Drew University

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In Vitro Exploration of Androgen Receptor Mediated Neuroprotection of Testosterone in an Oxidative Stress Model of Alzheimer's Disease.

A Thesis in Neuroscience

by

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder commonly understood to be affected by age, oxidative stress, Tau hyperphosphorylation and β-Amyloid plaques. Recent literature has begun to explore the impact of the sex hormones on neuron resistance to oxidative stress, and the impact that those hormones have on neuron viability under Alzheimer's disease like conditions. The literature surrounding this hypothesis has been largely focused on the impacts of estradiol, and research into the effects of testosterone is sparing. This study aims to build upon the literature exploring testosterone's *in vitro* neuroprotective effects. These experiments explored testosterone's impact on neuron viability and morphology under the Ferrous-Amlyoid-Butionine (FAB) *in vitro* cell model of AD. This model system generates reactive oxygen species, which cause oxidative stress on neurons and is an integral component of sporadic AD. Neuron viability and morphology changes were analyzed via MTS and immunocytochemistry. Our findings indicate that testosterone caused an increase in cell metabolism in unstressed cells, and that it promoted neurons to group together in culture more frequently. Testosterone had no significant effect on cells when stressed with FAB, and the clustered pattern of cells was still observed in stressed cells. We further attempted to assess the pathway by which the actions of testosterone occur by inhibiting the estrogen receptor with tamoxifen. No significant change in cell viability was observed as a result of tamoxifen treatment. Cotreatment of neurons with testosterone and tamoxifen resulted in a statistically significant rescue of cell viability, suggesting that androgen receptor activation results in neuroprotection from oxidative stress and β-Amyloid. These

experiments, when considered with the broader literature, show that testosterone is involved in neuroprotective pathways in neurons. Clinically, this suggests that the loss of testosterone may play an important role in the incidence and pathology of AD in aging men.

Contents

Introduction

Alzheimer's Disease Epidemiology and Brief Overview

Alzheimer's disease (AD) is a progressive neurodegenerative disease which currently has no cure and few treatment options (Crous-Bou et al. 2017; Vaz and Silvestre 2020; Alzheimer's Association 2021). AD is accounts for 60% to 80% of all dementias and is the fifth leading cause of death in Americans aged 65 or older (Alzheimer's Association 2021). The worldwide prevalence of AD is predicted to affect over 150 million individuals worldwide by 2050 (Vaz and Silvestre 2020). With populations living longer, and a general trend of decline in other chronic illnesses such as heart disease, increases in instance of AD and other age-related dementias must be expected.

The increase in those affected by age-related dementias results in a subsequent increase in the number of seniors unable to care for themselves. In addition to the physical, emotional and psychological effects AD has on those affected by it and their loved ones, AD is estimated to cost almost $500 billion annually in healthcare related expenses (Wells and Budson 2018). In addition to this cost, unpaid care equating to approximately $257 billion for individuals with AD from family and friends was provided in 2020 (Alzheimer's Association 2021). Diagnosis also represents a significant increase in out-of-pocket healthcare costs for families. A net increase of $137,280 lifetime medical costs is predicted to be incurred on average by someone diagnosed with AD, accounting for unpaid care provided by family members (Jutkowitz et al. 2017). Of

these additional expenses, 86% of this cost is paid by families in addition to any unpaid care they provide (Jutkowitz et al. 2017). This immense socioeconomic burden supports AD as a serious and significant public health concern.

AD is generally classified into two categories: late-onset, or sporadic AD, and early-onset, or genetically-linked AD. The primary risk factor identified for developing sporadic AD is age, although it is not a cause of AD (Apostolova 2016). Cardiovascular risk factors, moderate or severe traumatic brain injuries, family history and low educational and occupational attainment are other major risk factors (Apostolova 2016). The most prevalent genetic predisposition for sporadic AD is apolipoprotein (APOE) ε4 allele, which may present up to a 12-fold increased risk of developing AD in homozygous individuals (Michaelson 2014; Loy et al. 2014). Patients with early-onset AD will be autosomal dominant for amyloid precursor protein, Presenilin-1, and Presenilin-2, and are more likely to experience atypical symptoms including seizures and pseudobulbar palsy (Mendez 2019). Sporadic AD comprises of about 95% of all cases (Mendez 2019, Alzheimer's Association 2021).

The clinical presentation of AD can be varied; however, the main manifestation of the disease is memory loss. Patients may also experience a large variety of cognitive impairments affecting working memory, executive function, mood, and language. Symptoms will digress over time and new symptoms may appear later into the progression of the disease. As disease progression is more aggressive, symptoms will consequently increase.

Hallmarks of Alzheimer's Disease

Definitive diagnosis of AD requires the examination of brain tissue post-mortem. However, a differential diagnosis of dementia due to AD can be assessed by a physician via a battery of memory and cognitive tests. The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, and the National Institute on Aging-Alzheimer's Association outline the clinical criteria required for the diagnoses of mild cognitive impairment or major cognitive impairment, the broad categories that encompass dementia conditions (Apostolova 2016). In addition to cognitive tests and symptomatic diagnoses, the 2011 revision to the National Institute on Aging-Alzheimer's Association criteria include the use of disease biomarker screening for diagnosis (Apostolova 2016; Weller and Budson 2018). Biomarker screening includes the use of diagnostic imaging and evaluating cerebrospinal fluid (CSF) for β-amyloid and hyperphosphorylated tau protein (Weller and Budson 2018). These diagnostic screening methods allow for improved differential diagnosis and present an opportunity for earlier, more accurate diagnosis, and earlier treatment.

β-Amyloid Plaques

The first hallmark of AD is the aggregation of β-amyloid (Aβ) peptide into oligomeric plaques. Aβ is the sequential cleavage product of APP by β-secretase and γ-secretase into an extracellular 37-42 amino acid peptide (Xu et al. 2016). APP can also be cleaved by ADAM10, the primary α-secretase in neurons, which will cleave it into a

soluble fragment (Manzine et al. 2019). The insoluble 1-42 amino acid Aβ peptide fragments have been reported to be the primary component of the characteristic plaques of AD, and Hardy and Higgins (1992) hypothesized them to be the major causative agent of AD. Other studies into the functions of Aβ within the brain and ineffective AD treatment strategies that deplete Aβ have contradicted this hypothesis (Brothers et al. 2018).

Differential cleavage of APP by competing α- and β-secretases respectively produce the soluble 40 amino acid Aβ peptide (Aβ40) and the insoluble 42 amino acid Aβ (Aβ42) (Xu et al. 2016). Aβ is typically cleared from the brain by CSF and the vascular system, and Aβ42 in CSF can be used as a biomarker for AD (Perrin et al. 2009). Aβ42 binding molecules can also be administered and used in correlation with positron emission tomography for visualizing plaque formation in patients (Perrin et al. 2009). Recent research into the physiological role of Aβ has indicated that it is involved in several brain functions such as memory consolidation (Brothers et al. 2018). Oligomerized Aβ has also been suggested to aggregate extracellularly to "seal the leak" following a break in the blood brain barrier (Brothers et al. 2018). Currently the physiological and pathological roles of Aβ in the brain and in AD are still unclear. However, Aβ oligomerization is still largely considered to be a predominate causative agent in AD pathology, alongside the formation of neurofibrillary tangles.

Neurofibrillary Tangles

Neuroplasticity is essential in learning and memory. Dynamic microtubule restructuring is an essential component of neuroplasticity. An observed hallmark of AD is hyperphosphorylation of human tau. Tau is a microtubule associated structural protein encoded by the *MAPT* gene on chromosome 17 (Guo et al. 2017). Tau is a developmentally regulated gene product, expressed in six major isoforms corresponding to different neuron types and development stages (Avila et al. 2004; Guo et al. 2017; Pîrşcoveanu et al. 2017). These isoforms are regulated via alternative nuclear RNA splicing of exons 2, 3 and 10 (Pîrşcoveanu et al. 2017). Isoforms differ in N-terminal insert number, microtubule binding domain number and protein size (Avila et al. 2004; Guo et al. 2017; Pîrşcoveanu et al. 2017). Biochemical studies of human tau have identified it to be natively unfolded and contain few well-defined secondary structures (Guo 2017). Human tau is suggested to be one of several microtubule-associated proteins responsible for maintaining microtubule stability (MAP1A, MAP1B, MAP2, tau) and therefore influencing neuron morphology (Avila et al. 2004). In healthy neurons, it is observed to primarily be expressed in axons and is subject to several post-translational modifications affecting microtubule binding, including glycosylation, glycation, and phosphorylation (Avila et al. 2004; Guo et al. 2017; Pîrşcoveanu et al. 2017). Tau dysregulation and abnormal modification are associated with several pathologies, and its effects are referred to as tauopathies (Avila et al. 2004).

In AD, the primary tauopathy observed is hyperphosphorylation resulting in a loss of microtubule binding ability and tau aggregation. Microtubule binding is regulated by

phosphorylation of tau and several kinases have been suggested to be responsible for regulating phosphorylation at various levels, including GSK3β, Cdk5, PKA, and MARK (Johnson and Stoothoff 2004; Iqubal et al. 2005). However, the molecular mechanisms responsible for regulation of tau and the pathological mechanism of hyperphosphorylation are not well established (Johnson and Stoothoff 2004; Avila 2004; Pîrşcoveanu et al. 2017). One important aspect of hyperphosphorylation tauopathy is a lack of ubiquitin reactivity and hyperphosphorylated tau degradation (Pîrşcoveanu et al. 2017). The absence of tau degradation and abnormal hyperphosphorylation allow for tau to aggregate into tangles of twisted straight ribbons, paired helical filaments or straight filaments (Pîrşcoveanu et al. 2017). These tangles have been believed to be neurotoxic, however recent studies demonstrating survival of neurons containing tangles in P301L tau-expressing transgenic mice suggest that neurotoxicity of tangles may vary by tau isoform (Guo et al. 2017). The vast range of observed tauopathies and lack of consensus on pathological mechanism of tau, despite a plethora of literature exploring it, support continued research to better understand tauopathy mechanisms in AD and other tau-related disorders.

Neurodegeneration

A final hallmark of AD is cerebral atrophy (Pini et al. 2016). The progressive changes in in physiological conditions observed over the course of AD are accompanied by cell death, and therefore a reduction in cerebral volume. Over the course of the

disease and even before the onset of symptoms, atrophy can be observed via volumetric MRI in the medial temporal lobes and hippocampus (Scahill et al. 2002). Cerebral atrophy, loss of synapses and cell death helps explain the cause of cognitive and memory deficits observed in AD patients.

One hypothesized cause of this atrophy is dysregulation of neurotransmitters. Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system (Olloquequi et al. 2018). Glutamate activity is facilitated by ionotropic and metabotropic receptors, which result in depolarization of the post-synaptic neuron (Olloquequi et al. 2018). Synaptic concentration of glutamate is regulated by the glutamate/glutamine cycle (Conway 2020). Astrocytes associated with the glutaminergic synapse remove glutamate from the synapse via glutamate specific transporters (Conway 2020). This rapid clearance from the synapse prevents toxicity from overstimulation of post-synaptic cells. Glutamate excitotoxicity is suggested to be mediated by dysregulation of intracellular calcium (Ca^{2+}) via the N-methyl-D-aspartate (NMDA) receptor (Zádori et al. 2018; Olloquequi et al. 2018; Armada-Moreira et al. 2020). Dysregulation of intracellular Ca^{2+} concentration results in the formation of a non-specific pore in the inner mitochondrial membrane, causing a loss of the pH gradient required for oxidative phosphorylation and ATP synthesis (Armada-Moreira et al. 2020). Aβ is also hypothesized to contribute to excitotoxicity via the NMDA receptor (Zádori et al. 2018). Aβ is suggested to promote an increase of extracellular glutamate and downregulate glutamate clearance by astrocytes (Zádori et al. 2018). Disruption of the

intracellular Ca^{2+} concentration in neurons is also proposed to cause oxidative stress via increasing production of radical oxygen species (ROS) (Olloquequi et al. 2018).

Oxidative stress results in damage to lipids, proteins and nucleic acids via the generation of ROS and superoxide radicals (Zádori et al. 2018; Olloquequi et al. 2018; Armada-Moreira et al. 2020). These compounds are produced by side reactions during oxidative phosphorylation in mitochondria by complexes I and III (Tönnies et al. 2017, Cheignon et al. 2018). In order to control the proliferation of ROS and protect cell components from oxidation, cells contain endogenous antioxidant enzymes (Tönnies et al. 2017, Cheignon et al. 2018; Jiao et al. 2018). These enzymes scavenge ROS and prevent cell damage from radical oxidization of lipid membranes and genomic material (Tönnies et al. 2017). In the context of AD, Aβ has been demonstrated to increase oxidative damage *in vitro* using SH-SY5Y neuroblastoma cells (Singh et al. 2017). This study by Singh et al. indicates that Aβ helped induce cell death and inhibited neuron growth which was rescued with rapamycin (2017). This is significant, as other studies have suggested that rapamycin promotes upregulation of antioxidant gene transcription, namely Nuclear erythroid-2-related factor 2 (Nrf2) (Calap-Quintana et al. 2015). ROS present as a lucrative therapeutic target for intervention in AD, however further research of these pathways is necessary (Singh et al. 2017; Jiao et al. 2018).

Testosterone

Recent literature has begun to investigate the role that sex steroids have in neurodegenerative disease. Of this existing research, an overwhelming portion of the focus of study is on estrogens (Fisher et al. 2018; Zhu et al. 2021). Testosterone has begun to be investigated and is suggested to have neuroprotective activity.

Androgens are steroid hormones which typically exert genomic effects via binding to the androgen receptor (AR). Testosterone is the principal androgen expressed throughout development and adult life. It is synthesized from pregnenolone, which in turn is synthesized from cholesterol, or derived from other steroids, such as deoxycortone, in both males and females. In males, testosterone is primarily synthesized in the testes in Leydig cells, and in the ovaries of females (Celec et al. 2015). In addition, a small amount of testosterone is produced in the cortex of the adrenal glands of both sexes. In males, testosterone produced by fetal Leydig cells is responsible for promoting growth of the Wolffian duct, and maintaining *in utero* development of male sexual organs in combination with other hormones (Siiteri and Wilson 1974). Levels of testosterone in both sexes increases at puberty and declines as age increases (Davis and Wahlin-Jacobsen 2015; Feldman et al. 2002).

In theme with development of the sex organs, sexual differentiation of the brain has been classically suggested to be caused via sex hormone activity (Nadler 1968; McCarthy and Arnold 2011; Wu and Shah 2011). Throughout development, testosterone, acting through the AR, and estradiol synthesized in neurons from testosterone by

aromatase, have been demonstrated to impact brain organization and differentiation into male and female patterns (McCarthy and Arnold 2011). Cell culture models have also suggested that testosterone promotes neuron growth in sexually dimorphic nuclei, and accelerates arborization and dendritic outgrowth (Reddy et al. 2015). Behavioral patterns, as well as broader physiological effects, have been demonstrated to be at least partially dependent on sex hormone activity and upstream cascade changes (Nadler 1968; Wu and Shah 2011). These patterns do not represent unique dimorphous circuits, only that anatomical differences of dimorphous nuclei and brain regions supporting sex-specific responses (McCarthy and Arnold 2011). In addition, the classical understanding of sexual differentiation in the brain has recently been shifting to provide a greater focus on environmental and epigenetic factors supporting differentiation (McCarthy and Arnold 2011; Gegenhuber and Tollkuhn 2019). Current understanding of sexual differentiation is not limited hormonal and genomic effects, rather sexual differentiation is a cumulative effect of endogenous and exogenous factors promoting development.

Testosterone also exerts multiple effects on the body beyond sexual differentiation. Testosterone has been shown to promote increases in bone density and increased lean muscle mass (Rahman and Christian 2007; Tyagi et al. 2017). Its hypothesized impact on cognitive abilities is currently unclear and studies addressing cognitive effects of testosterone are controversial, but longitudinal testosterone replacement therapy studies have suggested that it promotes improved mood in hypogonadal men (Celec et al. 2015; Tyagi et al. 2017). There is evidence of testosterone having positive effects on maintaining synaptic volume in aged animals (Fattoretti et al.

2019). Data from a study by Fattoretti et al. suggests that testosterone may promote hippocampal neurogenesis and supports preservation of synapses in the hippocampus of aged animals (2019). This study, however, did not report any cognitive or memory related assays of the mice, limiting the conclusions able to be drawn from this study (Fattoretti et al. 2019). Although the current literature suggests that testosterone may promote beneficial effects on the aging brain, there is little conclusive evidence or consensus, supporting its further research. Additionally, there is little research exploring the effects of gradual decline in testosterone concentration.

The steady decline in bioavailable testosterone in older men has been well documented, and the decrease in testosterone in women over time has recently begun to be investigated (Feldman et al. 2002; Davis and Wahlin-Jacobsen 2015). The suggested beneficial effects of testosterone on the brain have prompted recent exploration of the relationship between testosterone and neurodegenerative disease. Much of this research has focused on identifying an association between testosterone and Aβ. Numerous *in vitro* and *in vivo* studies have suggested that testosterone is able to mitigate neurotoxicity from Aβ (Lau et al. 2014; Yao et al. 2017). In several studies, testosterone was identified to reduce synthesis of pathogenic Aβ fragments (Gouras et al. 2000; Wahjoepramono et al. 2008; Rosario et al. 2012). In another study, testosterone was demonstrated to protect against synaptic effects of Aβ (Lau et al. 2014). In this study by Lau et al., testosterone was identified to promote the integrity of synapses in primary hippocampal neurons stressed with Aβ via the AR, rather than an estrogen receptor pathway via the use of a competitive inhibitor of aromatase to prevent intracellular conversion of testosterone

(2014). Despite a growing body of research, the effects of testosterone within a model of AD are largely restricted to investigation of its interaction with Aβ. This focus, although beneficial, is insufficient to properly characterize the relationship between testosterone and neurodegeneration as observed in AD. Animal models are unable to remediate the holes of current *in vitro* research, as they rely on gonadectomized animals which are not comparable to the gradual loss of testosterone observed in an aging population (Wahjoepramono et al. 2008; Rosario et al. 2012). Attempts to remediate the issues of gonadectomized animals via exogenous testosterone dosed to replicate the gradual decline in testosterone observed in aging has not been published in the literature. Globally, the inability of model systems utilized in the literature to reproduce the gradual loss of testosterone and the subsequent upstream endocrine changes represents a need for extended development of these model systems. Furthermore, this underlines the importance of understanding the cellular effects and mechanisms of action within the question of how testosterone relates to AD. For this reason, *in vitro* models provide a very controlled and manipulable system by which the cellular mechanisms can be experimentally tested and understood. From this basal knowledge, *in vivo* models can be refined and organismal level effects can be identified. Despite their limitations, the use of these existing models, and broadening them to address multiple hallmarks of AD will allow for better identification of the relationship between testosterone and the pathology of AD at a cellular and organismal level.

Current Research

Although the pathological mechanisms of AD are not fully understood, it is essential that model systems of the disease encompass multiple physiological conditions observed in AD patients. A variety of *in vitro* and *in vivo* model systems are currently utilized in attempt to study both genetically linked and sporadic AD, including immortalized cell lines, primary cultures and transgenic mouse models. In attempt to overcome the limitations of a model system utilizing exclusively Aβ, or in conjunction with glutamate, this study utilizes the FAB oxidative stress model described by Lecaneu et al. (2006). This model attempts to represent multiple characteristics of the pathologic mechanisms of AD as they are currently understood. Ferrous sulfate generates ROS via catalyzing the Fenton reaction, buthionine sulfoximine inhibits synthesis of glutathione, an endogenous antioxidant, and contains Aβ in sufficient quantities to produce cellular effects without extracellular plaque formation (Lecaneu et al. 2006). By encompassing several neurotoxic pathways of AD, the FAB model can recreate two major hallmarks of AD, and serve as an efficient and manipulable way to model AD in *in vitro* primary neuron culture.

Several studies have hypothesized that testosterone plays a role in promoting cell growth and survival under stress (Reddy et al. 2013; Lau et al. 2014; Yao et al. 2017). These studies have explored *in vitro* potential rescue capacity of testosterone on neurons when in the presence of Aβ (Gouras et al. 2000). The primary flaw within this design is that these studies largely rely upon toxicity induced by Aβ, and do not account for other disease-causing factors, such as oxidative stress. In order to combat this issue and

thoroughly evaluate the relationship testosterone has in neurons within the context of AD it is important to continue this research while addressing this primary flaw. The existing literature addressing this question is somewhat sparring, further reinforcing the importance of identifying the role and mechanism of action of the hormone.

The current research seeks to explore the neuroprotective potential of testosterone within an oxidative stress model of sporadic AD. These experiments aim to explore and assess the hypothesis that physiologically relevant concentrations of testosterone promote increased resistance to oxidative stress, and a subsequent increase in cell survivability and viability. The potential gross pathway by which these neuroprotective effects, if any, are manifested will also be explored. This paper will explore the following hypotheses developed from a review of the available literature:

1. Testosterone will increase cell survival when in the presence of oxidative stress.
2. Global neuron size increases as a result of treatment with physiologically relevant concentrations of testosterone.
3. The genomic effects of testosterone on neurons under oxidative stress occur via the androgen receptor, not the estrogen receptor following intracellular transformation by aromatase.

Methods

Primary Neuron Culture

Primary neurons were cultured in 96 and 24-well clear, flat bottomed sterile cell culture plates (Greiner Bio-One CELLSTAR). Plates were prepared in sterile Class II, type A2 biological safety cabinets (NuAire), and treated with Poly-L-Lysine (brand, lot number) for 4-24 hours. Poly-L-Lysine solution was collected following treatment and reused. Plates were washed with sterilized distilled water for three, 15-minute washes. Washing water was removed from the wells between each wash via aspiration. Plating Media was added to the wells following the final wash and plates were stored in an incubator at 37 ℃, and 5% carbon dioxide (CO_2) until plating of collected cells.

Two liquid media, Plating Media and Growth Media, were used in the plating and maintenance of cell cultures. Plating Media is produced from Neurobasal (Thermo Fisher), Fetal Bovine Serum (Thermo Fisher), and Primocin (InvivoGen). Growth Media is produced from Neurobasal (Thermo Fisher), B-27 Supplement (50X) (Gibco), and Primocin (InvivoGen). Both media are lightly agitated, aliquoted into sterile 50 mL centrifuge tubes through a sterile Corning filter (0.22 µm, sterilizing, low protein binding), and stored at 20 ℃. Media is warmed in a 37 ℃ water bath prior to use for dissociation or dilution of dissected cells or feeding of cell cultures.

Primary cortical neurons harvested from E18 Sprague-Dawley rat embryos were harvested and plated for all experiments. Pregnant Sprague-Dawley rats sourced from Envigo were sacrificed via carbon dioxide asphyxiation. Embryos were immediately

dissected out and decapitated. Decapitated heads were maintained in Hank's Balanced Salt Solution (HBSS) on ice until dissection. The heads were dissected in cold HBSS, and the cortices were collected. The midbrain and hindbrain were discarded and cortical tissue was used in all experiments. Any red film tissue was designated as meninges and removed from the cortices, and any red tissue within the cortices was also removed and discarded. Meningeal tissue will disrupt primary cell plating and differentiation and is therefore discarded. Cortical tissue was dissociated in 3mL Trypsin and incubated for 5 minutes at 37 ℃. The dissociated cells were transferred to 3mL HBSS two times and washed at 37 ℃. After washing, the cells were transferred to Plating Media and mechanically dissociated using a flame polished glass Pasteur Pipette. The cells were counted using a hemocytometer, and diluted to a concentration of 1×10^6 cells/mL. Cells are plated and incubated in a sterile room. Following dissociation, cells are plated, fed and stimulated within a UV-sterilized Class II Biological Safety Cabinet wiped down with 70% ethanol. In each 96-well plate, 100 µL of diluted cell solution was plated in each of the 60 inner wells, plating 1×10^5 cells in each well. In the 24-well plates, 30 µL of diluted cell solution was plated in each well, plating 3×10^4 cells in each well. Plates are incubated at 37 ℃ for one hour following plating. The Plating Media is then aspirated and replaced with Growth Media. Cultures are then fed on a Monday, Wednesday, Friday feeding schedule. Feeding was performed by aspirating approximately 50% of the media, and the addition of 100 µL (96-well plates) or 550 µL (24-well plates). Cultures are maintained for 12 days before pre-treatment at 37 ℃ and

5% CO_2. Neurons were then pretreated for 2 days, then stimulated for 2 days before assaying.

Pre-Treatment and Stimulation

Testosterone and Tamoxifen were prepared for cell culture pre-treatment and stimulation via dissolution in dimethyl sulfoxide (DMSO). Testosterone stock aliquots of 100 µM were prepared and stored at -20 ℃. Tamoxifen stock aliquots of 134.6 µM, or 0.05 mg/mL, were prepared and stored at -20 ℃.

Cell culture plates were divided into 3 experimental groups and 3 control groups. The DMSO and FAB control groups were not pre-treated. The experimental group receiving treatment with testosterone and the testosterone control group were pretreated with testosterone diluted in Growth Media to a final concentration of 10 nM. The experimental group receiving treatment with Tamoxifen were pre-treated with Tamoxifen diluted in Growth Media to a final concentration of 50 ng/mL (13.5 nM). The experimental group receiving both testosterone and Tamoxifen was treated with a solution containing both testosterone and Tamoxifen at the aforementioned respective concentrations.

The FAB model system utilized for stimulation was adapted from Lecanu et al., 2006. Stock concentrations of 10 mM ferrous sulfate and 1 mM buthionine sulfoximine in Growth Media, and 100 µM $A\beta_{1-42}$ in sterile water. These stocks were then diluted in Growth Media to yield a final concentration of 100 µM ferrous sulfate, 50 µM buthionine

sulfoximine, and 500 nM Aβ. In this model system ferrous sulfate generates reactive oxygen species, buthionine sulfoximine inhibits endogenous antioxidants.

Cell Viability Assay

Cell viability and proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay using cell cultures in 96-well plates. In 6 mL of Growth Media, 1 mL of MTS was diluted. The media in each well of the 96-well plate was aspirated completely and 100 μL of the diluted solution was plated in each well. The plates were incubated for 1-4 hours at 37 ℃. MTS metabolism was assessed via transillumination imaging using an Amersham Imager 600 (General Electric Healthcare). This compound is metabolized into a colored formazan product by Pyruvate Dehydrogenase, allowing for quantification of cell survival via colorimetric assay (Cory et al. 1991). These images were subsequently processed using ImageQuest TL Array (v8.1), to generate numerical colorimetric data. The resulting data was calibrated to MTS solution plated in empty wells, then normalized to the control wells so that it can represent cell viability as a percent of the control. Two-way analysis of variance and independent samples T-Tests were conducted with IBM SPSS 26 to assess statistical significance. Independent samples T-Tests were performed with a corrected $\alpha=0.01$, to account for the increased number of assays performed.

Immunocytochemistry

The effects of testosterone on cell morphology were assessed via visualization of acetylated tubulin. Following stimulation, 24-well plates were fixed using 4% paraformaldehyde for 20 minutes at room temperature. Cell cultures would then be washed with phosphate buffered saline (PBS) three times for 3 minutes. Cells were then permeabilized by incubating for 10 minutes with 0.5% Triton X-100. Wells were briefly washed again with PBS. The primary monoclonal mouse anti-acetylated tubulin antibody (Sigma) was diluted 1:400 in PBS, then added to the wells and incubated between 2 and 5 hours at room temperature while shaking. The antibody was removed and the plates were briefly washed in PBS three times. Cells were then incubated with anti-mouse IgG conjugated to FITC, produced in goat (Sigma), following the same incubation procedure as the primary antibody. Following incubation, cells were washed briefly with PBS and plates were stored wrapped in parafilm at 4 °C until imaging.

Cell were visualized using a Zeiss LSM 510 Inverted Fluorescent Microscope (Hitech Instruments, Inc.) and images were captured using AxioVision Se64 Rel. 4.9.1 (Carl Zeiss Microimaging Inc.). FITC fluorescent dye, antibody conjugated to acetylated tubulin, was excited at 490 nm. Emission at approximately 525 nm was recorded. Approximately 10 images per condition were recorded at a magnification of 20x. Images were taken from randomly selected sections of wells and fluorescent exposure was minimized. Cell growth and stability was measured via acetylated tubulin antibody binding. Mean pixel illumination was measured using NIS-Elements Advanced Research (Nikon Instruments Inc.) software and data was normalized to a percentage of the control

in Microsoft Excel. Statistical significance was assessed by two-way ANOVA and independent samples T-Tests in IBM SPSS 26. Independent samples T-Tests were performed with a corrected $\alpha=0.01$, to account for the increased number of assays performed.

Results

Testosterone Promotes Increased Neuron Viability

The cellular effects of testosterone on *in vitro* primary neuron cultures were assessed. Viability was measured photometrically via MTS assay in 16-day old cultures. Cultures were stimulated with 10 nM testosterone for 4 days beginning on day 12. Cultures stimulated with FAB were treated for 2 days beginning on day 14. A two-way ANOVA indicated effects of testosterone F (1,236) = 5.302, $p<0.05$, and FAB F (1,236) = 184.651, $p<0.001$, on cell viability as indicated in **Figure 1**. No significant interaction between FAB and testosterone was observed F (1,236) = $p>0.05$. This suggests that testosterone did not help improve viability on cells stimulated with FAB. The effect of testosterone suggests that testosterone may promote increased cell metabolism in unstressed cells.

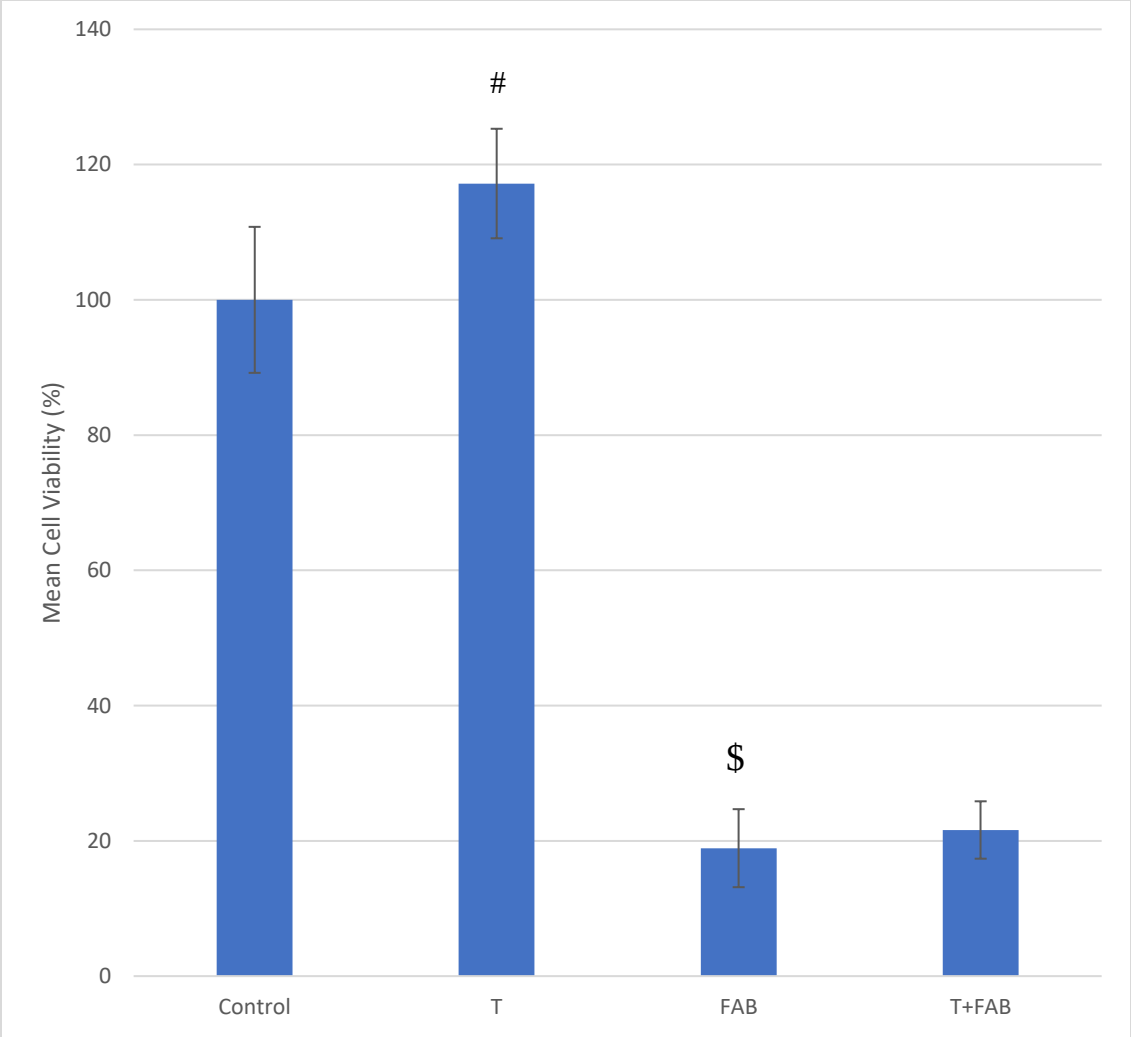

Figure 1: Testosterone improves neuron viability Cell viability as measured by MTS absorbance of cells treated with testosterone, designated as T, and stimulated with FAB. Cells were treated and stimulated for 48 hours. Data are mean ±SEM. N=40 for each condition. # denotes significant effect of T ($p<0.05$). $ denotes significant effect of FAB ($p<0.001$).

The observed effects of testosterone were further investigated and confirmed by immunocytochemistry. Cultures were fixed with paraformaldehyde and stained against acetylated tubulin. The average intensity of fluorescent images collected were recorded and assessed by two-way ANOVA (**Figure 2**). There was no main effect of testosterone

F (1,36) = 0.658, p>0.05. A main effect of FAB was observed *F* (1,36) = 7.454, p=0.01. An interaction between FAB and testosterone was not observed *F* (1,36) = 0.012, p>0.05. This result is similar to the results of viability assessed via MTS. It is notable that the FAB condition is less than one SEM of the control.

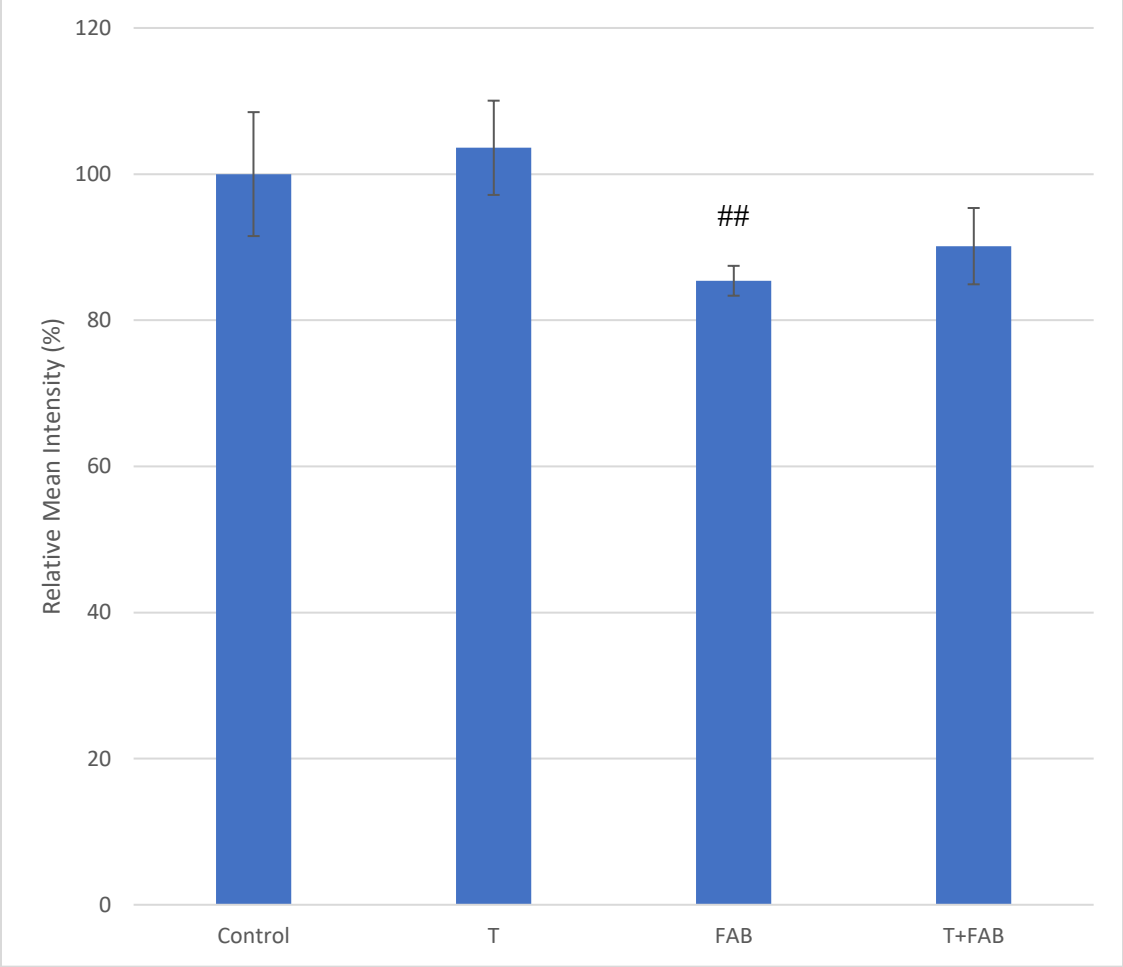

Figure 2: Testosterone and FAB do not statistically affect fluorescent labeling of acetylated tubulin Mean pixel intensities of randomly selected fluorescent images of cells immunostained for acetylated tubulin. Data are mean ±SEM. N=10 images for each condition. ## denotes significant effect of FAB (p<0.05).

Qualitative assessment of the images does reveal that FAB treated cells had less consistent fluorescence, suggesting less acetylated tubulin (**Figure 3C**). When compared to the controls (**Figure 3A**), neurons treated with testosterone (**Figure 3B**) appeared to have greater arborization, however the cells were often "clustered" together and few cells grew in isolation in any condition. Neurons treated with testosterone and FAB (**Figure 3D**) more closely resembled control neurons than FAB condition cells, and the gross morphology of individual neurons is more easily observable. Axons and dendrites were observed to have slightly greater antibody binding, and soma identification was more difficult in FAB treated conditions compared to control. Neuron "clustering" was commonly observed in testosterone and FAB treated conditions.

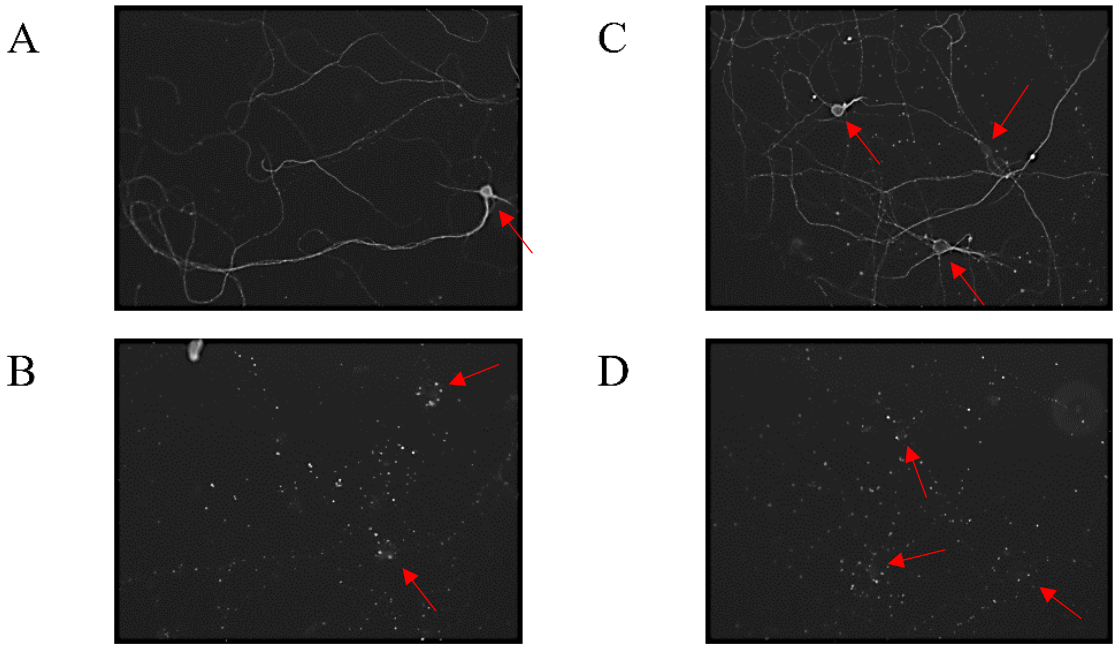

Figure 3: Fluorescent images of neurons stimulated with FAB and/or testosterone (**A**) Control condition neuron treated with 0.2 µL/mL DMSO (**B**) T condition neurons, cell clustering was common and isolated cells were rare (**C**) FAB condition neurons, minimal antibody binding was observed, individual cells are difficult to identify (**D**) TFAB condition neuron, cell processes are more easily identified compared to FAB, however extensive degradation is still observed. Red arrows indicate identified cell somas.

Tamoxifen Does Not Promote Neuron Survival Independent of Testosterone

Tamoxifen, a SERM, was used to competitively inhibit estradiol activity. In order to assess any affects that estrogen receptor inhibition via use of tamoxifen may have on stressed neurons independent of exposure to testosterone, cells were treated with tamoxifen and stressed. **Figure 4** shows the effect of treatment with tamoxifen on cells stressed with FAB. Planned comparisons using independent samples T-Tests indicated a significant difference between the Control condition and FAB condition $t(78) = 6.624$,

$p<0.001$. Additionally, a significant difference was observed between the Control condition and tamoxifen and FAB (TMX+FAB) co-treated group $t(52.460) = 6.058$, $p<0.001$. No significant difference was observed between the FAB condition and the TMX+FAB condition $t(78) = -1.373$, $p>0.05$. This suggests that treatment with tamoxifen alone does not result in rescue of cell viability as measured by MTS.

Testosterone Neuroprotection Operates via the Androgen Receptor Synergistically with Tamoxifen

Co-treatment of FAB stimulated cells with TMX and testosterone was performed to investigate the receptor pathway of testosterone's effects on cell survival observed in the above experiments. Tamoxifen was used to competitively bind the estrogen receptor in effort to isolate testosterone activity to the AR. The neuroprotective effects of testosterone as mediated by the AR were assessed by MTS assay and immunocytochemistry. In **Figure 4**, the effects of testosterone on cell survival were restricted to the AR mediated pathway by treatment with tamoxifen. Comparison of the Control group with the testosterone, tamoxifen and FAB treated group (T+TMX+FAB) by independent samples T Test indicated a significant difference in cell viability as measured by MTS $t(48.643) = 4.718$, $p<0.001$. Additionally, comparison of the FAB condition and the T+TMX+FAB condition indicated a significant difference $t(67.736) = -3.907$, $p<0.001$. A significant difference between the TMX+FAB group and the T+TMX+FAB group was also observed $t(78) = -2.85$, $p<0.01$. This indicates that co-

treatment of testosterone and tamoxifen partially rescues cells from FAB mediated cell death.

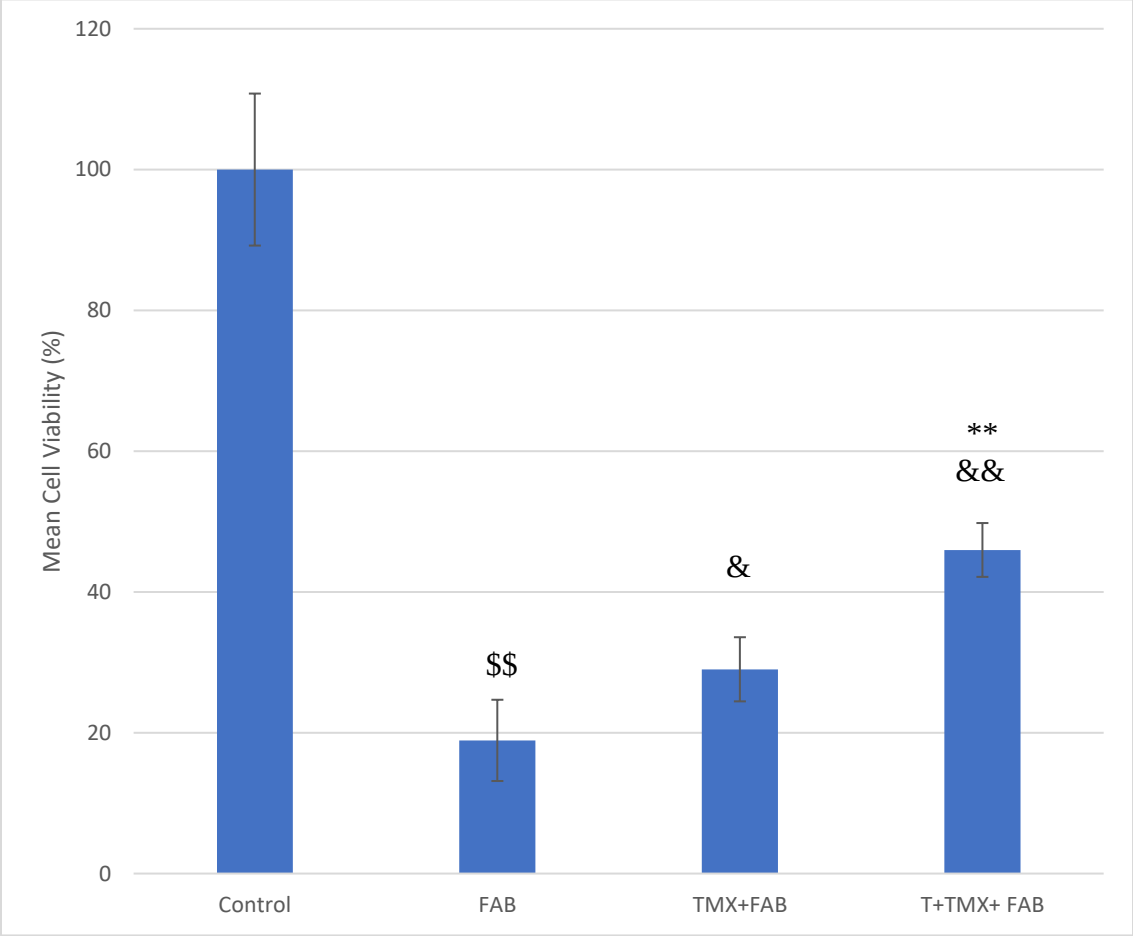

Figure 4: Testosterone improves cell viability in conjunction with a selective estrogen receptor modulator Cell viability of neurons treated with tamoxifen, denoted TMX, and testosterone, denoted T, for 48 hours. Treated cells were stressed with FAB and co-treated with TMX or T and TMX for 48 hours. Data are mean ±SEM. N=40 for each condition. $$ denotes significant difference of FAB from the Control ($p<0.001$). & denotes significant difference of FAB+TMX from the Control ($p<0.001$). ** denotes significant difference of T+FAB+TMX from FAB ($p<0.001$). && denotes significant difference of T+TMX+FAB from TMX+FAB ($p<0.01$).

To further explore the effects of testosterone via the AR pathway, treated cultures were stained and the relative intensity of fluorescent images were taken as previously. Illustrated in **Figure 5,** an increase in mean fluorescence is observed in the cells treated with testosterone and tamoxifen. An independent samples T Test comparing the Control group with the FAB treated group indicated an insignificant difference in mean pixel intensity $t(12.083) = 2.490$, $p<0.05$. No difference was observed between the Control and TFAB groups $t(16.881) = 1.480$, $p>0.05$, nor between the Control group and the T+TMX+FAB group $t(20) = -1.463$, $p>0.05$. Comparison of the FAB and TFAB groups indicated no difference between the groups $t(8.889) = -1.296$, $p>0.05$. An insignificant difference between the FAB and T+TMX+FAB groups was observed $t(14) = -2.876$, $p<0.05$. A similar insignificant difference was observed between the TFAB and T+TMX+FAB groups $t(16) = -2.629$, $p<0.05$. Although some differences would be deemed significant when using the standard $\alpha=0.05$, due to the increased number of statistical tests performed on this data the α was corrected to 0.01. Qualitative assessment of images indicates that cells from this condition more closely resembled the neurons treated with testosterone alone, and were heavily clustered (**Figure 6**).

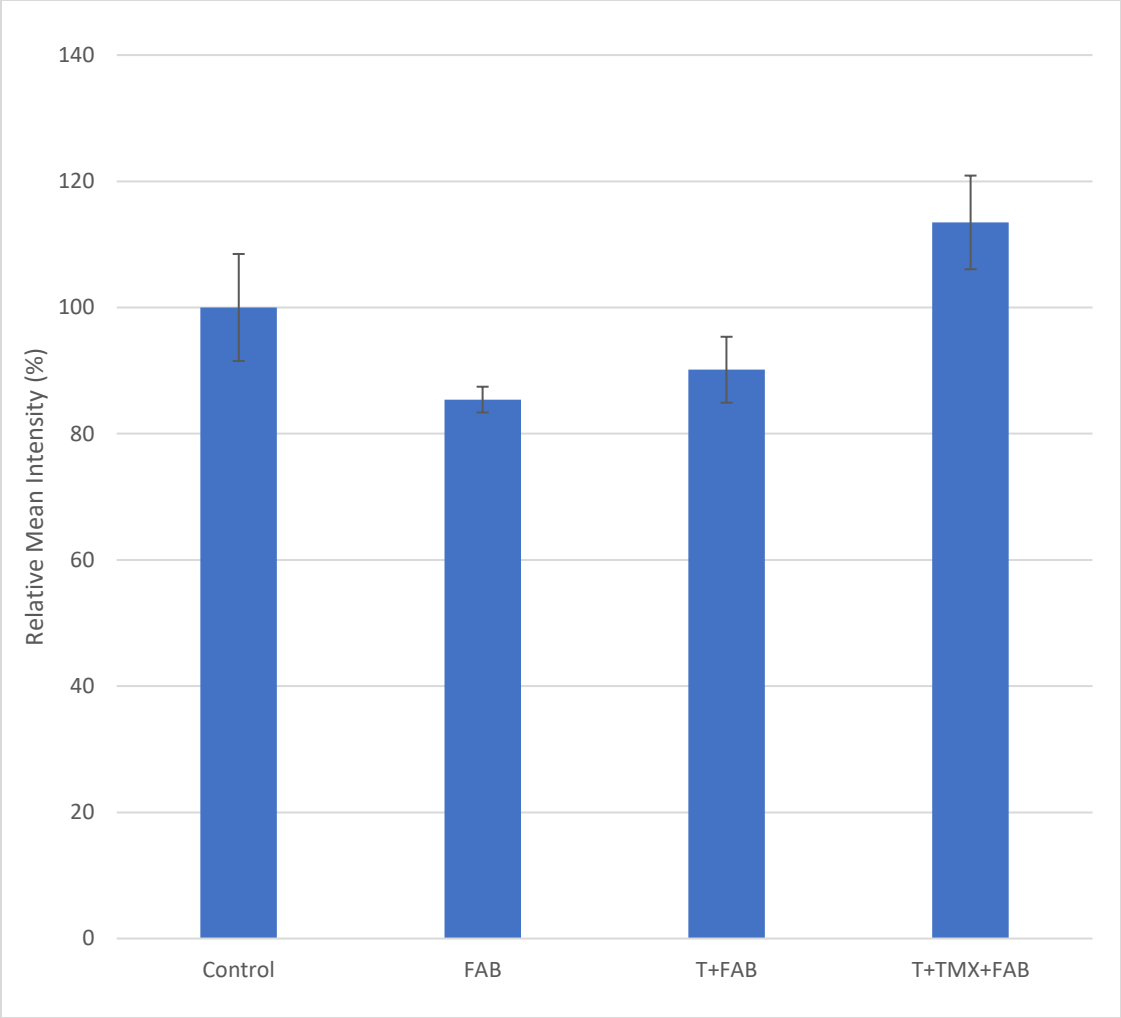

Figure 5: Fluorescent imaging indicates increased antibody binding to acetylated tubulin in tamoxifen treated cells Mean pixel intensities of randomly selected fluorescent images of cells immunostained for acetylated tubulin. Data are mean ±SEM. N=10 images for each condition.

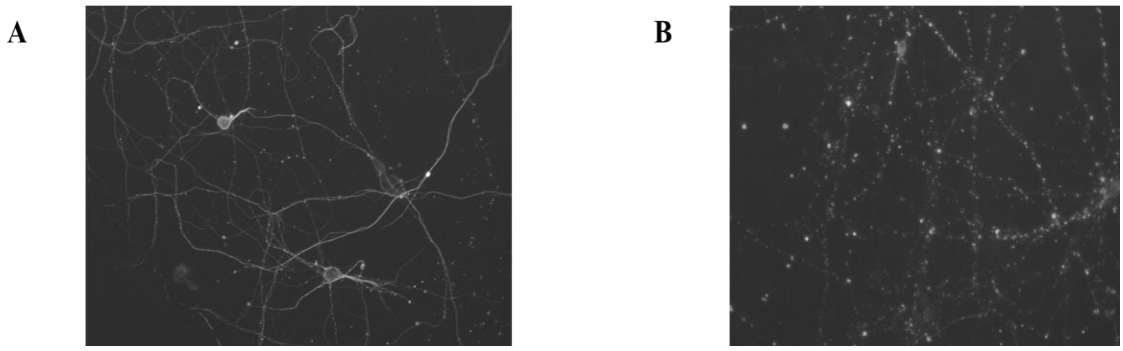

Figure 6: Co-treatment of testosterone and tamoxifen helps maintain tubulin stability when stressed with FAB (**A**) Testosterone treated control cells. Neuron clustering was commonly observed in conjunction with increased arborization. (**B**) Tamoxifen and testosterone co-treated cells stressed with FAB. Clustering and arborization are observed similar to **A**, however decreased antibody binding is observed. Neuron morphology appears to be relatively better preserved with compared cells treated only with FAB (**Figure 3C**).

Discussion

This study explored the effects of testosterone on viability of primary neurons under an oxidative stress and β-Amyloid model of Alzheimer's disease. The role of sex hormones in the pathology and incidence of AD is not currently well understood, however a significantly higher incidence rate of AD in women strongly suggests that sex is involved in risk for and onset of AD (Alzheimer's Association 2021; Vegeto et al. 2020). This has prompted research into the roles and pathways of sex hormones in AD with the bulk of this research has focused on estrogens (Fisher et al. 2018; Zhu et al. 2021). Current research investigating the impact of sex hormones on AD is insufficient, and few studies investigating the role of testosterone and other androgens in AD exist in the literature (Vegeto et al. 2020; Fisher et al. 2018; Zhu et al. 2021). The existing pre-clinical research supports testosterone as having neuroprotective functions, although the

mechanisms of neuroprotection are not well established. Additionally, clinical trials of hormone replacement therapy or supplementation of testosterone in men with AD has not been explicitly explored as of 2019 (meta-analysis by Buskbjerg et al. 2019). Due to the complexity of both AD and the up- and downstream pathways of sex hormones, additional research is required in order to understand the relationship between testosterone and AD.

Testosterone improves primary neuron growth *in vitro*

The effect of testosterone on cell viability and growth were assessed spectrophotometrically and semi-quantitatively using MTS assay and fluorescent visualization (**Figures 1-3**). As age is the primary risk factor associated with AD, it is important to establish model systems which more closely reflect the populations at increased risk of incidence (Alzheimer's Association 2021). Cells were treated with 10 nM concentration of testosterone in order to more closely resemble physiological concentrations observed in middle-aged adult men (Feldman et al. 2002). This concentration was chosen in attempt to partially replicate the physiological conditions of older men. It was deemed as the optimal concentration for exploring the neuroprotective effects of testosterone to prior literature supporting an effect on viability (Lau et al. 2014). Higher concentrations have also been explored in the literature; however, these are not representative of the populations at risk of AD, thus limiting their value for understanding disease mechanisms (Yao et al. 2017). By assessing the effect of

testosterone in the presence of an oxidative stress condition, and without stress, this study aimed to both reproduce prior results and explore testosterone's effects against oxidation.

Cell viability was assessed in this study primarily via MTS metabolism, which directly measures mitochondria activity (**Figure 1**). An increase in mitochondrial activity of approximately 20% following testosterone treatment was observed in MTS assays when compared to a DMSO treated control (**Figure 1**). This control was chosen as testosterone was dissolved in DMSO. This result is inconsistent with current literature, which has suggested a statistically insignificant effect of testosterone on the viability of unstressed cells (Pike 2001, Nguyen et al. 2005, Massa et al. 2017). This difference, however, can be explained by a difference in model systems. Our experiments utilized MTS metabolism to indirectly measure viability as a function of mitochondrial activity. The literature has largely utilized cell counting methods using viability dyes such as calcein acetoxymethyl ester as performed by Nguyen et al. (2005) and Pike (2001). A more recent study by Massa et al. utilized 7-amino actinomycin D staining, a membrane impermeant dye, measured by flow cytometry to directly assess the number of viable cells (2017). This study's results, however, were recorded from 16-day old primary neuron cell cultures; this method of culture is expected to result in approximately 10-20% cell death following day 14. This suggests that testosterone may protect neurons from natural death in the absence of stressors. Additionally, this result may be an artifact of testosterone's anabolic effects. The MTS assay measures mitochondrial activity, and testosterone, an anabolic steroid, is known to stimulate the resting metabolic rate, which would likely increase MTS metabolism and absorbance.

Visualization of acetylated tubulin was used to support MTS results (**Figures 2** and **3**). Mean pixel intensity of collected random images showed no statistical difference between testosterone and control groups (**Figure 2**). This result is more consistent with the literature, and supports the hypotheses that the observed increase in MTS metabolism is a result of anabolic effects on the neurons, or that testosterone is able to delay natural death of neurons in culture. Acetylation of α-tubulin has been associated with long-term microtubules stability (Baas et al. 2016). Therefore, acetylation can be used as a marker of cell structure integrity, allowing for comparisons of general neuron morphology in addition to cell viability. Qualitative assessment of visualized cultures suggests that testosterone does have effects on cell growth. Plated cells treated with testosterone were observed to "cluster" together and grew less frequently in isolation when compared to control cells (**Figure 3**). This is notable, as cell cultures intended to be immunostained and visualized were plated at a lower density of $3x10^4$ cells in each well in order to promote isolated growth of neurons. As all cells were plated from the same diluted stock of harvested cells, this "clustering" effect cannot be due to incomplete dissociation, rather is an effect of growth conditions. Greater arborization was observed in testosterone treated neurons, which suggests more synapse formation. Increased synaptic density as a result of testosterone has been observed *in vivo*, suggesting that increased arborization may be present despite a lack of quantitative analysis (Fattoretti et al. 2019). This result may be inaccurate, as arborization was not quantitatively measured, however it suggests future investigation of *in vitro* synapse density increases as a result of testosterone treatment. Additionally, the observed "clustering" effect may be an artifact of

testosterone protecting neurons against the natural death observed in culture around the 2-week period. This supports the observed increase in MTS metabolism as a higher number of cells are may be present in testosterone treated culture wells as compared to other conditions.

Neuroprotective effects of testosterone were assessed using FAB to mediate oxidative stress in conjunction with Aβ mediated toxicity. MTS assay of cell viability indicated no statistically significant rescue from FAB (**Figure 1**). FAB treatment produced an approximately 80% reduction in viability as compared to control cells. This degree of induced cell death is greater than expected and greater than that observed in prior *in vivo* models utilizing the FAB model system (Lecanu et al. 2006). These results limit the conclusions able to be drawn, as the neurons are experiencing a very high degree of oxidative stress that may not be representative of physiological conditions of AD. A non-significant rescue of average viability by testosterone is observed, which was not expected. Prior experiments have observed a 25% rescue of markers of neuron viability following testosterone treatment when stressed with Aβ (Lau et al. 2014). This study's results, however, are not directly comparable to the existing prior literature due to a discrepancy in model systems. In prior models of testosterone's interactions with Aβ, non-genomic effects were often assessed. Non-genomic pathway investigations were performed by treating culture with testosterone for 1 hour prior to stimulation, as reported by Lau et al. (2014). However genomic pathway investigations utilized 24–48-hour pretreatments of testosterone prior to stimulation (Ahlbom et al. 2001). Neuroprotection via the genomic pathway is of most interest in modeling of AD as it is present in the brain

throughout life, and therefore exhibiting genomic and nongenomic effects well before the onset of disease pathology. Additionally, any potential integration of testosterone therapy into AD treatment models would likely contain consistent dosing and prolonged bioavailability, suggesting both genomic and non-genomic effects.

Immunofluorescent staining of FAB treated cells indicated a statistically significant difference in average pixel intensity of randomly selected images (**Figure 2**). This mean was notably lower than the control, which is consistent with MTS data. Qualitative assessment following fluorescent visualization indicated a significant degree of degradation of FAB treated cells (**Figure 3**). Cells had spotted appearance, and fluorescence was inconsistent when compared to control cells. This appearance is caused by a decrease in acetylated tubulin spread throughout the cytoskeleton, which suggests less cellular integrity and a heavily damaged state. Cell outlines were difficult to make out and cells appeared to be irregular in shape, suggesting advanced cytotoxicity and increased apoptosis, which is consistent with MTS and immunofluorescent pixel data. Mean pixel intensity of FAB and testosterone treated cells was slightly higher than the FAB group, but this difference is insignificant ($p>0.05$). This suggests that testosterone alone was unable to promote significant protection of the cytoskeleton from damage. Visualization revealed easier to identify neurons and more preserved linear qualities of neuron processes (**Figure 3**). Spotted antibody staining was still observed; however, antibody adherence appears to be more distributed throughout cells. A greater volume of antibody binding within the soma region was observed in testosterone treated cells as compared to FAB alone. This suggests that testosterone may promote improved

stabilization of cell body microtubules, however this conclusion would require further investigation.

The existing literature relies on Aβ induced stress, as reported by Lau et al. (2014), however the FAB model utilized in this study combines Aβ induced toxicity with antioxidant suppression and Fe^{2+} mediated oxidation. Another study exploring neuroprotection of granule cells from oxidative stress found testosterone to have an approximately 30% rescue of cell death (Ahlbom et al. 2001). This study utilized H_2O_2 mediated formation of ROS, however it is comparable to FAB mediated generation of ROS. In short, our results suggest that physiological doses of testosterone do not significantly rescue neurons from FAB mediated apoptosis. Comparison to existing literature support further investigation into why significant protection is observed in ROS and Aβ based models of stress, but not when both are applied simultaneously.

Treatment with testosterone and tamoxifen results in rescue from FAB mediated cell death

Tamoxifen was used to inhibit the estrogen receptor (ER), in order to control for testosterone conversion to 17-β-estradiol and subsequent activation of ER-dependent mechanisms. Cells were treated with tamoxifen and FAB to identify any inherent effects tamoxifen treatment may have within the FAB model (**Figure 4**). MTS analysis revealed no significant effect of tamoxifen on cell viability when compared to FAB treated cells (**Figure 4**). Tamoxifen is a selective estrogen receptor modulator (SERM), and does not

function only as an ER antagonist (Dhandapani and Brann 2003). As such, tamoxifen has been observed to also exhibit partial or complete agonist actions as activation of non-genomic ER pathways (Dhandapani and Brann 2003, Lonard and Smith 2002). In previous *in vitro* experiments, tamoxifen was shown to have neuroprotective effects at 1 µM and 10 µM doses (Dhandapani and Brann 2003; Zhang et al. 2008). In the latter of these experiments, Zhang et al. showed that 10 µM concentration of tamoxifen in neuron cultures acts as a non-steroidal ER agonist (2008). In this study, tamoxifen was intended as an ER antagonist, in order to inhibit activity of aromatized testosterone. Clinical studies using much lower SERM doses have found tamoxifen to exhibit antiestrogen effects at the level of the hypothalamus (Tsourdi et al. 2009). The dose of 13.5 nM tamoxifen utilized in these experiments was used to replicate the antiestrogenic effects demonstrated *in vivo* by Tsourdi et al. (2009). Additionally, the selected dose is approximately 3 orders of magnitude lower than the 1 µM dose used by Dhandapani and Brann, comparatively minimizing any agonist effect it may have (2003). The antagonist effect of tamoxifen was not experimentally verified in this study, limiting the conclusions able to be drawn about its activity.

Co-treatment of neurons with testosterone and tamoxifen was performed in order to isolate the effects of testosterone to the AR. Testosterone is subject to conversion into estradiol by the aromatase enzyme, which demethylates testosterone into the aromatic steroid of estradiol. There are several methods of stopping estradiol activity in an *in vitro* model investigating testosterone, including: use of 5α-dihydrotestosterone, a testosterone metabolite, use of an AR agonist, inhibition of aromatase, or inhibition of the ER. The

last method was selected, and tamoxifen was used as an ER antagonist due to cost and availability. Co-treatment of FAB stimulated cells with tamoxifen and testosterone indicated a significant rescue of cells (**Figures 4, 5,** and **6**). MTS assay indicated an approximately 25% rescue of cell viability to 45% of the FAB control (**Figure 4**). This suggests that cell death had been reduced compared to FAB treatment alone. This was expected, as prior literature had suggested that genomic pathway activation of the AR is necessary for protection from ROS (Ahlbom et al. 2001). Other studies have suggested that non-genomic mechanisms of testosterone modulate preservation of synaptic terminals when exposed to Aβ mediated insult (Lau et al. 2014). It is important to note that much of the literature investigating testosterone's effects do not control for aromatization and ER activation (notably Lau et al. 2014). In the case of experiments by Lau et al. aromatization is not controlled for as they focus on the rapid effects of testosterone, and pretreatment of 1 hour is insufficient for any meaningful transcriptional changes following seroconversion. After this pre-treatment, cells are stimulated with both Aβ and testosterone for 24 hours. This is important, as the continued application of testosterone allows for both its later genomic effects and for effects of ER activation following seroconversion, introducing some mechanistic uncertainty to the results of their toxicity experiment (Lau et al. 2014). Another *in vivo* study addressing Aβ toxicity and testosterone utilize 5α-dihydrotestosterone to control for aromatization, however the same study determined that 5α-dihydrotestosterone's effects were mediated by an AR independent pathway (Yao et al. 2017). 5α-dihydrotestosterone was found to largely

have the same AR-dependent effects as testosterone, however this study decided to forego using 5α-dihydrotestosterone to avoid activation of AR independent pathways.

No statistically significant increase in fluorescence from anti-acetylated tubulin was observed in testosterone and tamoxifen treated groups stimulated with FAB (**Figure 5**). Some tentative conclusions can be drawn from this data, since significance would have been observed were the α not corrected to 0.01. An increase in relative image fluorescence of the T+TMX+FAB group when compared to the FAB group was observed (**Figure 5**). This assay potentially suggests that this treatment resulted in improved cytoskeletal stability. However due to the lack of significance, these conclusions require further support. Qualitative data collected from these images, shown in **Figure 6**, support the hypothesized improvement in cytoskeletal stability. Image analysis shows the bespeckled cells are more similar to the FAB and T+FAB group than the controls. Qualitative analysis shows very marked improvements when compared to FAB cells, more so than that of testosterone and FAB cells (T+FAB) shown in **Figure 3**. Improved α-tubulin stability would suggest that cells are able to better withstand FAB mediated insults to cell morphology and potentially support protection of synapses.

The limitations of this experiment restrict the conclusions that are able to be drawn from this data. This data does, however suggest that, in combination with a SERM, testosterone is neuroprotective in an advanced oxidative stress and Aβ toxicity model. One such limitation is that MTS data was required to be inverted. Typically, MTS assays would be analyzed using a dedicated spectrophotometer, such as an SpectraMax M3 (Molecular Devices), that can assess absorbance at 490 nm, with the

instrument zeroed to a set of blank wells. For these experiments, this tool was unavailable, resulting in less accurate results and introducing greater systemic and operator error into data collection. An additional limitation of this study lies in the FAB stimulation. Overstimulation of neurons results in very high rates of cell death, which is counteractive to understanding the rescue effects of testosterone. These experiments are limited by the approximately 80% cell death rate observed in FAB conditions, which may have reduced the concentration of cells below a critical point for observing meaningful effects. This does not detract from the effects observed, it simply indicates that some effects of testosterone or tamoxifen may not have been observed due to the overwhelming amount of toxicity observed. This suggests further exploration of these effects in a further developed model that is able to explore both high and more moderate degrees of induced toxicity.

Despite these issues, much of these results followed a general trend observable in the limited literature, that testosterone is able to promote neuroprotection when operating through genomic pathways. The significant improvement in cell viability when treated with tamoxifen and testosterone suggests that activation of AR-dependent cascades have neuroprotective activity in the presence of ROS and Aβ. This is consistent with the existing literature suggesting broad neuroprotection against Aβ and oxidation by testosterone. Further data and exploring this relationship are needed to confirm these conclusions. Unique from the existing literature is the investigation of testosterone's effects in the FAB model system. To date, there do not exist any studies exploring this effect under conditions of both Aβ induced stress and oxidative stress. This represents an

important intermediate of *in vitro* modeling of testosterone in AD. This suggests that testosterone is neuroprotective under conditions modeling two of the three major hallmarks of AD, suggesting that testosterone may be an important component of AD pathology in men.

Treatment of AD has largely failed, and combination and immunotherapies have become a shown the most clinical promise (Vaz and Silvestre, 2020). Recent clinical investigation into testosterone replacement therapy as a potential therapy for improving cognition have had mixed results (Corona et al. 2021). Testosterone deficiency is unlikely to be a central component of either AD pathology or its treatment. This study and the existing literature, however, support testosterone, and, more broadly, the sex hormones, as an important additional component of AD pathology. Changes in sex hormones as a result of age and other sexually dimorphic characteristics may potentially be involved in susceptibility to the molecular mechanisms of neurodegeneration (Fisher et al. 2018; Zhu et al. 2021). For this reason, it is crucial that the various supporting mechanisms of AD pathology are investigated alongside its major hallmarks of Aβ, Tau hyperphosphorylation and oxidative stress.

Future Work

The future of treating AD lies in a better understanding of the disease mechanisms on a cellular and systems level, coupled with effective combination therapies and personalized treatment. The gradual loss of sex hormones in advanced age appears to be

coincidental, however is more likely to be a component of AD pathology (Fisher et al. 2018). It is important to continue developing AD model systems that replicate not only the types of damage observed, but also the subtle physiological changes associated with AD and aging.

To better establish the mechanism by which the apparent neuroprotective effects of testosterone are mediated, the downstream genomic effects of AR activation must be assessed. This could be explored through transcriptome analysis of *in vitro* cell cultures via ChIP-Seq assay to identify changes in gene product expression. Furthermore, various siRNA or synthetic upregulation experiments can be performed building on this data to explore the essential gene products associate with testosterone treatment. This would allow for development of a molecular library of protein products involved in AR-mediated neuron pro-survival signaling or protection.

This study would also benefit from confirmation of its results via a different reporter assay. The primary conclusions regarding cell survival were derived from MTS assay data, which measures pyruvate dehydrogenase activity (Cory et al. 1991). Therefore, this is a direct assay of mitochondrial activity, and in the case of assessing the effect of testosterone, may cause some error in analyzing the data due to its functions as an anabolic steroid. Analysis of cell survival via a cytosolic metabolite or another global cell survival reporter would help support the observations made in this paper and in the existing literature. Furthermore, it would provide more global observations regarding changes in cell viability.

While a significant “clustering” effect was observed as a result of testosterone treatment, changes in synaptic connections was not assessed in this paper. Symptoms of AD are manifested by both the death of neurons and the degradation of synaptic connections and plasticity in the cortex. By exploring how the interactions between cells changes as a result of testosterone treatment, observations regarding changes in synaptic density can be made. This has been explored *in vivo* by Fattoretti et al. with positive results suggesting that testosterone promotes preservation of synapses in the dentate gyrus (2019). It is important to explore this within the context of an *in vitro* or *ex vivo* model, as a way of identifying the pathways associated with the supported protection of synapses. It is also important to explore this within a model system that addressed Tau protein hyperphosphorylation, which contributes significantly to synaptic degradation.

Conclusions

This study found that treatment of primary cortical neurons with physiologically relevant concentrations of testosterone improves neuron growth *in vitro*. This was observed independent of additional stressors associated with the modeling of AD. This paper also observed that, in conjunction with tamoxifen, testosterone is neuroprotective under ROS and Aβ induced stress. This effect was observed to be dependent on the genomic pathway of the AR, and implies that the neuroprotective effects of testosterone are dependent on promoting changes in the transcriptome. This finally suggests that sex steroids, namely testosterone, are likely important components of AD pathology, and that

their gradual reduction may be involved in the pathology of AD. Future work is required to verify these conclusions, as the literature is sparring, despite over 15 years of investigation. Elucidation of the specific molecular effects of testosterone and SERM cotreatment are necessary to understand the fundamental changes that are promoted to illicit neuroprotection in neurons when under oxidative stress.

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