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Investigating the Role of mTOR Inhibition in Neurodegeneration: Rapamycin's Impact on an Alzheimer's Disease Model System

Thesis in Biology
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Abstract:

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline, neuronal loss, and hallmark pathological features, such as amyloid-beta plaques and neurofibrillary tangles (Scheltens et al., 2021). However, despite extensive efforts in disease research, AD remains without a cure, with current treatments either merely relieving symptoms rather than disease modification or fall short in efficacy, suggesting more to uncover about the AD pathogenesis (Malik, 2017; Chhabra et al., 2024). The mammalian target of rapamycin (mTOR) pathway plays a central role in cellular metabolism, protein synthesis, and autophagy, and its dysregulation has been implicated in AD pathogenesis, particularly in its inhibitory effects on neuronal autophagy and clearance of toxic protein aggregates (Laplante et al., 2012; Cai et al., 2015). The goal of this study was to investigate the neuroprotective potential of the drug rapamycin, an mTOR inhibitor, in mitigating oxidative stress-induced neurotoxicity within an in vitro cell culture AD model system. Primary cortical neurons were exposed to AD-like stressor conditions through Ferrous-Amyloid-Buthionine (FAB) exposure, to generate oxidative stress similar to the AD mechanism. Assessment of rapamycin's therapeutic effects was done through comparing quantified reactive oxidative stress produced and cell viability. MitoSOX Red assays quantified reactive oxidative stress levels, while MTS assays measured neuronal survival under various treatment conditions. Existing research has demonstrated that oxidative stress plays a crucial role in AD pathology, and targeting reactive oxygen species provide therapeutic benefit and neuroprotection (Tamagno, 2012). The findings suggest no definitive results, due to limited data, but offer interesting trends suggesting rapamycin pretreatment can reduce oxidative stress in combination with a stressor, supporting its potential role in counteracting AD-related neurodegeneration. The trends seen align with previous studies

demonstrating rapamycin's ability to enhance cell autophagy clearance and reduce neuroinflammation (Meijer et al., 2015). Future research, more trials, and comparison to cell viability and neuronal morphology is necessary to confirm precise molecular mechanisms and effects underlying rapamycin's application to an AD model system and, in turn, its efficacy as a treatment.

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Introduction:

Medical Application of Disease Research

Through continuous innovation and technological advancements, disease research leads the way in transforming the modern healthcare industry. Trial and error plays a vital role in hypothesis testing, driven by ongoing widespread discussion by scientists and healthcare professionals. From the basis of understanding the root cause of disease mechanisms at the fundamental level of a molecular, cellular, or genetic basis, development of various treatments are established. This knowledge gained from studying the behavior of principle proteins in a diseased state can enhance our understanding of how they contribute to the disease process within this framework.

Molecular changes, such as their tendencies of activation of certain pathways or affinities to certain binding sites, can guide scientists in developing targeted therapies specific to combat or enhance these mechanisms effectively. This allows researchers and healthcare professionals to pinpoint the underlying pathways that could potentially serve as driving factors of certain diseases. Pathogenic knowledge of a particular disease prompts the development of numerous therapies, vaccines, and prevention strategies aimed to target critical steps in its mechanism, in turn halting disease progression or reversing the effects. As one uncovers the pathological process of a disease mechanism, specific biomarkers – indicators of the disease – can be utilized to detect and prevent the spread of illness early in its progression. Historical advances reinforce this process of pathogenic knowledge leading to treatment, as during the 1950s, Enders, Weller, and Robbins discovered that the poliomyelitis virus was capable of growth in cultures composed of various tissue types, facilitating the development of inactivated and live polio vaccines (Norrby, et al., 2007). This groundbreaking work not only revolutionized vaccine development,

but their research has contributed to the development of therapies that not only address symptoms, but tackle diseases at their very root. This targeted approach is exemplified in treatments for psoriasis, an immune-mediated inflammatory skin disease, as intervention is acted upon by the use of JAK inhibitors, as they target the signaling pathways that contributes to the inflammatory response associated with that disease (Tokuyama et al., 2020). In this treatment strategy, researchers address the disease's root cause to alleviate the symptoms at large.

Conversely, some infamous diseases, such as gastric cancer, have too complex of a disease mechanism. Given its ability to constantly evolve, its drug-resistant properties, and the intricateness of targeting cancer cells over healthy ones, merely understanding the pathogenesis of gastric cancer alone is insufficient to discover an effective cure (Zeng, et al., 2022). However, with such advancements highlighting past achievements, we must also acknowledge the progress that remains to be accomplished in overcoming challenges that unaddressed diseases pose at large. Although scientists have successfully eradicated various conditions, such as smallpox and rubella within the United States, through advancements in medical knowledge and advancements, there are other pressing challenges today in the area of leading diseases that remain without a cure. Many diseases instill deep fear upon prognosis due to their rapid declining nature, severity of course, and the few, if any, effective treatment options available.

This is particularly evident within the area of neurodegenerative diseases, which are becoming increasingly prevalent across various populations today, presenting an array of challenges (Lovrečić et al., 2013). These brain disorders significantly impair quality of life, as they gradually erode key aspects of personhood, such as memories, emotion, and cognitive function. Additionally, beyond affecting the individual suffering from the neurological condition, caregivers and providers experience increased emotional and financial burdens. Despite more

than 100 years of research efforts, the mechanisms underlying these diseases remain elusive, hindering the development of truly effective treatments.

To address a lack of mechanistic understanding, an alternative approach to the process of disease treatment development could be taken, considering not only the early stages of molecular pathogenesis, but going beyond that to evaluate the other end: focusing on the symptoms and the commonalities observed within affected individuals. Since the intermediate steps of the disease mechanism remain unclear, relating shared characteristics and their links to other diseases or areas of study could provide an opportunity to work backwards, utilizing trial and error to bridge the gaps in knowledge. By identifying the common denominators and their correlations with what we do know, we may be able to relate and apply treatments across various diseases, assuming they share similar mechanistic elements, acting in a similar manner. Through means of recognizing connections between symptoms presented, we can adopt treatments intended for different conditions to address similar symptoms, potentially discovering effective interventions through what could be a multi-use treatment. This idea of drug repurposing through common symptoms can be seen through the use of antidepressants being applied as a treatment option for chronic pain, ultimately bypassing traditional drug development procedures (Vasconcelos et al., 2024). It was linked that those with chronic pain conditions often had symptoms of depression co-occurring. By utilizing drug classes such as tricyclic antidepressants and serotonin-norepinephrine reuptake inhibitors, typically used to combat depression, analgesic effects were observed. These drugs work by modulation and alteration of neurotransmitter levels, leading to changes in the nervous system's processing of pain. Serotonin and norepinephrine play a role in both mood regulation and pain perception, thereby leading to improvement in pain modulation, highlighting the potential for drug repurposing (Wise et al., 2005).

Alzheimer's Disease in a Broader Context

One such condition in which this approach could be particularly valuable is Alzheimer's Disease (AD), a prominent neurodegenerative disorder that exemplifies the challenges of treatment development. Given the lack of effective treatments available, combined with the rapid decline of neurocognitive function, AD highlights the need for innovative strategies that go beyond the scope of the molecular origins of the disease, instead focusing on symptoms and their commonalities with other conditions. By incorporating a broader, symptom-based approach, as discussed, we may uncover potential treatments that could offer benefits across various diseases, including AD.

As a leading cause of dementia, AD is a progressive neurodegenerative disorder with an average survival period of only 6 years following the initial diagnosis, factoring in the elderly population affected (Scheltens et al., 2021). Not only is memory loss of an individual a key characterization of the disease, but behavior and emotions are heightened or stripped in the unpredictable and sporadic decline. The disease mechanism broadly manifests in neurocognitive impairment, behavioral alterations, and loss of motor function, said to be induced by amyloid beta plaque deposits and neurofibrillary tangles - abnormal proteins within the brain that interfere with neuronal function. However, difficulties remain in deducing whether these pathological features display root causes driving the disease process or alternatively a downstream result of consequences. This distinction is crucial and will be explored later in greater detail. For instance, further unidentified underlying mechanisms can also potentially contribute to this chronological inconclusiveness.

The pathologies and root causes of AD involve complex mechanisms that are seen to accumulate in specific individuals. In an attempt to understand these mechanisms, it is beneficial

to ask who is most at risk of developing AD. Identifying high risk groups can point towards clues regarding disease driving factors that contribute to onset and progression of AD. Specifically, those at risk for acquiring onset AD are categorized as individuals above the age of 65, as increasing age is a significant factor in this condition. Additionally, those with certain genetic predispositions, such as mutations in the APOE gene make one especially susceptible to AD. Furthermore, those with a familial history factor and those with certain existing health conditions are at a higher risk as well (Lopez et al., 2019). In regards to treatment strategies, because of the complex nature of the AD pathogenesis, there exists no remedy or cure, despite ongoing research. As the average human lifespan extends due to advancements in other areas of disease treatments, the percentage of the elderly population is on the rise, exceeding previous years, and alongside it, so is the risk of AD. Previously, a large number of intervention strategies such as acetyl cholinesterase inhibitors and the N-methyl-D-aspartate receptor antagonist Memantine failed to physically stop or reverse the progression or spread of AD on a cellular and molecular level, but instead merely serve as aids for better coping through symptoms (Malik 2017). Following this plateau of development, recent efforts have turned to monoclonal antibody treatments that physically lower plaque levels and delay cognitive deterioration (Chhabra et al., 2024). Despite this improvement, however, they remain in their infancy stages of clinical trials and are accompanied by potential side effects, such as brain abnormalities. As a result, AD continues to lack a definitive cure. Given the problems associated with uncovering the pathogenesis of AD, such as its multifactorial mechanism and the elusiveness of the upstream and downstream effects, proposed treatments are seeing minimal results. If more can be done to uncover the exact pathogenesis, more effective treatments can be applied in various methods, including the use of model systems. Since there exists both ethical and practical limitations of

direct human experimentation, model systems, such as animal models or cell cultures, allow for the simulation of the disease and testing of potential treatments, ultimately aiding affected individuals through application.

The affected population of those with AD exists not only in developed countries, but in developing countries, as well. According to the Global Burden of Disease, recent data indicates that approximately 51.6 million people suffer from AD, nearly doubling statistics from 1990 to 2019 (Javaid, 2021). This is a concern not only for individuals, their families, and caregivers, but from an economic point of view in healthcare as well. Societies worldwide are impacted, as the rising prevalence of AD places not only individual strain with this life altering condition, but also stresses public health resources on a global scale, as well.

AD Pathogenesis

The key pathological features of AD include the deposition of amyloid plaque, the formation of neurofibrillary tangles, neuronal loss and cognitive decline; however, it is important to note that while these four features are commonly seen, the exact relationships and interactions between them are not fully understood. Theories are widespread, however no definitive mechanism that is complete has been confirmed. It is hypothesized that the amyloid beta cascade involves the abnormal extracellular accumulation of amyloid beta peptides that forms amyloid plaque, triggering neurodegeneration. The aggregation of a protein derived from a larger amyloid precursor protein (APP), which plays a role in neuronal growth, repair, and synaptic communication, occurs. Accumulation of plaques and toxicity in amyloid beta aggregates arise during the cleavage of APP to form oligomers, which are smaller peptides that interfere with the synaptic function (Luo, 2016). Cleavage of APP can be through one of two pathways: a non amyloidogenic pathway, using processing enzymes α- and γ-secretases, and a cleavage pathway

utilizing the β- and γ-secretases (Sadigh-Eteghad, 2015). The latter enzymes cut APP at different sites, resulting in various lengths of Aβ peptides, such as Aβ42, that are toxic species fragments of amyloid plaques. Only the amyloidogenic pathway produces amyloid beta that contributes to the propagation of AD. A major initiation factor of β-secretase activation is the presence of Beta-Site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1), a specialized protein common in aging or cellular stress. The formation of amyloid beta results from the events following BACE1's cleavage of APP, as the remaining fragment is further cleaved by γ-secretase, producing amyloid-beta peptides, including the aggregation-prone Aβ42, responsible for the plaque present driving AD. However, it's important to recognize that within healthy individuals, it is seen that amyloid beta, including the Aβ42 form, is continuously being produced, but mitigated with a clearing out mechanism, causing no harm.

Reactive oxidative stress from reactive oxygen species (ROS) is known to stimulate the β-secretases cleavage by the increased expression of BACE1, promoting amyloidogenic processing (Tamagno, 2012). ROS are a normal byproduct of cellular metabolism and are generated during mitochondrial oxidative phosphorylation, the process of a cell making energy. These ROS free radicals are unstable and produce a state imbalance of reactive oxidative stress. As one ages, the production of ROS tends to increase, associating ROS damage with the age factor of AD. Specifically within the brain, it is susceptible to ROS toxicity, as it consumes a large amount of oxygen and has fewer levels of antioxidants for protection against the harmful superoxides of ROS. Although mechanisms of clearing amyloid beta naturally exist such as cellular uptake and the involvement of the immune system, overproduction of Aβ42 and other amyloidogenic peptide variants are a primary indicator of AD and its likelihood of neurodegenerative decline. In an attempt to predict and monitor the disease, a study was

performed utilizing a peptide of amyloid beta, 42-residue-long amyloid β isoform. Through its use of multimodal neuroimaging techniques of cerebrospinal fluid samples, the structural, functional, and metabolic processes of brain pathophysiology were successfully indicated, revealing biomarker indicator tendencies for detecting both early-onset familial and late-onset sporadic AD (Lista et al., 2014).

In addition to amyloid beta and the deteriorating effect of ROS, neurofibrillary tangles are another leading component that drives the development of AD. Such tangles are present in tau, a protein with a primary role of stabilizing microtubules, a structural component of the cell assisting in transport with neurons. Within an individual affected by AD, tau undergoes conformational pathological changes on a molecular level that lead to microtubule destabilization and toxic aggregates of the tau protein called neurofibrillary tangles. While in a healthy brain, some degree of phosphorylation and microtubule destabilization is normal, contributing to memory support and learning through neural plasticity or connection remodeling, the level of extent seen in AD is abnormal (Rissman, 2010). Hyperphosphorylation alters the structure of the protein, causing its detachment and aggregation. This abnormal intracellular accumulation of either hyperphosphorylated, conformationally changed or shortened tau in the brain, is associated with mechanisms contributing to the development of AD. Enzyme kinases, such as CDK5, CAMK2A, GSK3β are involved in tau phosphorylation; however, their precise role in AD pathogenesis remains a topic of ongoing research and debate (Drummond, 2020).

A relationship between misfolded tau protein and neuronal dysfunction has been demonstrated through various neuronal axon transport analysis tests, as tauopathy diseases demonstrate neurotoxic properties, damaging the cell and interfering with neuronal function (Folwell, 2010). The tau protein abandons its normal form and, in turn, its function of stabilizing

microtubules as well. Without a proper structure, tau inhibits its ability to carry out this function, disrupting cellular transport processes essential for neuronal survival. Neurofibrillary tangles present themselves as tau aggregates accumulating and leading to a disruption of neuronal function. Common driving factors between tau and amyloid beta proteins arise when it comes to forming harmful oligomers: post-translational modifications, such as adding excess phosphate groups to the protein, or forming disulfide bridges, which are like chemical interactions that lock proteins into shape (Mamun, 2020). However, the exact sequence of molecular events, intermediate key players in between, and causal chains in AD pathogenesis remain uncertain. Although theories exist that link amyloid beta to tau, a clear understanding of this relationship is still elusive, however the extracellular plaque is thought to induce the hyperphosphorylation of tau microtubules, which occurs intracellularly within the neurons, thereby presenting the neuronal loss associated with the dementia. Yet, there remains an issue in this hypothesis in that current treatments designed to bind and clear amyloid beta aggregates are not seen to have a drastic effect, suggesting more intricate processes are also involved from that limited efficacy. In addition to the amyloid plaques and neurofibrillary tangles, other hypothesized factors expand to a neuroinflammatory response, other cascades, dietary factors, and genetic factors as well. With the limitations of current knowledge combined with the complexity of the disease, the pathogenesis cannot be explained adequately by a single hypothesis, as it involves multiple interconnected series of events.

In addition, contributing to the complexity of AD pathogenesis is the involvement of genetic predispositions. The role of genetic factors is a critical area of research in understanding the progression of AD and identifying potential treatment options. The gene apolipoprotein E's allele, APOE 4, is a genetic risk factor that influences the likelihood of one developing AD, and

impacts the disease severity. Those with just one copy of this allele have an increased risk, as it is expressed in nearly 40-65% of AD patients, associated with both amyloid beta and tau pathologies. The risk is even greater for individuals with two copies who face a higher likelihood of developing AD. Given its role in lipid and energy metabolism within neurons, previous studies have suggested that APOE 4 may act as a transcription factor, regulating the expression of mitochondrial related genes and others linked to neurodegeneration. However, this is one out of many proposals of the role of APOE 4 in the disease pathway, as its interactions with amyloid beta aggregation and clearance, as well as direct interactions with tau, are more broadly researched through means of positron emission tomography radioactive tracing scans (Therriault et al., 2021). It also believed that the allele may directly interact with mitochondria, affecting function and through modified metabolism of the membrane, alter energy production and health due to imbalance (Pires et al., 2023). Additionally, this mitochondrial dysfunction and neurotoxicity is also found to be associated with increased mitochondrial calcium levels, as well as ROS levels, adding to the many factors that make the pathogenesis so intricate.

Furthermore, it is widely seen that age is a significant risk factor within AD, particularly in late-onset AD, alongside genetic predispositions. Studies have illustrated a strong positive correlation between age and AD incidence, with rates increasing significantly with older populations, reaching up to 28% prevalence in individuals 90 and above (Breitner et al., 1999). According to the “2024 Alzheimer’s Disease Facts and Figures”, 1 in 9 Americans aged 65 years and older have AD, with prevalence increasing to nearly 1 in 3 among those 85 and older (Alzheimer’s Association, 2024).

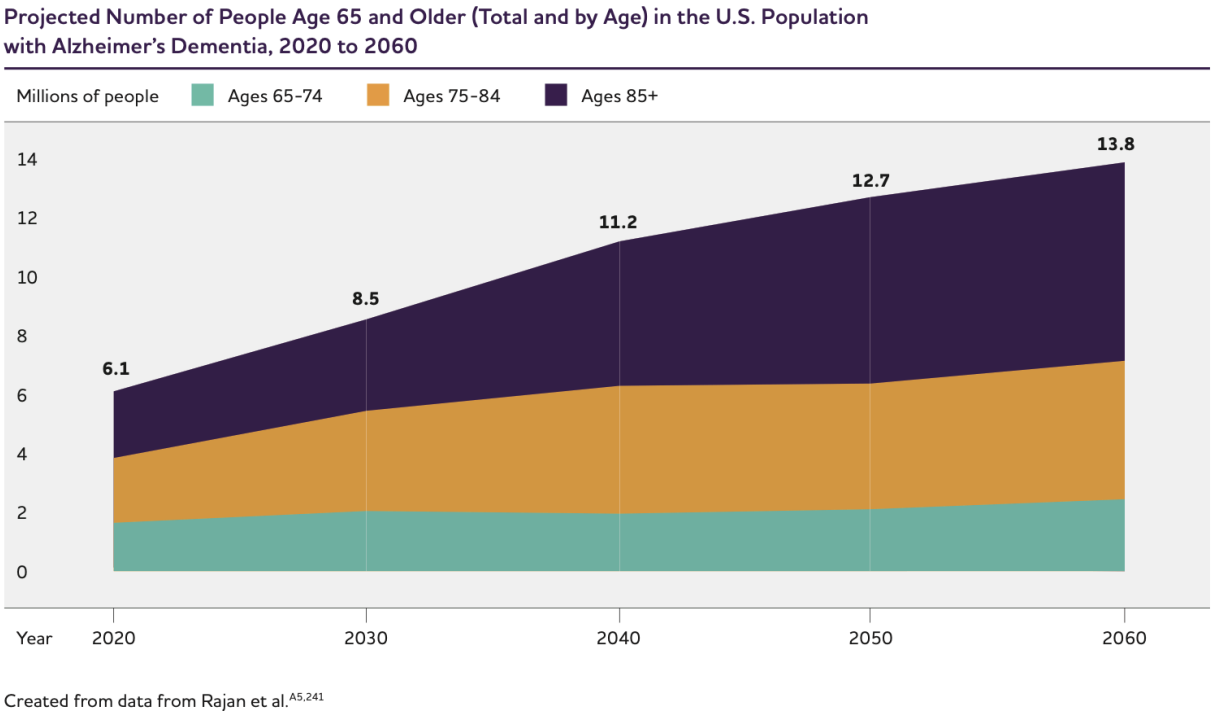

Figure 1. Graphical Representation of the Projected Number of People Aged 65 and Older in the U.S. with AD from 2020 to 2060 sourced from "2024 Alzheimer's Disease Facts and Figures" (Alzheimer's Association, 2024).

Several theories that may explain this association between the aging phenomenon and AD. One mechanism suggests the idea that age-related vascular changes accompany or even induce AD pathology, as modifications in cerebral and heart vessels occur. Atherosclerosis due to aging translates into neurological deficits (Cortes-Canteli et al., 2020), hence age being a common risk factor in AD. In addition, age acts as a risk factor of AD by an additional mechanism of age enhancing ROS. Aging through ROS enhancement may accelerate processes involving the formation of disease hallmarks, therefore contributing to AD lesions (Harman, 2002). Finally, the overall process of cellular senescence, decrease in activity in the cell cycle

that is associated with aging, lowers the brian's threshold for neurofibrillary tangle formation and neuronal loss (Musi et al., 2018). A molecular interplay can be seen between aging and AD, as genetic susceptibility serves as a key linking factor. For instance, as seen through genome wide association studies, the APOEε4 allele is associated with increased AD risk, while the FOXO3A has been linked to age related changes in the brain (Xia, et al., 2018). These findings emphasize that aging itself may exacerbate genetic risks. Furthermore, that study also references the use of DNA methylation to estimate one's epigenetic clock, closely linked to one's chronological age. In conjunction with a previous report, the epigenetic age of the prefrontal cortex reveals that faster epigenetic aging is associated with AD-related brain damage, such as plaques and amyloid build up (Levine et al., 2015). The stronger the link between their epigenetic age, the more significant the association with cognitive decline of AD. Together, these processes highlight the possible critical roles of aging driving AD development and advancement.

Neurodegeneration is characterized by the gradual loss of cognitive functions, including memory, decision making, and reasoning. This process is accompanied by the appearance of neuronal atrophy, where dendrites - the receiving processes of neurons - are deteriorated, leading to synaptic dysfunction (Seeley et al., 2009). As neurons shrink and lose their connections, the breakdown of cognitive abilities accelerates, leading to the progression of decline. Synapses, the regions connecting neurons, are damaged, hindering crucial communication between cells, and eventually, leading to neuronal death. It is seen that in AD, functional and structural impairment of synapses located in the neocortex and hippocampus are linked to cognitive decline within the patient (Gillingwater et al., 2013). In advanced stages of AD, the affected neurons have features that are significantly reduced, with fewer synaptic connections, and a decreased neuronal network, further impairing brain function.

What we do know is the end result of the disease leads to memory loss and neurodegeneration in every instance, with age being the common denominator. Aging is a multifactorial process consisting of compounded molecular and cellular damage over a duration of time. This accumulation of changes manifests in declined biological functions and increased prevalence of age related diseases. It was found that the effect of age on AD shows a feedback loop between the two, as AD and aging both contribute to the acceleration of each other's pathology (Liu et al., 2024). Key contributors to the aging process are increased cellular stress, including oxidative stress, DNA damage, and mitochondrial stress and dysfunction. Furthermore, as age increases, association with hormonal changes, chronic inflammation, and decreased brain plasticity can be seen as well (Park et al., 2021). Aging can make the brain more vulnerable to age associated diseases, such as AD. With that being said, since age is the most consistent risk factor, by examining the aging process and associated diseases, and turning to their successful treatment methods, we may be able to identify therapeutic approaches from other age-related diseases that could help reduce harmful effects associated with AD.

To compensate for the inconclusiveness within the exact mechanism of the disease pathway and the various gaps in effective treatment options, it is important to stress the development and application of various medications. While the groundwork for AD has been loosely established and some evidence is promising, more discoveries have yet to be made. New approaches have been investigated, focusing on neuronal protection, and combating the spread of neurodegenerative decline, however the results fall short.

Current Treatments

Traditionally, the focus of AD treatment has been centered on symptom management as opposed to addressing and modifying the underlying disease mechanisms. This approach using

drugs such as donepezil, galantamine, rivastigmine and memantine has helped alleviate cognitive decline and improve functioning in the lives of some patients through a cholinergic system agonism or N-methyl D-Aspartate (NMDA)-receptor agonism mechanism, improving neurotransmitter activity, chemical signals that boost neuronal communication (Pardo-Moreno et al., 2022). While these strategies have assisted in some learning ability, memory, and overall brain function processing, they have not been effective in halting or reversing the progression of the disease, allowing further brain degeneration to persist. There are very few promising FDA approved treatments available, as few are seen to reduce the rate of the progression of the disease itself. Additionally, each has their drawbacks; for example, the acetylcholinesterase inhibitors have efficacy that is limited to only mild stage AD cases and carries gastrointestinal and neurological side effects, to a point where some users have discontinued use.

Turning towards other types of treatment, such as monoclonal antibodies, Aducanumab targets amyloid beta plaques, reducing their formation, by enhancing clearance, and preventing its aggregation. This new form of treatment also brings forth concerns regarding side effects and safety. Issues such as amyloid-related imaging abnormalities (ARIA) exist, which can cause brain swelling or microhemorrhages. Other emerging treatments in the monoclonal antibody therapy field, such as Lecanemab and Donanemab, have demonstrated some promise as they aim to modify the disease by targeting plaques via immune system activation (Bentley et al., 2024). These novel therapies, however, are not financially feasible, offer little to no cognitive benefits with only moderate success, and subsequently induce dangerous brain swelling. Developing effective treatments for AD has proven particularly challenging due to the complex pathology of the multifactorial disease and the slow onset symptoms leading to late diagnosis. Therapies are not consistent in proving long-term clinical benefits, prompting researchers to believe that there

are other factors involved in the amyloid hypothesis (Vaz et al., 2020). The combination of these challenges highlights the need for more robust and further developed treatment options that can effectively halt or reverse the progression of AD and do not merely put a bandaid over it rather than serving as a lasting form of intervention.

Today's current treatment options are lacking in a number of areas and are not well developed enough to be effective, facing significant limitations. Even in combination with each other, they prove to be insufficient in addressing the intricate, multifaceted nature of the condition. Most existing therapies focus on symptom management, such as mitigating memory loss or cognitive decline, but such strategies only provide temporary relief, while simultaneously creating major risks, complicating their widespread use. Meanwhile, therapies that do target and reduce amyloid plaques show limited effectiveness in improving cognitive function, implying a lacking component in the AD disease hypothesis. Therefore, a new therapeutic approach is needed that compensates for what the previous drugs have lacked and have been unable to attain due to knowledge gaps. An innovative therapeutic strategy is required to address these shortcomings and provide a more comprehensive and effective solution to the neurodegeneration that occurs during the pathogenesis of AD.

Considering that the development success rate for novel treatments in AD is as low as 0.4%, with 99.6% of treatments failing in clinical trials, it is clear that a new approach is urgently needed (Cummings et al., 2014). One promising avenue could be to explore existing treatments from other disease areas that show potential for addressing AD, particularly based on symptom similarity and working backwards, as opposed to the root of fundamental disease mechanisms. As discussed previously with the alternative approach to disease research and treatment, we can turn to diseases that share the factor of age-related characteristics, and address AD concerns

through that linked commonality. We can turn to the protein mammalian target of rapamycin (mTOR) and the drug rapamycin itself as a novel strategy to potentially alleviate the neuronal damage caused by AD.

mTOR

In this context, one possible approach involves mTOR and its corresponding pathway. mTOR is a protein kinase that controls a variety of essential cellular functions such as both metabolic and catabolic activity, immune response, autophagy, survival, and proliferation. These cellular responsibilities are critical to regulate optimal internal cellular balance and maintain a state of homeostasis. The protein operates through two complexes, mTORC1 and mTORC2, which play a role in phosphorylating key proteins involved in translation within protein synthesis, lipid and nucleotide formation, and growth factor signaling (Panwar et al., 2023). Malfunction and disruption of the mTOR pathway is caused by genetic modifications and often associated with aging, neurological diseases, and cancer. The regulatory network of mTOR has an effect on various cellular processes that are vital to survival (Laplante et al., 2012). The consequences followed by the downstream effects of cellular function, as a result of mTOR activation ultimately determine how a cell functions, grows, or adapts to changes in its environment. This is achieved through how the cell processes energy and nutrients, the formation of proteins, lipids, and nucleotides, breaking down and recycling cellular components, and through cell survival mechanisms with immune system interaction (Saxton et al., 2017). mTORC1 has a role of regulating these processes as it promotes the first effectors of each metabolic pathway, such as p70S6 Kinase 1 leading to protein synthesis, therefore controlling balance of anabolism and catabolism. Its significance lies in its ability to integrate and process

various signals such as nutrient availability, energy status, oxygen levels, and growth factors, serving as the upstream signals that prompt activation.

Specifically within the pathway, TOR exists as two separate complexes, TORC1 and TORC2. While both complexes play important roles in cellular processes, this discussion will primarily focus on mTORC1 due to its relevance in the sensitivity to rapamycin, the drug of interest. Within mammals, mTORC1 is composed of mTOR and Raptor, Regularly Associated Protein of mTOR, which mediates TORC1's activation. mTORC1's binding substrates include proteins such as S6 kinase, S6K, and 4E-BP1, which play roles in regulating protein synthesis and cell growth (Dazert et al., 2011). Growth factors are known to activate mTORC1 through receptor tyrosine kinases and various cell signaling pathways leading to phosphorylation. As mTORC1 is activated, in turn a regulatory complex known as tuberous sclerosis complex 1, TSC1-TSC2, is inhibited. TSC1-TSC2 responds to multiple signals to downregulate mTORC1 (Child et al., 2014). This complex's normal role is to serve as a GTPase-activating protein, converting a molecular switch, Rheb, from its active GTP-bound state to its inactive GDP-bound state. Without Rheb active, mTORC1 deactivates, a regulatory process essential for maintaining proper cell growth and metabolic homeostasis. When Rheb is in the active GTP-bound state, it will directly bind to and lead to the activation of mTORC1, promoting protein synthesis and lipid production (Yang et al., 2017). Low cellular energy turns on AMP-activated protein kinase, AMPK, which through a cascade, ramps up the TSC1-TSC2 complex, ultimately preventing the activation of mTORC1.

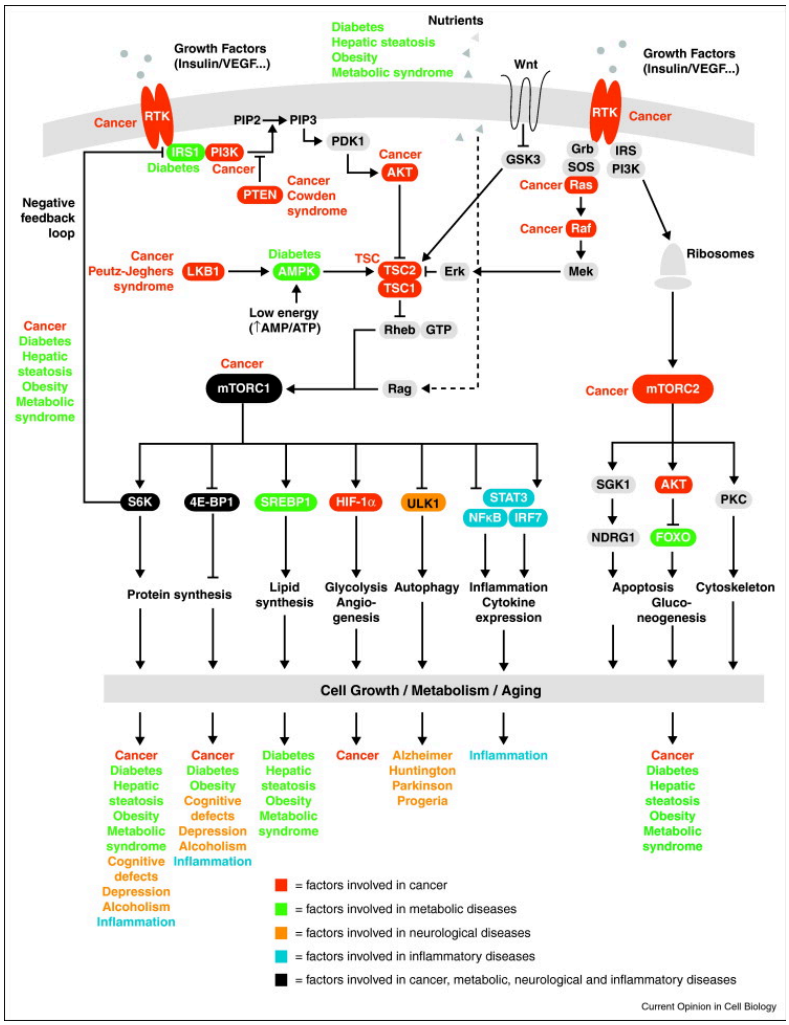

Figure 2. Graph of mTOR Pathway and Various Signals. Image sourced from "mTOR Signaling in Disease" (Dazert et al., 2011).

Dysregulation of mTOR signaling has been linked to various serious diseases. Mutations of key regulatory genes within the mTORC1 protein result in brain abnormalities, seizures, and cognitive dysfunction (Crino, 2015). Recent findings have shown abnormal mTOR signaling in relation in cancer, metabolic disorders, inflammation, as well as neurological diseases. The wide cast of clinical correlations highlight the importance of mTORC signaling on protein and lipid

synthesis, specifically within RNA translation. Within the realm of neurodegenerative diseases, mTORC signaling is crucial for neuronal survival; however, the excessive use of signaling in some cases may impair autophagy, a critical mechanism essential for removing misfolded proteins and aggregates (Ebrahimi-Fakhari et al., 2017). The hyperactivity of mTORC1 can lead to significant disruptions associated with pathological conditions. The drug rapamycin helps to counteract these consequences, supporting the preservation of cellular function.

Exposure of the rapamycin drug leads to an inhibition of mTORC1 only. Rapamycin binds to the intracellular FK506 binding protein 12 (FKB12), forming a complex that interacts with Raptor and prevents mTORC1 kinase functionality. Rapamycin impairs mTORC1's activity by disrupting the assembly of mTOR protein complexes (Bové et al., 2011). The Rapamycin-FKBP12 complex prevents mTORC1 from phosphorylating its downstream targets, such as S6K. Rapamcyin use coincided with the loss of S6K1-induced gene expression, as in a study, it was found that activation of S6K in a Drosophila fly fat-body blocked extension of lifespan by rapamycin, indicating that they counteract and oppose each other (Zhang et al., 2024). In addition, the multifaceted drug suppressed mRNA translation during mTORC1 inhibition, a process associated with slower aging by reducing oxidative stress (Panwar et al., 2023).

Upon further research of rapamycin and in addition to it promoting autophagy, the treatment exhibited strong anti-aging effects across a variety of species. Within genetic mouse models that had lower levels of mTORC1 signaling, it is suggested that mTORC1 signaling promotes aging (Arriola et al,. 2016). Rapamycin administered was found to increase the lifespan of mice, invertebrates, yeast, and fruit flies, delaying the natural process of aging. When tested within a model system of C. elegans worms, suppressed expression of the mTOR homolog

ceTOR and Raptor daf-15 were associated with a nearly doubled increase in lifespan (Vellai et al., 2003). An additional component includes rapamycin increasing the self-renewal ability of hematopoietic stem cells, and thereby the life span of mice, suggesting that the aging process is driven by constantly differentiating adult stem cells (Chen et al., 2009).

Rapamycin is hypothesized to be beneficial in treating neurodegenerative diseases by prolonging the lifespan of an individual. However, contrary to the generalized theory that increased lifespan leads to a higher incidence of age-related diseases, rapamycin is proposed to mitigate age-related symptoms and molecular changes associated with aging. Rapamycin is thought to increase lifespan with minimized age related disease prevalence. Rapamycin can clear aggregated proteins by promoting efficient autophagy clearing mechanisms within neurons, as tested in nutrient and growth factor-deprived conditions, it was found that mTORC1 activity was suppressed, indicating an inverse relationship between autophagy and mTORC1 activation (Meijer et al., 2015). Additionally, there is believed to be some interplay with mTOR signaling and regulation of neural plasticity, strengthening the communication and ties between processing cells within the brain (Panwar et al., 2023).

History of Rapamycin

Structurally, rapamycin is a macrolide antibiotic characterized by its serine and threonine kinase inhibitor catalytic domain. The first traces of rapamycin date back to the 1970s where terrestrial bacteria *Streptomyces rapamycinicus* on Easter Island was tested for antifungal properties (Yoo et al., 2017). Initially, following its isolation and biosynthetic modifications performed by Dr. Arnold Demains, rapamycin was intended solely to be used as an antifungal agent, as it possessed the ability to disrupt protein synthesis in yeast. Going beyond its original use, more applications were uncovered demonstrating inhibitory effects against fungal growth

within different contexts. Over time it was further tested for its efficacy within in vitro conditions against the fungal pathogen *Mucor circinelloides*, an infection prone to transplant recipients. Early clinical applications were approved by the FDA, focusing on preventing organ transplant rejection of the kidney, liver, and heart (Cai et al., 2024). Subsequent research using animal models revealed rapamycin possessed immunosuppressant and antiproliferative properties. These unintended discoveries sparked interest in application of rapamycin to treat many broad medical cases.

With its success in heart transplants and in combination with other immunosuppressants for a synergistic effect, rapamycin soon became approved in the U.S. and Canada by the 21st century. Additional benefits included reducing kidney toxicity, antitumors to treat in the brain and colon, and eventually as an anti-cancer treatment, as it prevented the growth of multiple cancer cell lines. mTOR overexpression is more prevalent in skin cancers, urinary tract cancers, soft tissue cancers, and cancers of the stomach (Murugan, 2019). Rapamycin derivatives or analogs, known as rapalogs, improved the survival of patients with renal cell carcinoma, and were effective in treating patients with lymphoid malignancies as well.

Furthermore, using rapamycin as a form of treatment for rare genetic disorders has been looked into specifically with tuberous sclerosis complex (TSC) and its associated epileptic seizures. For example, a rapalog, everolimus, has been approved for TSC-associated renal angiomyolipoma and has demonstrated potential benefits for decreasing tumor size after a study revealed reduced seizure frequency by 25% (Sasongko et al., 2023).

In addition to these confirmed and approved applications, experimental research has explored rapamycin's potential effects in other areas of treatment. In regards to recovery and repair after spinal cord injury, rapamycin is found to mitigate pain within a mouse model system,

while also offering neuroprotective properties. In instances of ischemia reperfusion injury, areas of restored blood flow, rapamycin showed effects that preserve cells from damage and death by boosting protective mechanisms such as enhanced autophagy, reduced apoptosis, and stimulating phosphorylation of Akt, a signaling molecule involved in cell survival (Li et al., 2019). Rapamycin's efforts expand beyond the spinal cord into the intervertebral disk through another proposed mechanism, involving modulating the local inflammatory environment caused by ROS presence and enhancing tissue regeneration (Bai et al., 2020).

Experimental lines of usage to be more carefully evaluated in the future include cardiovascular disease treatment, and neurodegenerative conditions, as overall anti-aging effects and associated disease applications remain emerging. However, found in today's therapeutics and approved for certain conditions, rapamycin is promising due to its diverse properties including antifungal, antitumor, neuroprotective/neuroregenerative benefits and life span extension.

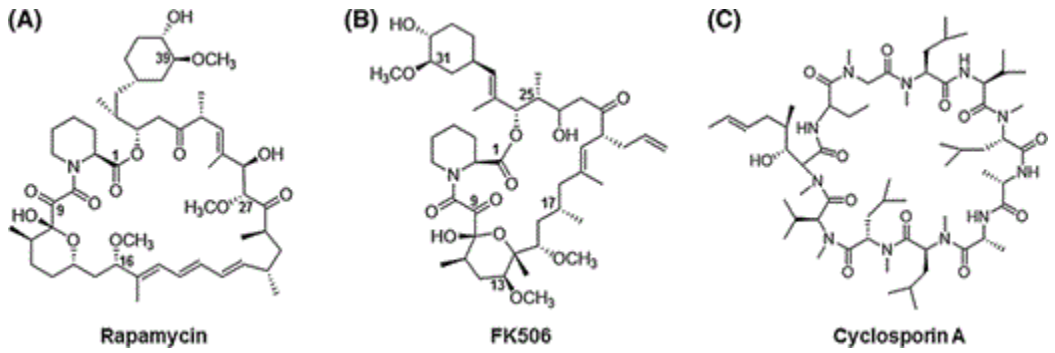

Figure 3. Structure of rapamycin and FK506, its intracellular binding site. Image sourced from "An Overview of Rapamycin: From Discovery to Future Perspectives" (Yoo et al., 2017).

Yet, minimal side effects exist, such as immunosuppression and glucose intolerance in some individuals. In a retrospective cohort study of kidney transplant recipients, 98% of patients

reported at least one side effect ranging from dyslipidemia, peripheral edema, cytopenia, acne, proteinuria, and oral ulcers (Verhave et al., 2014). It was reported that those presenting with dyslipidemia and cytopenia still managed the effects with continued drug administration, however the patients with the latter symptoms did not. Side effects appear to be mild, avoidable and reversible including hyperglycemia and starvation pseudo-diabetes (Blagosklonny, 2019). However, there exists some debate regarding the validity of the control group in comparison to those demonstrating symptoms, as there is often no true placebo group - individuals with the disease itself typically manifest symptoms, such as fatigue, regardless of treatment or not. Although resistance and limited efficacy may occur to a varying degree in some individuals, along with the possibility of infection risk and metabolic changes, these are standard symptoms, as with ordinary drugs on the market today. Overall, rapamycin's attributes reveal potential therapeutic benefits that appear to outweigh these challenges in its aspects of versatility and promising applications in age related diseases. With efforts to maximize potential while minimizing side effects, rapamycin could emerge as a promising alternative for treating AD pathogenesis.

The exploration of rapamycin as a potential treatment for AD stems from its success in other age related conditions and its role in targeting underlying cellular mechanisms (Selvarani et al., 2021). Age is a common factor seen influencing cancer, cardiac function, and neurodegenerative processes, with higher prevalence correlating with the higher one's age. Studies evaluating cardiac function show that rapamycin had persisting effects following treatment, suggesting it is beneficial not only in prolonging the onset of disease but improving conditions later (Quarles et al., 2020). Therefore, based on the previously used applications in age related diseases, combined with mTOR's role in cellular mechanisms, it is plausible that

rapamycin could show improvements in an AD affected brain. By focusing on a shared common symptoms, such as age, researchers can adopt an alternative, symptom driven approach to disease research, shifting away from focusing solely on root causes and instead identifying connections across conditions. Symptom commonalities may provide more insight through relating shared mechanisms of varying diseases, potentially uncovering knowledge gaps and addressing unknown mechanistic features from a solution-oriented lens.

Age is a significant risk factor in AD, as described previously, with the likelihood of diagnosis increasing substantially. Similar trends of aging, cellular function, and disease progression are observed in cancer, as an increase in age puts an individual more at risk, with the exception of the mutations that present in cancer. A connection between disease and the aging process can be drawn, likely linked by modifications in cellular function or immune response. For instance, increased inflammation and dysfunctional B cell response activity can disrupt normal defense mechanisms, leaving individuals to be at a greater susceptibility of infectious diseases, as these processes malfunction (Frasca et al., 2020). Given rapamycin's success in the uses for cancer treatment and mTOR inhibition's heavy association to anti-aging, rapamycin could be applied to AD patients to reduce cellular damage, possibly through mTOR interplay. Considering rapamycin's neuroprotective role within spinal cord regeneration, its promotion of autophagy, and the possible mitigation of immune response triggered by ROS, we can establish a connection between this anti-aging drug and the unknown pathogenic mechanisms underlying AD. Furthermore, given the success observed in age-related diseases, AD may share a mechanism related to the metabolic and cell differentiation functions of mTOR. It is possible that by prolonging the lifespan of an individual, the treatment could slow down the mechanism

causing the age-related increase in the pathogenesis of AD, bypassing the undetermined gaps that exist in the AD pathogenesis. Rapamycin may offer a means to do so.

mTOR can then be explored in the context of the AD pathway. Given mTOR's tendencies to inhibit neuronal autophagy, it can be proposed that when mTOR is active, amyloid beta is not adequately cleared. However, since rapamycin is the inhibitor of the mTOR signaling pathway, a restoration of neuronal autophagy can be expected, leading to a decrease in amyloid beta aggregates - a key pathological characteristic of AD. The possibility that mTOR regulates amyloid beta generation or clearance is explored through biomolecular evidence interaction among signaling pathways of PI3-K/Akt, GSK-3, insulin/IGF-1, AMPK, and p70S6K (Cai et al., 2015). These pathways consist of both the upstream and downstream components of mTOR, which are simultaneously involved in AD progression.

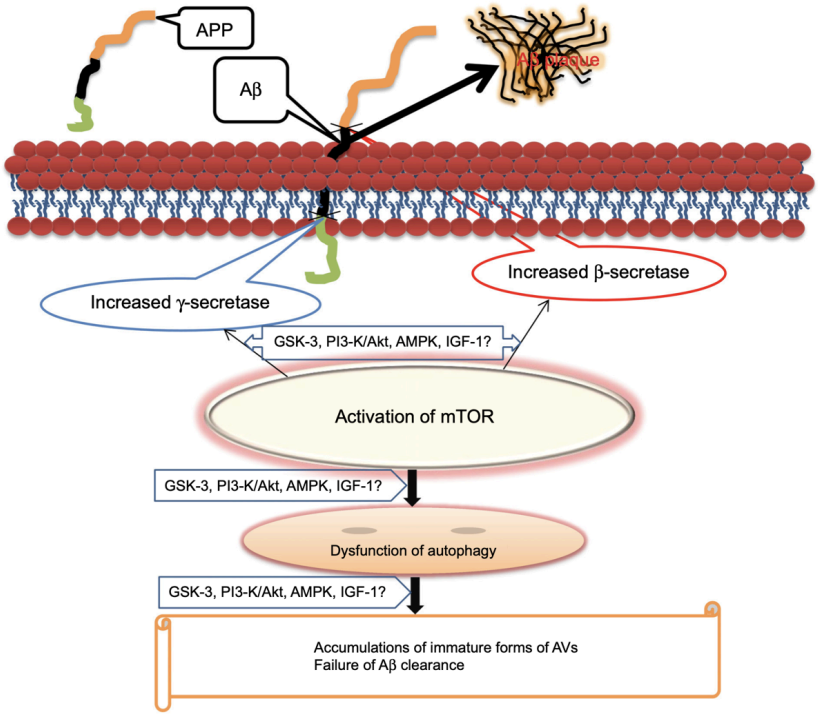

Figure 4. Diagram of potential mechanism of relationship of mTOR to amyloid beta production. Image sourced from "Activation of mTOR: a Culprit of Alzheimer's Disease?" (Cai et al., 2015).

For instance, AMP-activated protein kinase (AMPK) is a protein that regulates metabolic processes and enhances energy production, with mTOR as a downstream target that it represses (Kimball, 2006). AMPK inhibition reversed the hormone leptin's known ability to reduce amyloid beta levels, indicating that AMPK is a key mediator of leptin altering amyloid levels (Greco et al., 2009). Through this association, it is demonstrated that AMPK, an upstream component of mTOR signaling, can be linked to the amyloid beta of AD, providing evidence of relevance of mTOR in AD pathways. Interestingly, both AMPK and rapamycin share an inhibitory effect on mTOR, suggesting parallels in the mechanisms of action against AD pathology.

In addition, we can link the AD and mTOR pathways through the downstream components of mTOR signaling as well. S6 kinase is a ribosomal protein effector of TORC1 that phosphorylates other substrates in metabolism, learning, memory, and aging (Magnuson et al., 2012). Downstream mTOR signaling proteins can be linked to AD through the S6 kinase component, as the kinase can destabilize tau at sites S262, S214, and T212 (Pei et al., 2008). This tau destabilization contributes to neurofibrillary tangles, another characteristic of AD pathology, as well.

Evidently, the pathway of mTOR and AD can be seen to intersect at varying points, one way or another. It is not surprising that these intersections have prompted further research into targeting mTOR's inhibitor rapamycin as a therapeutic intervention for AD. Previous studies have investigated the use of rapamycin in AD models in order to test its potential in mitigating the disease. By means of in vivo animal model systems, through the use of cognitive function assessments such as the Morris Water maze, 3xTg-AD mice experienced improved learning and memory upon administration of rapamycin, as well as reduced amyloid beta and tau pathology

(Caccamo et al., 2010). Other model systems have include the use of two-photon imaging software to analyze the cortical vasculature and an immunoblot analysis of proteins among transgenic mice, finding that rapamycin offered neuroprotective effects, evading the blood brain barrier breakdown (Van Skike et al., 2018).

Aims of Study

Previous studies have shown that rapamycin can reverse age-related increases in ROS production, as observed in isolated liver mitochondria of CB6F1 mice (Martínez-Cisuelo et al., 2016). By applying rapamycin to the neuronal culture model, this current study evaluates its potential to mitigate ROS-induced neurotoxicity and its broader implications for AD pathogenesis. This connection between ROS and AD progression provides a strong rationale for targeting oxidative stress as a means for therapeutic intervention. By focusing the study on this part of the disease pathway, we aim to evaluate how oxidative stress contributes to neurotoxicity and AD pathogenesis.

The primary purpose of the following experiments was to observe the effects of rapamycin in an AD model system, focusing on oxidative stress and cell viability, in search of identifying neuroprotective properties in response to a stressor mimicking AD like deterioration. In order to observe the effect rapamycin had in alleviating ROS on the neurons themselves, a mode of assessment was needed. Parameters included measuring levels of reactive oxidative stress, which is both a driver and byproduct of neurodegeneration, and evaluating neuronal cell survival. A neuronal cell culture model was utilized to provide adequate parameters for neuronal growth, visualization, and effects of manipulation, as live neurons were isolated without being inside a host organism. In the past, postmortem brain samples have been used to make observations in regards to illustrate the involvement of ROS production in the disease

progression of AD (Birnbaum et al., 2018). The use of cultured neurons offers a more efficient alternative for examination, focusing on immediate cellular and molecular processes, unlike postmortem samples, which only reveal insights into later stages of disease progression. This system allows for direct testing of neuronal responses to rapamycin under controlled conditions, facilitating the study of it as a treatment option.

To replicate the diseased state of neurons, an effort was made to mimic aspects of the AD pathology using a known model system. The cell culture model offers a means to expose neurons to amyloid plaque components, with the FAB stressor inducing ROS. This ROS could then be quantified through the parameter of fluorescence, indicating stress. This model is based on the theory that amyloid beta contributes to neuronal cell death through the ROS-mediated ASK 1 signaling pathway (Kadowaki et al., 2005). The production of ROS, which leads to cellular damage and dysfunction, represents a key mechanism in AD progression. By focusing on oxidative stress, this study addresses a critical aspect in the disease pathway, supported by evidence that a weakened antioxidant defense system promotes ROS accumulation and facilitates AB42 activity (Lecanu et al., 2006).

To assess the efficacy and impact of rapamycin on ROS-induced neurotoxicity, a series of experiments were conducted to quantify oxidative stress and evaluate neuronal survival. Oxidative stress was measured as a marker of oxidative damage, with fluorescence serving as an indicator to detect change in stress levels. This approach provides insight into the extent of oxidative stress damage and the counteracting potential protective properties of rapamycin. By comparing neurotoxicity under conditions with and without rapamycin, this study seeks to determine its impact on AD related cellular processes.

Neuronal viability was assessed by means of a dimethylthiazol-carboxymethoxyphenyl-

sulfophenyl-tetrazolium (MTS) assay, allowing for the quantification of cell survival under conditions with and without rapamycin treatment. By comparing the quality and condition of the neurons in treated and untreated cells, this study aims to evaluate the therapeutic efficacy of rapamycin in mitigating AD-related cellular damage.

It was hypothesized that rapamycin could offer protective measures by reducing ROS-induced cellular damage, therefore preserving mitochondrial and neuronal health. Through these experiments, this study hopes to investigate the role of mTOR inhibition in neurodegeneration by assessing rapamycin's viability as a possible therapeutic approach for AD intervention. Through evaluation of oxidative stress levels and neuronal survival in response to rapamycin treatment, this study aims to elucidate the knowledge gaps in the AD pathogenesis, by testing possible treatment strategies.

Methods

The cellular model was obtained from conducting a primary cell culture of Sprague-Dawley rat embryo neurons. Application of FAB, a stressor containing iron, amyloid beta, and buthionine sulfoximine was used to stimulate the neuronal cells. A dye, MitoSox Red Mitochondrial superoxide indicator, was used to quantify the reactive oxidative stress produced in an assay that was read on a plate reader in the presence of both pre-treated rapamycin and FAB. Research was conducted through a series of analyses indicating rapamycin's impact on this AD model system, its therapeutic potential on mitigating oxidative stress and neurodegeneration, and to gain a deeper understanding the role of mTOR inhibition in neurodegeneration.

Harvesting and Performing Primary Cortical Neuronal Cultures

In order to ethically simulate the conditions of a brain affected by the pathology of AD, a primary cell culture was performed in which neuronal cells were derived. Brain tissue of a single selected fetal rat embryo was harvested, followed by subsequent chemical and mechanical breakdown, in order to acquire the appropriate cells needed for the chosen model system for experimentation.

Prior to the culturing of the embryonic cortical rat neurons, two 96 well plates were prepared in advance, by means of coating the bottom surface with poly-L-lysine, a chemical compound that aids in the adherence of cells. Following a duration of 24 h, the poly-L-lysine was removed, and sterile water was used to rinse the wells. A set of three water washes was performed, each sitting for 10 min to ensure the removal of the existing poly-L-lysine. A vacuum with a micropipette tip placed on top was used to remove the water, in efforts to prevent contamination. After the last water wash, plating media, a nutrient rich basal medium, was then placed in the wells and left to incubate until the time of the dissection. All measures and handling of plates and cells were performed in a sterile environment with 70% ethanol aseptic precautions, the use of gloves, masks, and working under hoods with blowers on.

Upon each dissection, a single female Sprauge-Dawley rat of an 18 day gestation period was humanely euthanized under controlled laboratory conditions using carbon dioxide exposure. An incision was made along the midline of the abdomen in order to access the uterus and obtain the sacs of embryos. The heads of each embryo were detached and placed in a dish full of saline solution, HBSS. Within the dissection of the individual embryo, the selected head was placed in a separate HBSS culture dish under the view of a light microscope. A pair of autoclaved forceps were used to puncture the membranous skull and maneuver the cerebral cortex to access the

forebrain's hemispheres. Once the hemispheres were isolated, they were transferred to a fresh HBSS culture dish. Forceps were then used to pick and peel the layer of meninges from the hemispheres. The hemispheres were transferred to its final fresh culture dish of HBSS where mechanical breakdown took place, once again using the forceps in a teasing motion.

In a 37 degree Celsius heated water bath, 3mL aliquots of trypsin, HBSS, and plating media were stored. The tissue fragments were pipetted to a tube of trypsin and placed in the bath for five minutes for chemical breakdown. The tissue was then pipetted to a tube of HBSS and placed in the bath for three minutes. This step was repeated in a subsequent fresh tube of HBSS. Finally, the last transfer was to 3mL of the same plating media, containing fetal bovine serum to promote growth. Additional mechanical breakdown was then performed using a flame polished glass pipette roughly 50 times. The cell concentration was then counted using a hemocytometer and a solution was made accordingly to a $1x10^6$ concentration. 100uL of the final cell solution was pipetted directly onto the plating media within the inner 60 wells of an prepared 96 well plate that was incubated. The cells were cultured and left to settle in the incubator at 37 degrees Celsius for a period of one hour. Finally, the plating media was removed and replaced with warm growth media, containing no serum, but a B-27 supplement. The cells were removed from the incubator three days a week to take out half the amount of old growth media and replenish it with fresh media, a process known as "feeding." Cells were left to be fed and matured for a duration of 14 days, until they were ready to be tested.

Toxic Conditions Mimicked by FAB

To replicate the conditions of AD within in vitro parameters, a stressor had to be created and applied to mirror the neurodegeneration that occurs within an affected brain. The mechanism by which FAB induces an AD-like state is multifaceted, as the incorporation of iron (II)

introduces a redox-active metal that can catalyze the formation of ROS. High levels of oxidative stress is a characteristic of AD, contributing to the neuronal damage and the pathology progression. Additionally, the natural presence of amyloid beta and its build up of insoluble fragments is an inherent hallmark of AD, as well. Buthionine was responsible for suppressing the neurons' natural antioxidant ability provided by glutathione to protect cells from damage caused by ROS.

Ferrous-Amyloid-Buthionine (FAB) was prepared by incorporating 7.95 mg of $FeSO_4$, 50 uL amyloid beta, and 133.5 mg of buthionine into 50 mL XM basal media, a growth media like solution that lacks growth hormones and vitamins. Following the dissolving of the reagents, the solution was then filtered to make a 3x stock solution. To create the experimental concentrations for the conditions used, this 3x stock was diluted accordingly to prepare two experimental conditions: High FAB and a Low FAB. The High FAB consisted of 250 uL of stock FAB to 3.75mL of XM. The Low FAB consisted of 100 uL of stock FAB in combination with 3.9 mLs of XM. FAB creates a model that mimics AD like pathology, replicating characteristics of an AD-like state.

Rapamycin Concentration

A 2 mM stock solution of rapamycin was made using dimethyl sulfoxide (DMSO). To prepare a 20 nM working solution, 2 mL of the 2 mM stock solution was diluted. Previous studies have seen promising results at this concentration of rapamycin implemented within the context of a cell culture (Radad et al., 2015). Specifically, 2 uL of the stock was added to 2 mL of XM media, yielding a 2 uM working solution to use as a base in rapamycin-related conditions. A 20 nM concentration was then prepared by adding 20 uL of the 2 uM working solution to 2

mLs of XM. Finally, 20 uL of the 2 uM working solution was added to both the Low FAB + Rapamycin and the High FAB + Rapamycin conditions.

MitoSOX Red Superoxide Indicator Assay

The production of superoxide was observed by using an indicative dye, Mitosox Red, and scanned for fluorescence detection. The performed assay was built off of previous work regarding whether such measures of oxidative stress could be physically quantified. This assay yielded the superoxide molecules within each condition, correlating the harmful oxidative stress released through neural mitochondria. A 50 ug MitoSOX Red dye was initially diluted to 1 ug/uL in DMSO and stored in the freezer. To prepare the stain solution mixture, 2 uL of the diluted dye was mixed with 3 mLs of XM. 10 uL of the stain solution was pipetted in each well and sat on the treated cells of various conditions for 10 minutes, incubated. The plate was analyzed on a SpectraMax M3 plate reader with an excitation wavelength of 510 nm and an emission of 580 nm. The plate was read from the bottom and a well scan was performed to reveal the fluorescence.

MTS Assay Measuring Cell Viability

The MTS assay is a common known procedure to deduce cell functionality and survival through metabolic activity. This assay functions by means of the conversion of the tetrazolium salt, visualized through a colored product. The metabolic activity of the living cells from the assay is correlated with the amount of viable cells present. This basis is used to assess the health quality of neurons, as those on the low viability end do not contain the necessary enzymes and means to undergo the conversion of MTS, therefore, not being read by the reader. The protocol entailed formulating an MTS dye consisting of a 1:5 ratio of MTS dye and XM. Following the course of exposure to treated conditions, all existing media was vacuumed and replaced with 100

uL of the MTS/XM solution. The plate was placed back in the incubator for a duration of one hour before the reading. On the SpectraMax M3 plate reader, an absorbance reading was done via endpoint at a wavelength of 490 nm, with higher values indicative of more viable cells.

Immunocytochemistry

In an attempt to visualize neurite growth in response to the stressors and rapamycin, 24 Well plates of neurons were prepared to undergo immunocytochemistry (ICC), a technique that uses antibodies to detect and label specific alpha-tubulin proteins within cells. This allows visualization under a fluorescence microscope based on intensity values. Changes in fluorescence intensity reflect the abundance of neuronal tagged proteins, used to assess appearance, localization and health of neurons, indicators of cellular response to conditions. The process entailed fixing cells in 4% a paraformaldehyde solution for a period of 20 minutes. Three rounds of washes with phosphate-buffered saline (PBS) were done, at three minutes each. Cells were then incubated with 0.5% triton-100 at room temperature for ten minutes. Both primary and secondary antibodies were prepared in a 1:500 dilution using PBS. A subsequent wash of PBS was then done before applying 150 uL of the primary antibody in each well to detect acetylated tubulin, a post translational modification seen on stabilized microtubules. The plate was placed on a shaker for a duration of one hour. Three washes of PBS were performed, before application of the secondary antibody, Cy-3, also at 150 uL and placed on the shaker for a duration of one hour. The final three washes of PBS were done, after which the remaining PBS in the plate was sealed with parafilm and stored in the refrigerator, until imaging. Imaging consisted of a collection of 20 images per condition using NIS-Elements software, and relative intensity values were calculated by subtracting the mean from the minimum values.

Treatment Conditions

A total of 6 conditions were assessed in both the MitoSOX Red Superoxide Assay and MTS Cell Viability Assay. They consisted of (1) a control parameter of just XM media, (2) pretreated rapamycin treatment, (3) a low concentration of FAB, (4) a high concentration of FAB, (5) pretreated rapamycin treatment with low FAB concentration, and (6) pretreated rapamycin treatment with high FAB concentration. Pretreatment, found optimal through previous work, was done 48 hours before the stimulation. Despite the pretreatment of the selected three conditions, the remaining three that were not pretreated were fed and maintained as normal until the time of stimulation. Exposure time was also deduced in previous experiments, following an optimal time curve of the ROS levels produced. Following applying the pretreatment, 48 hours later, the conditions were finalized with stressors added and immediately read for the MitoSOX Red Superoxide Assay. However, for the MTS Assay, after the 48 hour pretreatment, and application of stressors, the neurons were given an additional 48 hours post stimulation to properly assess the viability.

Data Extrapolation:

The plate readings given by both fluorescence readings and absorption readings in respect to each assay were performed using the software SoftMax Pro Data Acquisition 6.2.1. The data was converted and exported to a Microsoft Excel File. For data analysis, mathematical values were manipulated in order to get the mean and standard deviation of the experimental conditions. Given the small-scale and exploratory nature of the pilot study experiment, no statistical significance analysis was performed. Significant outliers were removed from wells that had values 5-8x greater due to the extremely small sample size of one plate.

Results:

Part I - Optimization of ROS Quantification in Cell Culture Model

Previous research done in the Knowles lab attempted to measure oxidative stress in neuronal cultures; however, the absence of a positive control led to inconclusive results, preventing confirmation of measurable ROS levels (Cevallos, 2022). In order to confirm that reactive oxygen stress levels could be measured, a known substance that was used to induce reactive oxygen stress was used, as opposed to FAB. Previous studies have determined that hydrogen peroxide and peroxyl radicals can be used to induce oxidative stress within neuronal cells (Othman et al., 2015). The same parameters of the cell culture model and MitoSOX Red dye were used, as it served as an indicator of mitochondrial superoxides. A series of experiments were conducted to ultimately deduce the optimal time curve of the neuronal cell culture model system to the hydrogen peroxide by determining both the concentration of the stressor and the duration of exposure times. A challenge in measuring ROS is the instability of oxygen free radicals, their high activity, very short lived lifespan, and low concentration. ROS production due to oxidative stress must be accurately measured at the short term lifespan. On the other hand, a prolonged exposure period can lead to cell death, skewing representation of accurate oxidative stress measurements (Pavelescu, 2015).

To find the optimal curve, exposure time of the stressor was needed. Several trials were performed, with exposure times ranging from 30 min to 48 h of short-term and long-term models of exposure. Through trial and error, a short-term time curve was found to be the most promising in terms of reactive oxygen stress production. By evaluating the levels of reactive oxygen stress produced at each reading point in time, an optimal time and concentration curve could be

identified. After validating the ability to measure ROS, and testing various conditions, such as time and concentration, this technique was then applied to FAB exposure and was tested across a time course to see how ROS levels changed over time. By optimizing conditions to accurately measure ROS in living cells, without causing cell death, initial trials informed the design of the current study. The goal was to capture ROS production at the precise moment when cells were still viable, ensuring measurements were reflective of oxidative stress, without prematurely killing cells. This approach allowed for determining the shortest exposure time needed to observe ROS effects in the neuronal AD model system, minimizing cell death and improving data reliability.

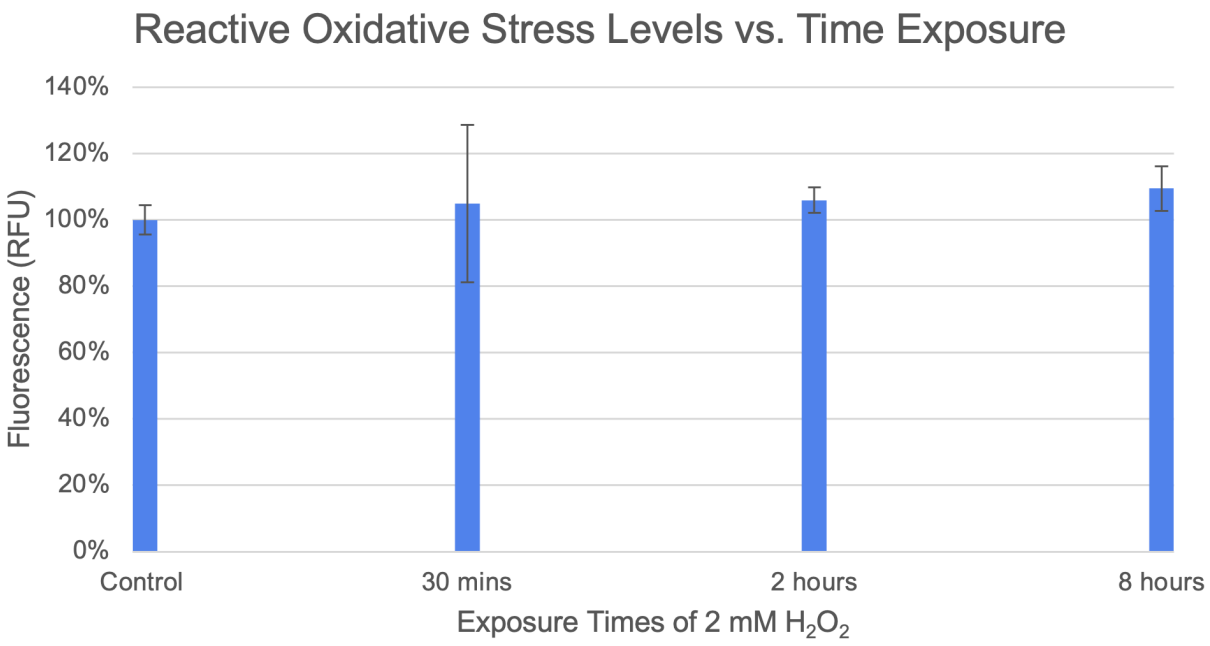

Figure 5. Detection of Relative Percentage of Oxidative Stress Levels over a Timeline of 2 mM H_2O_2 Exposure. This graph depicts the percentage of oxidative stress across short- and long-term exposure times, enabling the determination of an optimal time curve of stressor exposure time.

Neurons in cell culture were 2 weeks old. Samples included 10 wells of each condition, being the control of XM, 30 min, 2 h, and 8 h exposures.

It was observed that after 8 h of exposure time to 2 mM hydrogen peroxide, the relative percentage of oxidative stress produced increased from the control. Given that the 2 h exposure was the shortest period to observe sufficient and consistent stress production with the least amount of variability, the 2 hour exposure period was determined to be optimal for stress induction. Any exposure longer than that mark was found to induce cell death rather than superoxide radical production, as a previous study was done that simultaneously performed a cell viability with MitoSOX Red Mitochondrial superoxide indicator assay and found that cell death occurred at the longer exposure time period, with ROS being inconclusive (Cevallos, 2022). By determining the shortest duration in which neurons could withstand a stressor during the period of oxidative stress, we consistently detected stress levels at the 2 hour mark.

In addition, to determine the optimal concentration, various serial dilutions of 9.8 M hydrogen peroxide in growth media were created (0, 2, and 5 mM) and applied to the neurons to evaluate the degree of reactive oxygen stress produced. Through trial and error, a hydrogen peroxide concentration of 2 mM was found to be tolerable for the cells, inducing stress without overwhelming them or causing rapid cell death, which could have compromised the accuracy of measurements.

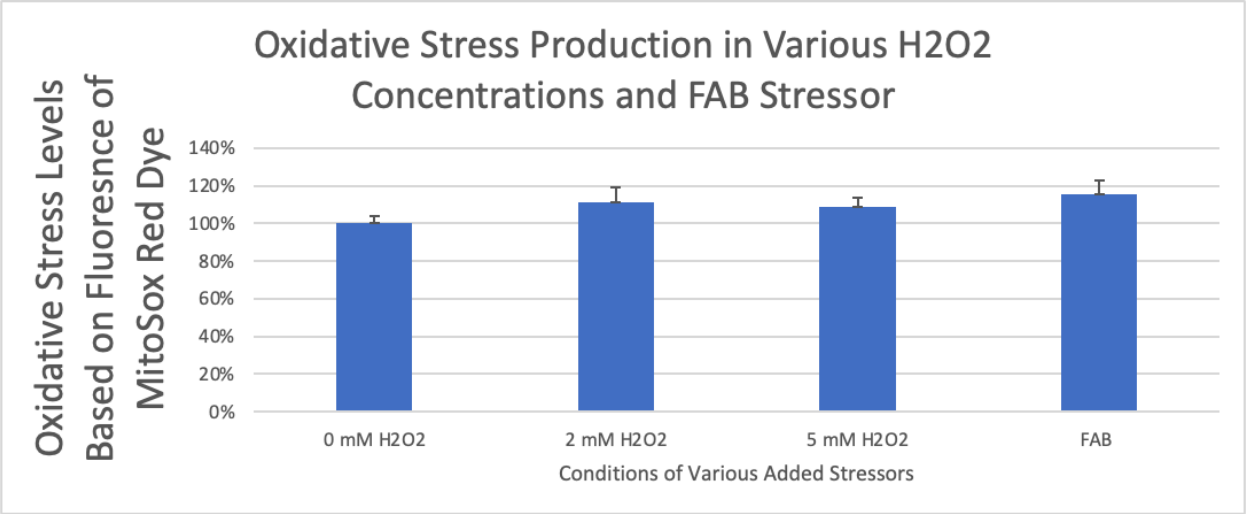

Figure 6. Detection of Relative Percentage of Oxidative Stress Levels at various concentrations of H_2O_2 Exposure (0, 2, and 5 mM) in addition to FAB. Neurons in cell culture were 2 weeks old and exposed to stressors for a duration of 2 h. This graph depicts the percentage of oxidative stress response to known ROS inducing substances, facilitating the determination of an optimal concentration, as well as the ability to quantify ROS.

As seen in **Figure 6**, a control value of 0 mM hydrogen peroxide was used to compare the varying concentrations of the serial dilution. With an increased concentration of 2 mM hydrogen peroxide, ROS increased slightly compared to the control. However, a higher concentrated stressor of 5 mM hydrogen peroxide yielded fluorescence that was within the range of 2 mM produced, suggesting the stressor was not concentration dependent in the production of oxidative stress. The most amount of oxidative stress produced was seen in the FAB condition. By combining the discovered 2 h time exposure with the FAB condition, the optimal conditions

for effectively detecting oxidative stress in the model system was utilized further for the main study. This work validated the use of the FAB conditions, as it yielded a similar amount of stress levels as the 2 mM hydrogen peroxide positive control, known to induce oxidative stress. With this ability to measure, application of a more representative AD model system was used with the FAB stressor moving forward.

Furthermore, when using the fluorescence scan plate reader for MitoSOX Red Dye detection, the goal was to determine the acquisition settings that yielded the best representation of the stress. Several trials were done varying the settings of well scan, end point, and direction of the points scanned. The most important finding determined was the setting change from a single endpoint to a wellscan of three horizontal points, leading to more constant results.

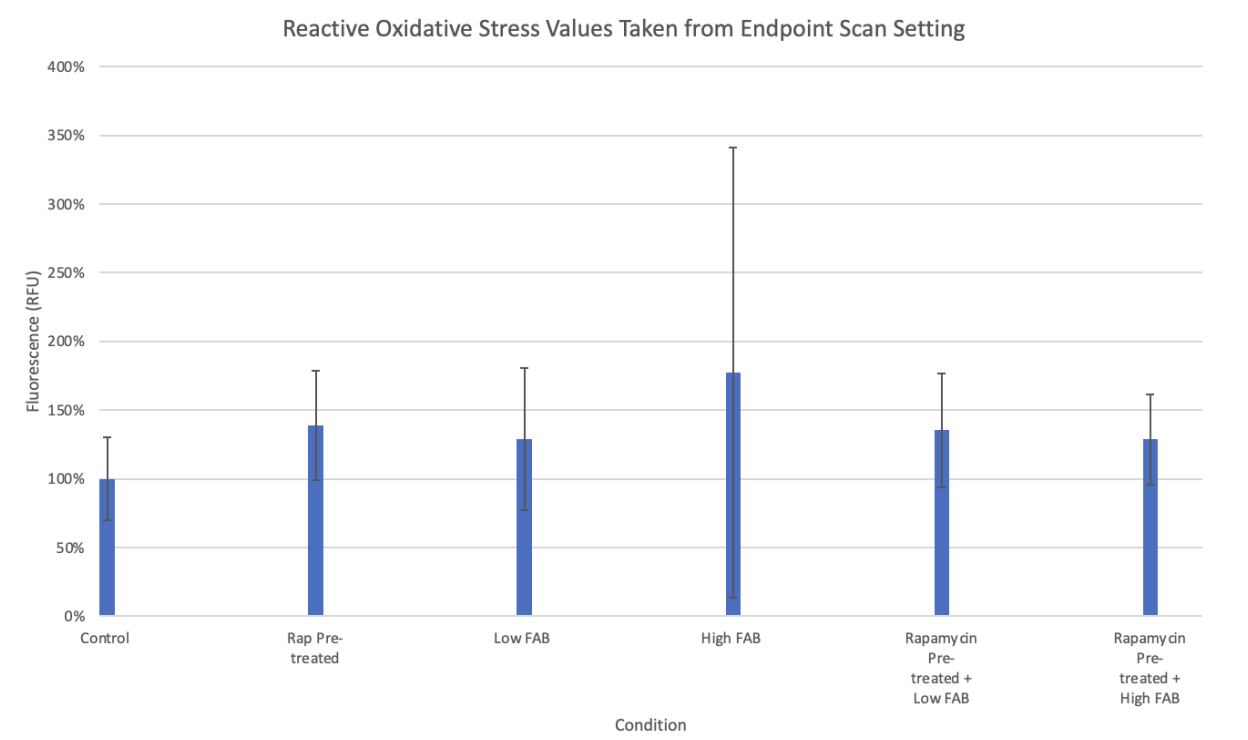

Figure 7. Mean Fluorescence of 96 Well Plate of MitoSOX Red Reactive Oxidative Stress Assay obtained through Endpoint Scan. 1.5-week old neurons at a concentration of $3.5x10^6$ were exposed to stressors, and reactive oxidative stress levels were measured using MitoSOX Red fluorescence. Fluorescence intensity acts as an indicator of oxidative stress. Conditions included 8 wells XM media as the control, 8 wells pretreated rapamycin (48 hours in advance), 8 wells Low FAB concentration, 9 wells High FAB concentration, 9 wells pretrated rapamycin in combination with Low FAB, and 8 wells pretreated rapamycin in combination with High FAB. Neurons were subjected to the specified conditions for two hours, after which the dye was placed and read.

From **Figure 7**, an excess amount of variability is seen among the same conditions, as indicated by the unusual error bars. When compared to the same plate, taken under 3 point horizontal well scan, as opposed to a single end point, the error bars seen on **Figure 9** had decreased variability. These settings allowed for reliable readings of reactive oxygen stress levels within each well. All preliminary work was done to ensure that experimental conditions effectively mimic AD-like deterioration as closely as possible, while allowing for a measurable therapeutic response.

Part II - Rapamcyin's Mitigation of ROS in Cell Culture Model

Another series of studies was conducted to investigate the neuroprotective properties of rapamycin based on the duration of administration, aiming to determine the most effective form of treatment parameters. The drug was applied 2 h prior to the dye placement and reading as well as 48 h prior. The 2 h duration was chosen as 2 h coincided with the time of stressor stimulation, as deduced from **Figure 5**. The 48 h duration was chosen to allow sufficient time for potential

molecular changes, including genetic transcription, mRNA production, protein translation, and transport to neuronal axons. 48 h gave ample time for the downstream consequences of rapamycin to occur and for the cells to be stable. Compared to the control, rapamycin had a greater ability to decrease reactive oxygen stress levels in trials that were administered in advance with a greater exposure time, rather than at the time of being introduced later with a shorter exposure time. From this, it was decided to pretreat the neurons 48 h prior to maximize optimal protective effects.

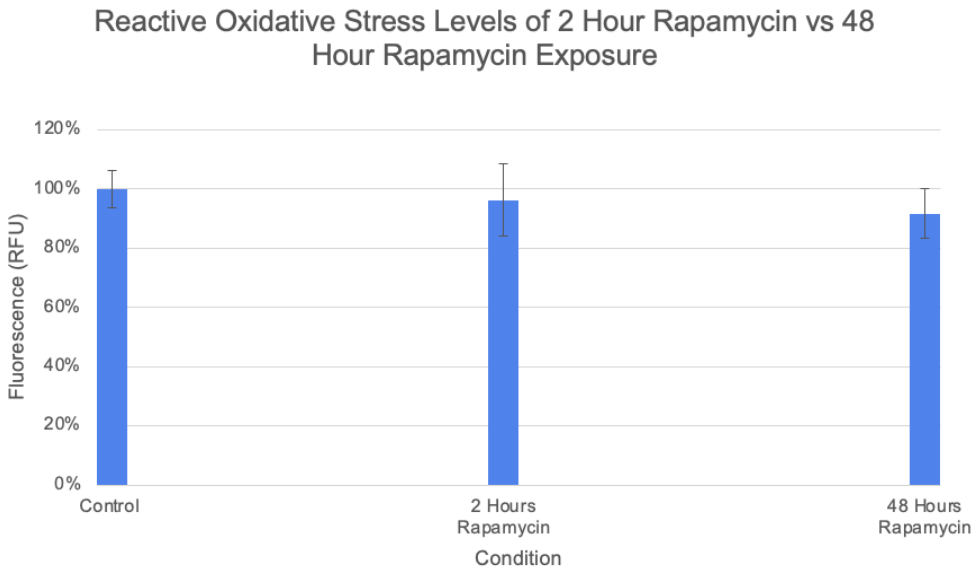

Figure 8. Detection of Relative Mean Fluorescence of 96 Well Plate of Rapamycin Over Immediate and Long Exposure Time in MitoSOX Red Reactive Oxidative Stress Assay obtained through well scan. 2-week old neurons were exposed to rapamycin administered 2 h prior to reading and 48 h prior to reading. Reactive oxidative stress levels were measured using MitoSOX Red fluorescence. Fluorescence intensity acts as an indicator of oxidative stress as a result of exposure time to rapamycin. Conditions included 10 wells XM media as the control, 10

wells rapamycin at 2 h in advance, and 10 wells treated with rapamycin 48 h in advance. Neurons were not subjected to any condition of stress.

Given that rapamycin exposure time variance had no stressor applied, it was concluded that rapamycin by itself did not cause reactive oxidative stress, as compared to the control value, as fluorescence values decreased seen in **Figure 8.** Since a 48 h exposure of rapamycin alone further decreased fluorescence values beyond the 2 h exposure condition, this revealed that pretreatment 48 h in advance could be beneficial to the neurons.

Table 1. Mean Fluorescence Values of 96 Well Plate of Rapamycin Over Immediate and Long Exposure Time in MitoSOX Red Reactive Oxidative Stress Assay

Condition	**Fluorescence (RFU)** (Mean +/- SD)
Control	100% (+/- 6%)
2 Hours Rapamycin	96% (+/- 12%)
48 Hours Rapamycin	92% (+/- 8%)

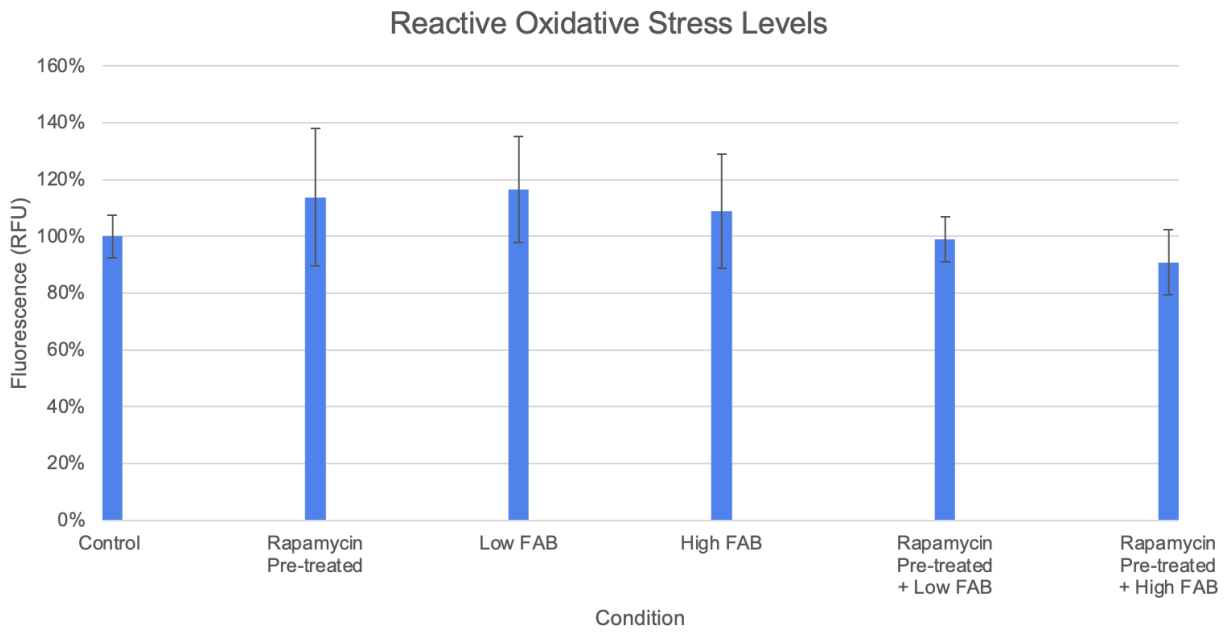

Figure 9. Mean Fluorescence of 96 Well Plate of MitoSOX Red Reactive Oxidative Stress Assay obtained through well scan. 1.5-week old neurons at a concentration of $3.5x10^6$ were exposed to stressors, and reactive oxidative stress levels were measured using MitoSOX Red fluorescence. Fluorescence intensity acts as an indicator of oxidative stress. Conditions included 8 wells XM media as the control, 8 wells pretreated rapamycin (48 hours in advance), 8 wells Low FAB concentration, 9 wells High FAB concentration, 9 wells pretrated rapamycin in combination with Low FAB, and 8 wells pretreated rapamycin in combination with High FAB. Neurons were subjected to the specified conditions for two hours, after which the dye was placed and read.

Fluorescence intensity, measured in relative fluorescence units (RFU) varied across condition treatments for the MitoSOX Red Reactive Oxidative Stress Assay. The control group exhibited a mean fluorescence of 100% (+/- 7%) when normalized to control conditions, serving as a baseline for comparison. Rapamycin pre-treated alone resulted in a slight increase, 114% (+/- 24%), as did low FAB exposure 116% (+/- 19%) and high FAB exposure 109% (+/- 20%).

In contrast, cells pre-treated with rapamycin in combination with FAB exposure showed a decreasing trend, seen in the modest reduction of the fluorescence units. Rapamycin pre-treated + low FAB resulted in fluorescence values lower than the control 99% (+/- 8%), while rapamycin pre-treated + high FAB exhibited the lowest fluorescence 91% (+/- 11%). To visualize the degree of protection rapamycin had, compared to FAB alone, it was seen that in the Low FAB concentration, FAB conditions yielded 116% fluorescence value, whereas rapamycin + Low FAB concentration reduced the fluorescence value to 99%, a 17% difference. Similarly, an 18% reduction is seen in the High FAB concentration as well, as with High FAB concentration alone fluorescence values of 109% were seen, followed by a 91% fluorescence value when rapamycin was introduced, revealing rapamycin's potential degree of protection.

The experiment was designed to conduct three trials of each parameter being tested: MitoSOX Red Reactive Oxidative Stress Assay, MTS Assay, and ICC, with all groups being normalized to the control. Although three dissections were performed, the success of the first two culture conditions faced contamination and neuronal growth issues, resulting in the collection of only one trial for the oxidative stress assay. Consequently, the results presented are only based on one trial from one assay, as additional trials were expected but could not be completed due to unforeseen neuronal culture conditions that were beyond control.

Table 2. Mean Fluorescence Values of 96 Well Plate of MitoSOX Red Reactive Oxidative Stress Assay

Condition	**Fluorescence (RFU)** (Mean +/- SD)
Control	100% (+/- 7%)
Rapamycin Pre-treated	114% (+/- 24%)
Low FAB	116% (+/- 19%)
High FAB	109% (+/- 20%)
Rapamycin Pre-treated + Low FAB	99% (+/- 8%)
Rapamycin Pre-treated + High FAB	91% (+/- 11%)

Although an MTS assay was conducted, no results were able to be extrapolated. 24 Well plate neurons were also prepared for ICC. The neurons were not adequately mature, therefore no results were collected.

Discussion:

AD remains one of the most complex and destructive neurodegenerative disorders, involving a pathogenesis of multiple interconnecting factors including amyloid beta accumulation, tau hyperphosphorylation, oxidative stress, mitochondrial dysfunction, and genetic predispositions. Despite many years of research and resources, no single hypothesis definitively elucidates the exact mechanism of the disease progression. Therefore, an understanding of the pathology and the development of a potential treatment is needed.

This study sought to investigate the neuroprotective effects of rapamycin in mitigating oxidative stress, a key contributing factor to AD pathology of neurodegeneration and cellular damage. Rapamycin, an antimicrobial agent, inhibits mTOR, a protein involved in cell growth and energy regulation. On the basis that age is a major risk factor seen in AD and rapamycin offers lifespan extending properties, it was thought that rapamycin could intervene in AD progression by addressing molecular changes associated with aging. It was hypothesized that rapamycin, when administered as a pre-treatment, would reduce the oxidative stress levels in neuronal cultures subjected to induced stress from FAB that simulate the diseased AD state.

The extent of reactive oxidative stress production was determined by the concentration of the stressor and quantified using the fluorescence plate reader in the MitoSOX Red Reactive Oxidative Stress Assay. With a known stressor concentration and exposure duration, the ability to measure reactive oxidative stress levels was confirmed, allowing for the comparison of treatments, such as rapamycin, in modulating reactive oxidative stress levels in the model system.

The results obtained from the MitoSOX Red Reactive Oxidative Stress Assay provide insight into the oxidative stress levels within neuronal cultures. It is also important to note that, due to the poor development conditions of the neurons, relatively few adequate wells were produced, therefore outlying data was discarded, offering a potential for bias to exist upon data selection, as up to 2 out of 10 wells were excluded. The selection was done on the basis of a value being 5x greater than the range of the other values within that same condition. This selection was done considering the restricted sample size, due to contamination issues, as an additional two trials were planned to provide more data but did not occur. The neurons were also stimulated on an accelerated time frame, as viability was decreasing, presenting a limited

window. Unusually high variability was also observed, especially in pre-treatment conditions, yielding high standard deviation values, possibly due to the reduced maturity of the neurons. Building on the previous oxidative stress analysis induced by hydrogen peroxide, optimized measurement methods were applied to evaluate rapamycin's impact. Mean fluorescence intensity serves as a key indicator of oxidative stress, as the MitoSOX Red dye specifically detects mitochondrial superoxide production. Fluorescence intensity varied across treatments, allowing assessment of the drug's effectiveness in mitigating oxidative stress, a key factor in neurodegeneration.

Because of the poor development of neurons, additional planned experiments to reinforce rapamycin's neuronal effects such as MTS assays for cell survival and ICC neurite growth were not able to be executed. Also due to the limited data from a single plate, statistical analysis was not performed. Instead, the focus remained on the comparison of trends in the quantified data without testing for statistical significant differences. While definitive conclusions cannot be accepted or rejected due to the study's pilot nature, observed trends offer valuable insight and suggest directions for further research.

Part I - Optimization of ROS Quantification in Cell Culture Model

The series of early trials confirmed that oxidative stress could be effectively measured using the fluorescence plate reader and MitoSOX Red, aided by the determination of optimal exposure times and stressor concentration. In deducing the varying exposure time, a constant 2 mM concentration of a known stressor, hydrogen peroxide, was determined through existing literature and utilized (Othman et al., 2015). The trends in **Figure 5** suggest that a short-term stimulation of neuronal cells is more representative of the AD model system, as prolonged stress

exceeds its relevant threshold of cell death at 24 hours seen in a previous study, going beyond the point of reactive oxidative stress production (Cevallos, 2022). These results indicate that exposure durations to the 2 mM H_2O_2 stressor longer than 2 hours surpassed the window of oxidative stress production, leading to cellular damage. This coincides with the previous works done to assess the effects of mild and severe oxidative stress on BACE1 expression, in which it was found that mild stress more closely resembles what happens in the aging brain and is more relevant to studying AD pathogenesis, as opposed to severe oxidative stress, risking significant cell loss (Tan et al., 2016). With knowledge of the optimal stressor exposure timeline from the consistency in detecting oxidative stress at 2 hours, the ability to measure reactive oxygen stress in a neuronal cell culture was established and implemented into the main study. **Figure 6** depicts various concentrations of a known ROS inducer, H_2O_2, created through serial dilution, used to produce and evaluate reactive oxidative stress levels. The 2 mM showed an increase compared to 0 mM, while 5 mM yielded a similar value. This suggests that the stressor did not have a concentration dependent effect on the production of reactive oxidative stress. Interestingly, FAB did exhibit the highest levels in that culture, confirming its ability to induce ROS in comparison to the hydrogen peroxide positive control, allowing for ROS quantification in the application of an AD model. H_2O_2 influences the levels of reactive oxidative stress produced in the system, however not in a concentration dependent manner. From the establishment of FAB's capacity to generate oxidative stress within this timeframe, the conditions of FAB at a 2 hour duration were determined to be used for effective ROS quantification in the subsequent rapamycin application.

Part II: Effect of Rapamcyin on Oxidative Stress within Cell Culture Model

Trials with different rapamycin exposure durations revealed that cells treated with a longer duration of the drug alone exhibited lower reactive oxidative stress than the control, as seen in **Figure 8**. Additionally, longer administration time resulted in a greater reduction in oxidative stress compared to the shorter 2 hour administration before measurement. This led to the thought that the protective properties rapamycin offered were most prominent when given more time with the neurons before the exposure of treatment. This coincides with rapamycin applied to immune-related models, such as rapamycin pre-treated and non pretreated arterial allografts. In a study using both human endothelial cells and a human-mouse chimeric model of allograft artery rejection, it was seen that pretreatment of the drug reduced rejection by increasing PD-L1/PD-L2 expression, lowering IL-6, and promoting regulatory T cells, ultimately leading to less damage (Wang et al., 2013). This suggests some connection between the timing of rapamycin administration and its protective properties, potentially through immune response enhancement, or a primed strengthening effect before exposure to the stressor. This immune response could have resulted from a cascade of molecular changes prompting an overall protective effect. Similarly, in neurons, prolonged rapamycin exposure may activate parallel protective pathways on a molecular level that reduce oxidative stress, possibly explaining the decrease in stress levels of the longer rapamycin exposure condition. Based on these findings, rapamycin administered 48 hours in advance as a form of pretreatment was thought to be beneficial for conducting the MitoSOX Red Reactive Oxidative Stress Assay in order to view the most optimal neuroprotective effects against the FAB stressor within our model.

Rapamycin's Counter Increase in ROS

The same condition of rapamycin administered 48 hours in advance as a form of pretreatment produced different results in two separate cultures, with one decreasing oxidative stress and the other later increasing oxidative stress. The discrepancy between pre-treated rapamycin's decrease in oxidative stress in **Figure 8** and the increase in oxidative stress in **Figure 9** is likely due to developmental differences of the neuronal state. Accounting for the poor development of the cells, variability with pretreatment conditions could have produced the high standard deviation seen in **Table 2**. Conducting more consistent trials with adequate neurons could help address this issue and determine whether rapamycin ultimately has a positive or negative effect in non-diseased neurons.

Compared to the control group, pre-treated rapamycin alone raised reactive oxidative stress values by roughly 14%, suggesting that rapamycin can increase stress in the absence of existing AD pathology. This can be explained by the fact that the neurons were in a healthy condition, subjected to no AD simulation and molecular changes as a result of aging. Under such scenarios, mTOR is thought to function optimally, regulating cell growth processes and metabolism in non diseased AD brains. When rapamycin inhibits mTOR under these specific circumstances, it may disrupt this cellular regulation balance, leading to the observed increase in harmful oxidative stress seen in **Figure 9**. It was interesting to note that the results of rapamycin administered alone as a form of pretreatment contrasted with the trends shown in **Figure 8**. Alternatively, previous work shows that ROS can be involved in growth and proliferation in certain contexts. For instance, ROS produced as a result of aerobic exercise are found to be advantageous and offer restorative properties through counterregulatory mechanisms (Jîtcă et al., 2022). In moderate amounts, oxidative stress may even have a strengthening effect on cells,

potentially explaining the observed impact of rapamycin, presented in **Figure 9.** It is interesting to consider that in contexts where oxidative stress may be healthy, rapamycin could potentially activate a growth mechanism, with oxidative stress emerging as a helpful byproduct.

To determine whether the oxidative stress observed in the neurons was ultimately harmful or beneficial to the neurons, the MTS Assay for cell survival and ICC for neuronal growth and microtubule stability would have been employed. Unfortunately, due to poor neuronal development, such assays were not conducted. However, if the increase in oxidative stress was seen to also coincide with positive values of MTS indications of metabolism, beneficial rapamycin properties would have been suggested. If the increase in oxidative stress was seen to also correlate with large intensities of microtubule stability, neurite growth could suggest rapamycin also would have protective effects.

The increase in harmful reactive oxygen stress suggests that for therapeutic applications, rapamycin should be administered closer to the age of peak AD prevalence, just before one approaches the onset of the disease. Unfortunately, this approach faces limitations due to a complex interplay of timing, safety concerns of invasive techniques, variability among patients, and the difficulty of identifying reliable biomarkers early, as initial symptoms often present in a subtle manner. The increase in reactive oxygen stress levels produced by pretreated rapamycin alone raises caution about the potential adverse effects when administered too early in life, without the immediate subsequent diseased state pathology. Although previous studies have not looked into the harmful effects of rapamycin being administered too early, studies have evaluated the damaging effects of rapamycin and risks that may exist.

Rapamycin's effectiveness is said to depend on the lysosomal degradative capacity of one's brain as explored in a study evaluating the potential dual response of the drug (Carosi et

al., 2023). It was found that since lysosome function is key to clearing toxic proteins such as AB, those with a decreased capacity to clear out the proteins exhibited a rapamycin-induced autophagy stress, exacerbating amyloid plaque rather than alleviating it. In contrast, brains with higher lysosomal function benefited from rapamycin's ability to decrease stress though means of autophagy enhancement. Future considerations should be taken into account based on the timing of rapamycin administration and the status of lysosomal capability of the patient, in order to prevent unwanted rapamycin effects that lead to higher stress.

In addition, rapamycin's dual opposing effect was demonstrated in another study analyzing kidney diseases, as cells of the glomerulus faced worsening damage with excess protein in urine, however, a nephro-protective effect within a chronic inflammatory tubulointerstitial model (Torras et al., 2009). As the latter outcome mirrors long term inflammation of the tubules, a similarity can be seen from the results obtained in **Figure 9**, in which rapamycin offers a protective effect only in the presence of disease.

Effectiveness of FAB Stressors

In comparison to the control, both the stressor conditions were successful in inducing an AD like state, supporting that FAB mimicked AB components to establish the disease model. The Low FAB concentration produced reactive oxidative stress levels that were approximately 16% higher than the control, whereas the High FAB concentration yielded a slightly smaller increase of 9% higher than the control. However, with high standard deviation of 19% and 20%, the variability suggests that repeated trials could yield overlapping results, making the difference between the two concentrations statistically insignificant, with equal variation being likely.

This Low concentration of FAB could have produced more stress because it provided sufficient components that replicated the disease pathology, without overwhelming the neurons.

In contrast, a higher concentration of stressors could alter the mitochondria's tolerance of the stress. A study examining the effects of the mitochondrial stressor rotenone in rat retinal cells found that mitochondrial dysfunction can lead to endoplasmic reticulum stress and glycogen synthase kinase-3-β activation, in a concentration and duration dependent manner (Han et al., 2014). This suggests that at lower concentrations, stressors may initiate adaptive cellular responses, allowing neurons to tolerate and respond to oxidative stress without immediate dysfunction, whereas higher stressor concentrations overwhelm mitochondria resistance, triggering cellular damage.

Similar to findings in the effects of mild and severe oxidative stress on BACE1 expression, where mild stress more closely mirrors the conditions of the aging brain and is more relevant for studying AD pathogenesis, severe oxidative stress, by contrast, poses a risk of significant cell loss (Tan et al., 2016). While both Low and High FAB conditions induced stress, Low FAB potentially had more of an effect due to a higher mitochondrial tolerance to the low concentration, as opposed to the High FAB concentration. However, considering the variability in equally high standard deviations of the two conditions, the variability suggests that the difference between the two concentrations would be relatively similar in future subsequent trials. This could be verified by MitoSOX Red Reactive Oxidative Stress Assays with consistently developed neurons, reinforced by both MTS survival assay and the ICC staining visualization.

Effect of Rapamycin on Modulating Stress

In order to assess rapamycin's effectiveness in combating the oxidative stress produced, the conditions in which pretreated rapamycin was combined with each level of stressors were compared. When evaluating the Low FAB to the Rapamycin Pre-treated + Low FAB, the applied treatment decreased oxidative stress levels by a 17% reduction. The High FAB conditions

experienced a decrease of 18% reduction. These values were calculated by subtracting the decreased relative fluorescence of the Rapamycin Pre-treated + FAB condition from the corresponding concentration of just FAB alone. The relatively similar values of reduction suggest that rapamycin does have potential to decrease reactive oxidative stress, under conditions in which a disease pathology follows, regardless of stressor severity concentration. By doing so, this implies that rapamycin's interaction with the mTOR pathway in relation to AD does play a role. This finding supports studies proposing that rapamycin has an ability to enhance autophagy on age-related oxidative stress and a number of other factors such apoptotic cell death, and markers of neurodegeneration, by inhibiting mTOR and facilitating the clearing of harmful AD proteins (Singh et al., 2019). Trends seen from the reactive oxidative stress assay show promise in rapamycin having a protective effect, as both conditions exhibited a decrease of 17-18% in oxidative stress. This trend shows that reduction is unlikely to be random, implying the involvement of possible underlying biological processes. However, future subsequent trials are needed to further support this theory. In addition, given the multifactorial AD pathogenesis, a multifactorial treatment may also be needed, allowing rapamycin to be a starting point.

Limitations and Improvements:

The results demonstrated that rapamycin pretreatment, particularly in combination with High and Low concentrations of FAB, exhibited a reduction in reactive oxidative stress levels compared to the control, suggesting a potential protective effect. However, given the limitations of the study, including a single successful trial due to contamination and growth issues, further investigation is required to validate these findings. Although these results suggest a potential neuroprotective effect of rapamycin, the study faced limitations that prevent definitive conclusions being drawn. MTS was anticipated to be performed, however the MTS had no

variability because there were not enough developed cells, yielding similar values that were too similar and too low. ICC was also anticipated to be performed, however the neurons were not developing and were not mature enough to be visualized and stained. Improvements to this study in the future would consist of implementing improvements to experimental methods, including optimizing neuronal culture conditions using fresh reagents, and possibly a stronger growth factor or supplement to ensure successful cell conditions for the MTS assay and ICC staining.

Future Line of Work and Expectations:

Execution of several MitoSOX Red Reactive Oxidative Stress Assays, MTS assays, and ICC stainings could reinforce the potential therapeutic benefits of rapamycin in the disease model and expand upon several parameters, such as microtubule stability and overall neuronal health. If rapamycin does in fact exhibit protective properties, alleviating the amount of reactive oxygen stress levels produced, it would be expected that conditions with rapamycin administered as a pretreatment would display higher metabolic activity functions within the MTS assay and display greater microtubule stability staining in the ICC, compared to the stimulated cells with stressors alone. This could expand upon the effects of mitigating the molecular changes in the aging process, possibly presenting results overall and in other areas of cellular function. As seen in a study analyzing primary cortical neurons and human astrocytes, rapamycin was found to increase neuronal survival and viability by reducing inflammation and astrocyte proliferation following the events of a spinal cord injury (Goldshmit et al., 2015). This reinforces the line of rapamycin providing an overall strengthening benefit to neuronal health, expanding to other areas of the inflammation process.

Expansion of this study could entail implementing the use of additional markers of oxidative stress and neurodegeneration, such as lipid peroxidation by-products like

malondialdehyde, 4-hydroxy-2-nonenal, and acrolein, to better assess rapamycin's effects (Singh et al., 2010). By exploring other oxidative stress indicators, the results obtained in this study using the MitoSOX Red Reactive Oxidative Stress Assay could be reinforced in response to rapamycin. Further, it would be interesting to see if alternative ways of promoting cellular clearance and autophagy have an effect as well. Finally, methods of strengthening neuronal mitochondria could also be useful, as mitochondria are a primary source of reactive oxidative stress. Previous studies have looked into mitochondrial health, as the organelle provides cellular energy necessary for neuronal activity and survival, with signs of dysfunction also seen in stages of AD (Ankarcrona et al., 2010).

Conclusion and Broad Application

In summary, an exploration of rapamycin's potential as a therapeutic agent to mitigate the pathology of AD was conducted, specifically through means of analyzing reactive oxidative stress produced. This logic was arrived at through evaluating common risk factors present in AD, particularly focusing on increased age, and reasoning that the process has some mechanistic involvement in the disease pathogenesis. A short term exposure was needed to observe the effects of reactive oxidative stress in that moment in time, before cell death occurred. The corresponding survival and neurite growth would be then analyzed, in conjunction to the effects and levels of various reactive oxidative stress produced, to confirm positive or negative effects of rapamycin. However, contamination and neuronal growth issues compromised two of the three planned trials, leaving only one successful oxidative stress assay. Additionally, while an MTS assay was prepared, insufficient neuronal development resulted in values that were too low and uniform across all conditions, preventing meaningful interpretation. The ICC assay was also unsuccessful due to inadequate neuronal maturity. Given these limitations, the findings of this

study cannot be accepted or rejected with confidence, but they do provide a valuable foundation for future investigations.

Interestingly, the obtained results suggest that rapamycin's neuroprotective effects may be more pronounced when applied as pre-treatment in combination with FAB exposure, supporting its potential role in modulating oxidative stress in AD. However, additional trials are necessary to confirm such trends and further investigate rapamycin's mechanism of action, and if the long term stress levels are ultimately beneficial or harmful. As reduced levels of oxidative stress were observed in conditions that combined each concentration of the stressor with pretreated rapamycin, this finding reinforces its potential effectiveness in AD pathology. It further contributes to the growing body of evidence supporting mTOR inhibition's mechanistic role in this context.

Extending beyond the scope of this study, broader applications include better understanding the role of oxidative stress in AD pathogenesis, which represents a key avenue in therapeutic intervention. The results contribute to the growing accumulation of research exploring rapamycin's therapeutic potential, reinforcing the need for a multifaceted approach to AD treatment. Further exploration of mTOR inhibition, combined with other forms of neuroprotective strategies, may be beneficial, offering additional advantages in modifying AD pathology.

Furthermore, these findings reinforce the need for continued investigation in experimental models, which can then be translated into clinical applications, aimed at identifying effective AD treatments in the future. Beyond AD research, adopting alternative approaches, beyond solely targeting the disease's root causes, may facilitate the discovery of viable treatment options, bypassing stumbles of unknown knowledge gaps. By demonstrating rapamycin's

potential in reducing oxidative stress, the research presented highlights the importance of targeting molecular changes associated with the aging process to counteract the mechanistic pathology of AD.

Given the increasing prevalence of AD and the absence of disease-altering treatments available, identifying effective interventions is imperative. As lifespan increases due to advancements in treating other diseases, greater emphasis should be placed on AD research and its implications for the aging population. If successful in providing neuroprotective properties, rapamycin could further aid in other neurodegenerative diseases as well, such as Parkinson's or Huntington's disease.

From a societal standpoint, this research holds significance for aging and quality of life, as discovering potential treatments can enhance cognitive health and could improve long-term well being for older populations. If rapamycin shows promise in demonstrating clinical efficacy, it could alleviate burdens on patients, caregivers, families, and healthcare systems. Moreover, if FDA-approved for AD purposes, rapamycin could address financial disparities in treatment accessibility, unlike monoclonal antibodies that cost tens of thousands annually, making them largely inaccessible to many patients. Since rapamycin is available as its generic form Sirolimus, originally developed as an immunosuppressant, it presents a more feasible option.

The trends seen in this pilot study propose the groundwork for future investigations into rapamycin's long-term effects and possible synergies with other neuroprotective agents. Further exploration into its mechanistic pathway interaction with the AD pathology could enhance the understanding of its role in aging and neurodegeneration, paving the way for a more targeted and refined interventional response. Ultimately rapamycin has the potential to advance neurodegenerative disease treatment, aging research, and healthcare outcomes. Further

exploration of the drug's inherent properties will make significant steps forward in improving patient outcomes at large, while simultaneously rethinking the way disease treatment research is carried out.

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