison et al., 2009; R. A. Miller et al., 2014) and rapamycin with metformin (Strong et al., 2016) are well documented, whether such lifespan extension is a result of slowed aging is controversial. It has been proposed that rapamycin extends lifespan in mice primarily by reducing the incidence of cancer, and that the effects of rapamycin on aging are minimal (Neff et al., 2013). However, experimental evidence is conflicting (Bitto et al., 2016; Wilkinson et al., 2012) and the notion that rapamycin limited effects on aging has been contested (Blagosklonny, 2013b; Johnson, Martin, et al., 2013). Previously, we have demonstrated an increased ratio of PRO:DNA synthesis in multiple tissues from long-lived model organisms (Drake et al., 2014, 2015; Miller et al., 2014), and have proposed that PRO:DNA synthesis ratios may be a biomarker for slowed aging (Miller et al., 2014). In the current study, RAP increased the PRO:DNA ratio in male mice, but RAP and RAP+MET decreased the PRO:DNA ratio in young female mice. It is plausible that the

decreased PRO:DNA synthesis ratio reflects a negative effect of treatment. This hypothesis should be tested with additional measurements and functional outcomes, and with methods that isolate the different cell types in the brain. Importantly, the levels of DNA synthesis in brain are extremely low compared to other tissues (Drake et al., 2013, 2015). Thus, the PRO:DNA synthesis ratio in the brain may only weakly implicate functional outcomes and aging, if at all.

Limitations

The use of genetically heterogeneous UM-HET3 mice in the current study is simultaneously a strength and weakness. The utilization of mice that are genetically distinct from one another increases the translatability of findings to human physiology. However, genetic manipulation is typically required to study human ND in mice, and in the absence of genetic intervention mice are not predisposed to neurodegeneration (source). In this way, the use of UM-HET3 mice in the current study limits inferences that can be made regarding human brain physiology. In addition, it is possible that important treatment effects on proteostatic maintenance in the brain that would be observed in humans were not detected. For example, both treatments had a minimal effect on the amount of protein aggregation in whole brain homogenates. This is somewhat unexpected, given that rapamycin promotes autophagy (source). A reasonable explanation is that RAP and RAP+MET generally failed to reduce protein aggregation because there was not an appreciable amount of protein aggregation to reduce. If similar measurements were made older humans, it is possible that treatment with rapamycin may have a marked effect on protein aggregation, as has been

demonstrated *in vitro* (Caccamo et al., 2013; Dehay et al., 2010) and in transgenic models of ND (Viscomi et al., 2012).

In contrast to protein synthesis measurements made in the young cohort, which utilized multiple timepoints, measurements of protein synthesis in the older cohort were made using single time points. This discrepancy in measurements made between the young and old cohorts opens the possibility that important differences in protein synthetic responses to treatment were missed. Further, we were unable to make comparisons of protein synthesis rates between young and old mice, which may have revealed an influence of age on neuronal protein synthesis, particularly following treatment with RAP and RAP+MET.

Finally, further insight on the effects of RAP and RAP+MET on brain health and aging could be gained if current data was coupled to functional outcomes. As discussed, it is possible that a decreased PRO:DNA synthesis ratio, or decreased brain protein synthesis in general, would reflect dysfunctional cellular processes that could impair motor and cognitive function. Alternatively, decreased PRO:DNA synthesis ratios in the brain may indicate a reduced need for protein turnover. The addition of functional outcomes to future studies would help to elucidate the significance of alterations in the PRO:DNA synthesis ratio in the brain. Incorporation of functional outcomes is particularly important as the influence of protein synthesis on ND is not straightforward. In some models of ND, decreased translation improves symptoms. At the same time, neuronal atrophy is a common characteristic of certain human ND.

Conclusions and Future Directions

Results from the current study are the first to demonstrate the effect of rapamycin and rapamycin plus metformin on various aspects of proteostatic maintenance in the brain. Additionally, these results show that deuterium oxide can be used as a stable isotopic tracer to measure protein and DNA synthesis rates in the brain. The principle finding is that measurements of proteostasis-related outcomes in the brain in response to treatment with RAP and RAP+MET are strikingly different between sexes. Future studies should address the relevance of these measurements as they relate to functional outcomes. Finally, comparable measurements made in transgenic models of ND could increase our understanding of the underlying causes of neurodegeneration, as well how they may be treated and prevented. The use of slowed aging treatments, such as RAP and RAP+MET, remains a promising and unexplored area of biogerontological research.

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