THE ROLE OF LIPID RAFTS IN ANDROGEN'S NEUROTOXIC EFFECTS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3α-17β-hydroxysteroid dehydrogenase (3α-HSD)

3β-17β-hydroxysteroid dehydrogenase (3β-HSD)

3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA)

Alzheimer's disease (AD)

Androgen Receptor (AR)

Blood Brain Barrier (BBB)

Dehydroepiandrosterone Sulfate (DHEAS)

Dihydrotestosterone (DHT)

DNA Binding Domain (DBD)

Hypertension (HTN)

Lewy Body Disease (LBD)

Ligand Binding Domain (LBD)

Low-density lipoproteins (LDL)

Membrane-bound AR (mAR)

Multiple Sclerosis (MS)

NADPH oxidases (NOX)

Oxidative Stress (OS)

Parkinson's disease (PD)

Reactive Oxygen species (ROS)

Sex Hormone–Binding Globulin (SHBG),

Vascular dementia (VaD)

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Sex Differences in Neurodegeneration

Sex differences have been observed in neurodegenerative diseases, such as Alzheimer's Disease (AD), Parkinson's disease (PD), and Vascular Dementia (VaD). In diseases like VaD, PD, and Lewy Body Disease (LBD), men have a higher risk, however women have been seen to have a higher risk for AD and Multiple Sclerosis (MS). Also, some diseases such as Huntington's disease affect both men and equally, therefore showing no sex bias [1, 2] (**Figure 1**).

Oxidative stress (OS) is a hallmark common to all these disorders [3]. It is possible that the effects of sex hormones on OS could mediate these sex differences. Estrogen, the major female sex hormone, is an antioxidant [4]. Many studies have shown that estrogen decreases OS by binding to estrogen receptors and upregulating the expression of antioxidant enzymes via intracellular signaling pathways[4]. Estrogen has been shown to have some protective effects in many neurodegenerative diseases [5].

Unlike estrogen, the major male sex hormone, testosterone, can have diverse effects on OS. Testosterone can be aromatized to an estrogen and protect cells from subsequent OS damage [6]. In contrast, testosterone can be reduced to dihydrotestosterone (DHT) and increase OS [6]. How testosterone impacts the brain is dependent on the health of the brain, such as OS levels. If

OS levels are low, then androgens are protective against subsequent OS related stressors [6]. If OS levels are high, then androgens can exacerbate OS damage [6].

Common risk factors for these neurodegenerative diseases are cardiovascular diseases, such as hypertension [7]. Cardiovascular diseases can increase OS [8]. Interestingly, cardiovascular disease is higher in men than pre-menopausal women [9]. After menopause, cardiovascular disease increases in women [10]. Therefore, men exhibit a higher OS load than pre-menopausal women. Further, this increased OS load in men is chronic as it generally occurs at middle-aged and is sustained throughout aging [11], whereas the OS load is more acute in post-menopausal women. Interestingly, hypertension that is higher in men than women[9] has been

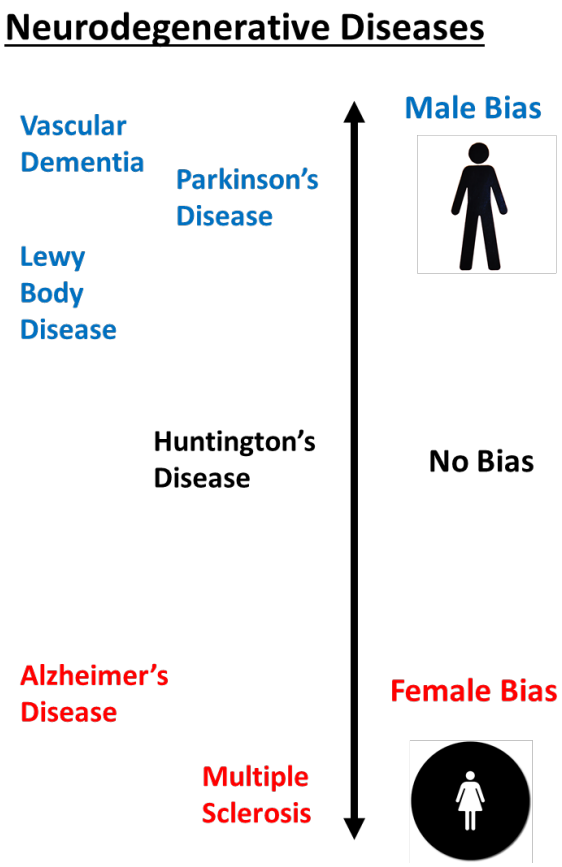

Figure 1: Male bias versus female bias neurodegenerative disorders. Men have a higher incidence of Vascular Dementia, Parkinson's Disease, and Lewy Body Disease while Alzheimer's Disease and Multiple Sclerosis show a greater bias for female. Huntington's disease affects male and female equally.

associated with cognitive decline later in life[12]. Therefore, chronic OS burden that is generally observed in men may increase the risk for neurodegenerative disorders, such as VaD.

1.2 Vascular Dementia

VaD, a heterogenous group of brain disorders, is a form of cognitive decline resulting from cerebrovascular disease in small or large vessels. Diseases such as hypertension, obesity, diabetes, and hyperlipidemia increase OS levels which can lead to a cerebrovascular impairment and consequently VaD (**Figure 2**) [13]. VaD includes an array of cerebrovascular cognitive impairments, which range from mild to severe cognitive impairment and consequently, dementia [14]. The cerebral blood vessels play a crucial role in maintaining brain health [15]. They are not only responsible for delivering oxygen and nutrients to the brain, but also to maintain the well-being of neurons and glia cells [15]. Thus, damage to cerebral blood vessels can lead to oxygen deprivation and consequently cell death in brain cells [15].

VaD is the second most common type of dementia [16], causing approximately 20% of known dementia cases [17]. It is also characterized as a neurocognitive disorder, which also encompasses behavioral symptoms, locomotor abnormalities, and autonomic dysfunction [16]. Some of the behavioral changes seen as VaD progresses include agitation and irritability [1]. According to The World Health Organization, an estimate of 35.6 million people live with dementia, and this number is expected to triple by 2050 [18]. Approximately 7.7 million new cases of dementia are diagnosed every year, therefore causing a great burden on families, caregivers, and society [15]. Dementia remains a devastating and costly disease [19]; in the US, the financial cost to society of VaD has surpassed the cost of that of cancer and heart diseases [19]. Of utmost concern, this cost is expected to dramatically increase as the number of aged Americans are expected to double by 2060, resulting in 23% of the total population being greater than 65 years of age [20]. The number of

demented patients worldwide is projected to increase to approximately 81.1 million in 2040 [21]. VaD, which often coexists with AD, is the leading cause of age-related cognitive impairment [15]. Age-related dementia is an irreversible condition that causes a progressive decline in cognition [15]. Importantly, age is a dominant risk factor of VaD [17] and the prevalence and incidence of cognitive impairment increases significantly after age 65 [17].

Previously, VaD was attributed to sclerosis of cerebral arteries leading to diffuse ischemic injury and brain atrophy; however, VaD was identified to be caused by multiple and discrete ischemic lesions in patients with vascular risk factors, such as hypertension, diabetes, atrial

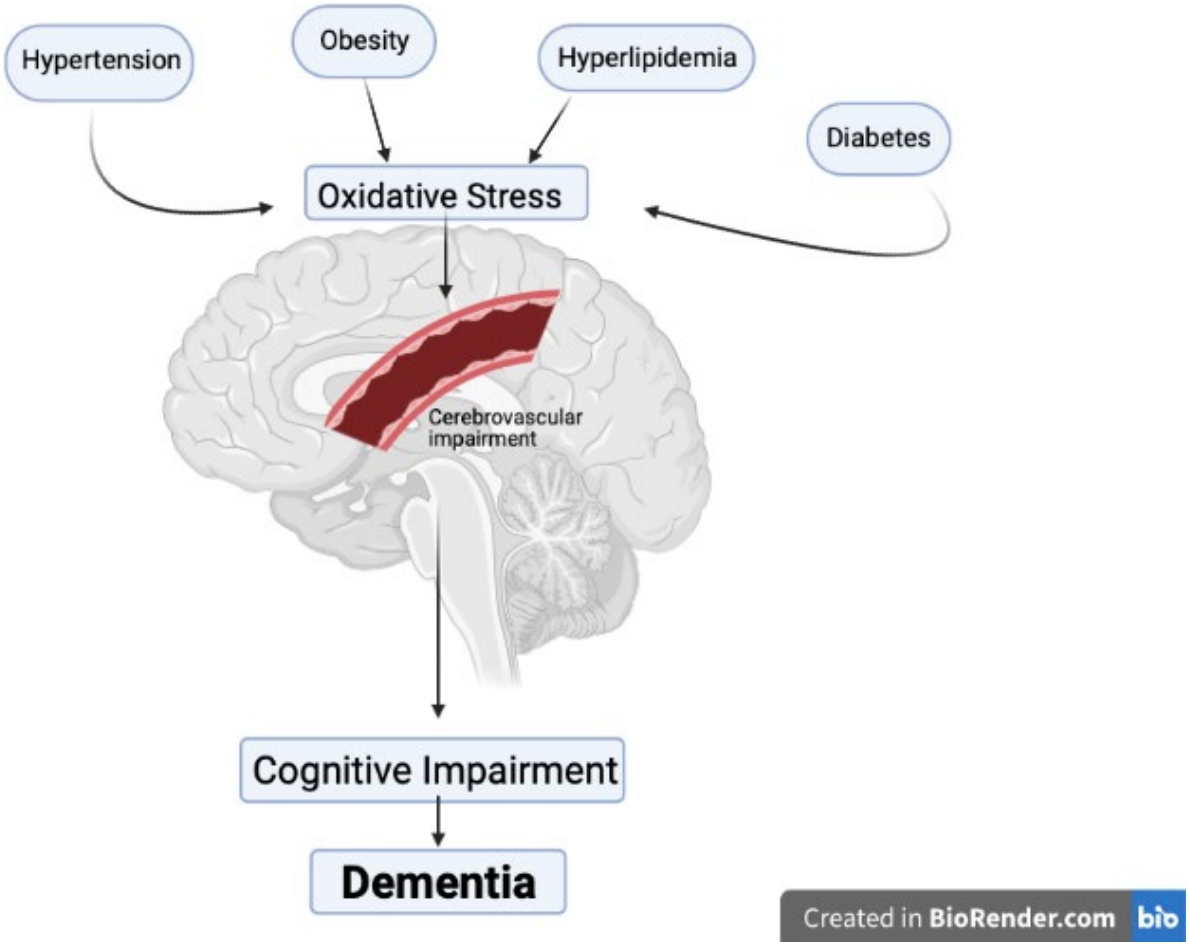

Figure 2: Cardiovascular disease can increase dementia. Hypertension, obesity, hyperlipidemia, and diabetes can increase oxidative stress levels and cause cerebrovascular impairments and eventually VaD.

fibrillation, hyperlipidemia, smoking, and cerebrovascular diseases [22]. Thus, suggesting that preventing these risk factors could also prevent dementia [22, 23]. Since lifestyle factors such as obesity and high fat diets can impact cardiovascular diseases, hence posing as risk factors for VaD, this puts approximately 35% of Americans at risk [24]. Unfortunately, regardless of the awareness that has been brought to obesity, the rates of sufferers continue to increase in the United States and the world [24]. These risk factors have been seen to increase the risk of VaD independently of the associated increase in stroke risk [25]. There is also evidence that shows that there is a reduction in the white matter cerebral blood supply in patients with VaD and that the ability for neural activity to increase blood flow in brain or retina is compromised in patients with VaD factors such as hypertension and diabetes. Currently, there is no cure for VaD, and current medications administered to patients are only palliative and just treat symptoms, but do not modify the underlying disease pathology [1].

1.3 Oxidative Stress Generation

OS plays a large role in aging associated diseases, such as vascular diseases and dementia [26]. Under normal homeostatic conditions, reactive oxygen species (ROS) play a role in immunity, homeostasis, and signal transduction pathways [27], however, increased OS can result in cell loss [28]. OS describes an environment where free radical species overwhelm antioxidant species [26]. Free radicals include peroxides, superoxide, hydroxyl radicals, and singlet oxygen [29, 30]. These radicals are extremely unstable, thus reacting quickly with many biological products, hence causing OS [27].

The age-related increase in reactive oxygen species (ROS) is partially attributed to the increased activity of NADPH oxidases (NOX) [31]. NOX family are multiunit enzymes that are abundant in cerebral blood vessels [32], and have been identified as an important OS generator in

vascular OS associated with aging [32]. NOX proteins transfer electrons across biological membranes. In this process, oxygen is the final electron acceptor which gives rise to a superoxide at the end of the electron transfer [33]. Therefore, the biological function of NOX is to generate ROS. Therefore, to improve VaD in patients, inhibition or genetic inactivation of the NOX enzyme has been shown to be effective [34]. In addition, vascular risk factors for VaD, such as hypertension, insulin resistance and diabetes, have been shown to lead to vascular OS and inflammation in both animals and in humans [35-37].

Interestingly, a sex difference has been observed with NOX expression. Increased NOX is observed in males compared to females [38-41]. An increase in OS and NOX expression was observed in the aortic and cerebral arterial cells of young healthy male rats compared to young healthy female rats [41, 42]. This disparity in OS in both sexes continue and is exacerbated with aging in both animal and clinical samples [43, 44]. Some studies suggest that sex hormones may underlie the observed sex differences in OS generation [28].

1.4 Steroid Hormones

Steroid hormones are a class of hormones that serve as chemical messengers in the body and are derivatives of cholesterol [45]. Steroid hormones include corticosteroids and sex hormones, which regulate different physiologic processes, including the development and function of the reproductive system [45]. The actions of steroid hormones are mediated through the steroid hormone receptors, which are intracellular proteins that belong to the nuclear family of transcription factors [45]. These receptors mediate signal transduction through genomic and nongenomic actions in a context-specific manner [45].

Androgens are the major male sex hormone that are responsible for developing and maintaining masculine characteristics [46]. These hormones are produced from cholesterol in

several steps which involve different enzymes as shown in (**Figure 3)**. Testosterone, the principal male hormone [47], and its metabolite dihydrotestosterone (DHT) are responsible for the development of the male sexual organs, secondary sexual characteristics, and fertility [46]. Testosterone is synthesized in the Leydig cells, found in the interstitium of the testis and to a smaller extent by the adrenal glands [47]. Testosterone has a four ring C18 steroid structure [46]. Testosterone is also involved in the production of sperm, promotion of muscle mass, strength, bone density and it is necessary to maintain normal sex drive [47]. Testosterone is also produced in minute quantities in the ovaries; however, testosterone levels in women are much lower than men [47].

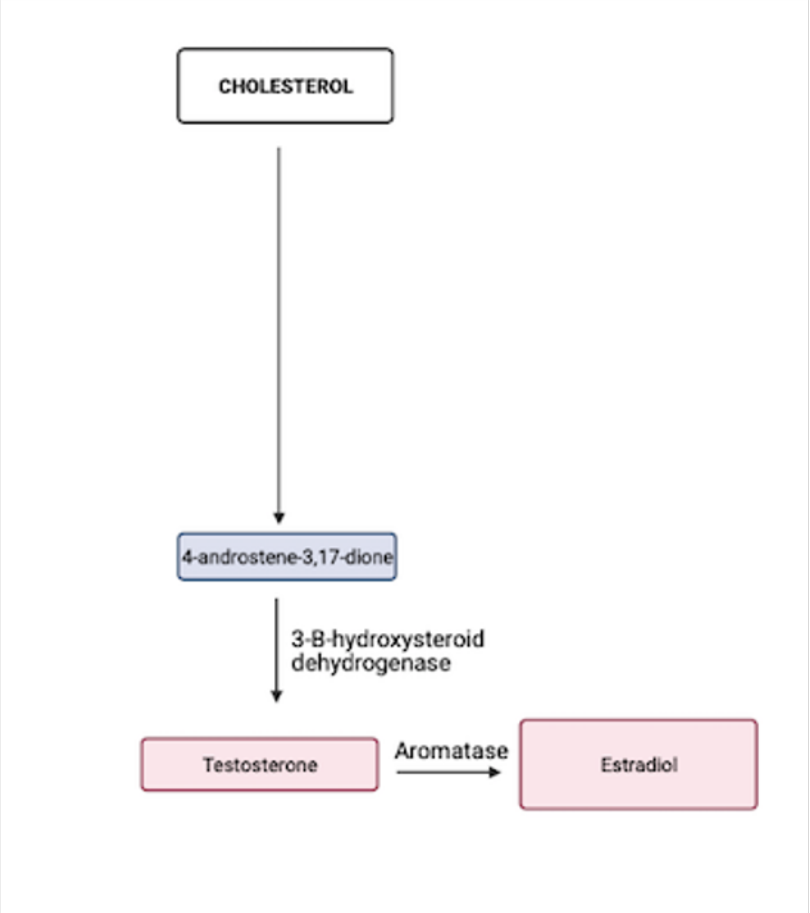

Figure 3: Testosterone steroidogenesis. Testosterone is synthesized from cholesterol in several steps and then, testosterone can be aromatized to estradiol which has antioxidant properties.

DHT is the 5α-reduced metabolite of testosterone [48]. Testosterone is converted to DHT in target organs, which include the prostate, skin, and liver. DHT can also be synthesized from other precursors as seen in the prostate; however, these other pathways are inconsequential [48]. Because DHT is a more potent androgenic agonist than testosterone, it is necessary in some tissues including the prostate for full organ development and function [48]. DHT is mainly synthesized through the irreversible action of Type I and II microsomal SRD5A [48]. Both types of SRD5A are localized to the prostate tissue, skin, liver, and hair follicles and these catalyzes the formation of DHT from testosterone in these tissues [48]. DHT is metabolized into inactive steroids by the initial actions of 3α-17β-hydroxysteroid dehydrogenase (3α-HSD) and 3β-17β-hydroxysteroid dehydrogenase (3β-HSD) in the liver, intestine, skin, and androgen-sensitive tissues [49].

1.5 Genomic and Non-Genomic Androgen Receptors

The effects of androgens are mediated via the androgen receptor (AR or NR3C4), a member of the steroid nuclear receptor superfamily [46, 50]. The AR is encoded by a single gene which is located on the X chromosome [46]. Androgens carry out their actions via the AR in a DNA binding-dependent manner to regulate target gene transcription or in a non-DNA binding-dependent manner to initiate rapid, cellular activities (**Figure 4)** [50]. The non-DNA binding-dependent signaling of the AR occur within seconds to minutes of androgen treatment and can be seen in different cells, including neurons [50, 51]. Based on evidence, it is known that some of the non-DNA binding-dependent actions of androgens are mediated through the activation of membrane-bound protein receptors such as membrane-bound AR (mAR) [51].

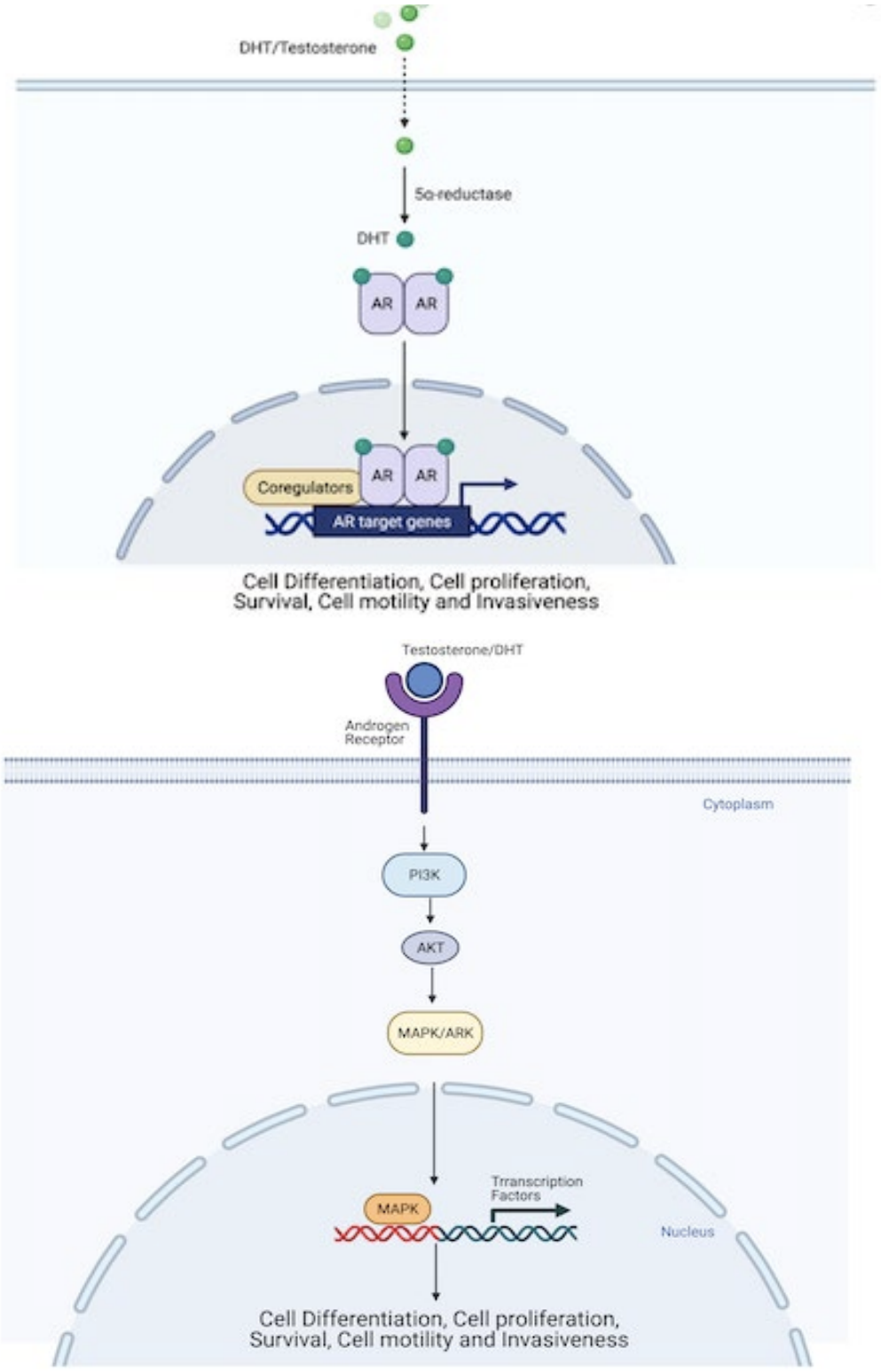

Figure 4: Cell signaling of Androgen Receptor Signaling. Cell signaling of androgen receptor showing Genomic Signaling (A). Cell signaling of Androgen Receptor Signaling showing the Non-Genomic pathway (B).

The AR comprises three main functional domains, which are the N-terminal transcriptional regulation domain, the DNA binding domain (DBD), and the ligand binding domain (LBD) [50]. The N-terminal domain of the AR has the most variability, while the DBD is most commonly conserved among the different steroid hormone nuclear receptor family [50]. The DBDs of all steroid hormone nuclear receptors contain two zinc fingers that ensure the direct DNA binding of the AR to the promoter and enhancer regions of AR-regulated genes, thus allowing the activation or repression functions of the N-terminal and LBD for gene transcription [50]. The LBD also has a similar structure to the nuclear receptors. The LBD mediates the interaction between the AR and other proteins such as the heat shock and chaperone proteins, while also interacting with the N-terminus of the AR to stabilize bound androgens and the DBD [50].

Testosterone and DHT are capable of binding to the same AR [49], however, at low circulating androgen levels, binding of DHT is favored over testosterone. DHT binding affinity for AR is significantly stronger, thus making DHT a more potent agonist than testosterone. Although, the stabilization of the AR is driven more by testosterone than DHT [49].

Some cell-impermeable androgens have been seen to have fast cellular effects and have been suspected to carry out their functions through membrane-androgen receptors (mAR) which are located outside the cell, usually in the lipid rafts of plasma membranes. In humans, a naturally occurring splice variant of the membrane androgen receptor, AR45 has been identified [52]. The *AR45* gene is made up of the AR DNA-binding domain, hinge region and ligand-binding domain, preceded by a novel seven amino-acid long N-terminal extension, and it lacks the entire region encoded by exon 1. A survey of human tissues revealed that AR45 was expressed mainly in heart and skeletal muscle [52]. Interestingly, the mAR splice variant AR45 was also seen in several brain regions including the substantia nigra pars compacta, entorhinal cortex, and hippocampus of the

dopaminergic neuronal cell line. It has a molecular weight of 45 kDa and lacks an N-terminal domain, indicating it is homologous to the human AR45 splice variant. In dopaminergic neurons, AR45 is also localized to plasma membrane lipid rafts, a microdomain involved in cellular signaling [51].

1.6 Androgen Receptor in Lipid Rafts

Lipid rafts are part of the plasma membrane that are made up of sphingolipids and cholesterol in the outer exoplasmic leaflet and phospholipids and cholesterol in the inner cytoplasmic leaflet of the lipid bilayer [53]. Flotillin and caveolin are support proteins, which are located within the planar lipid rafts[54]. Lipid rafts play an important role in many cellular processes, including signal transduction, membrane sorting and trafficking, and cell polarization [53]. Lipid rafts are first assembled in the Golgi complex in mammalian cells after which they are transported to the plasma membrane where they carry out their functions[55].

In some cell types, such as dopaminergic cells, hippocampal cells, and cells in the cerebral cortex[56], the mAR are localized to plasma membrane lipid rafts, a microdomain involved in cellular signaling (**Figure 5)** [51]. The androgen receptor variant present in the lipid rafts is the AR45, which has been seen in the lipid rafts of dopaminergic N27 cells and substantia nigral brain tissue from young and middle-aged rats in the absence and presence of sex hormones [51]. It has also been identified that AR45 forms a complex in lipid rafts with NOX1 and NOX2 [57], which have all been implicated in mediating androgen's negative effects [57]. The involvement of the lipid rafts have been identified in several diseases including AD, PD, and VaD [53].

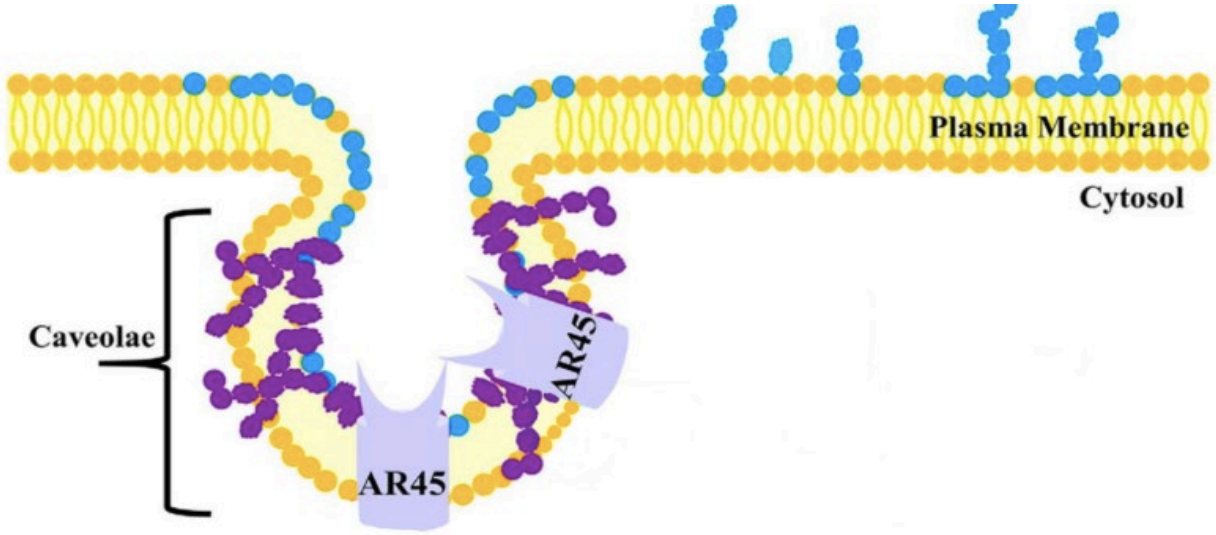

Figure 5: **Localization of Androgen Receptors in the lipid rafts within the plasma membrane.** The cholesterol-rich lipid rafts are involved in cellular signaling and are housed in the plasma membrane. The lipid rafts contain some receptors such as the androgen receptor variant (AR-45) that is involved in mediating the effects of androgens.

1.7 Cholesterol and Neurodegeneration

Statins are a class of drugs that blocks the conversion of Hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) to mevalonic acid in the mevalonate pathway (**Figure 6**), thus they are referred to as HMG-CoA reductase inhibitors [58]. They are used for lowering serum cholesterol levels and other cardiovascular interventions [59]. There is evidence showing that individuals with hypertension and hypercholesterolemia, resulting from high levels of cholesterol, are at a higher risk for VaD and AD [60, 61]. Since statins may decrease AD risk and progression by lowering cholesterol levels and delaying amyloid beta plaques production [62, 63], recent studies have focused on understanding the efficacy of statins in the prevention of neurodegenerative diseases [64]. Currently, there are no therapeutic drugs for many neurodegenerative diseases, such as VaD [62]. However, epidemiological studies indicate that statins are associated with decreased risk of these diseases [65]. In a meta-analysis that was comprised of observational studies and a

randomized control study, it was observed that statins were associated with decreased cognitive impairment disorders [65], thus, repurposing statins for neurodegenerative disease therapeutics could be advantageous.

1.8 Statins and the Brain

Even though the mechanisms of statin-associated neuroprotection are not well understood [64], lipophilic statins can impact brain function. Currently, there are no studies published on the uptake, distribution, and metabolism of statins in the brain, but lipophilic statins can cross the Blood Brain Barrier (BBB) more freely than hydrophilic statins (**Table 1**) by passive diffusion [62, 66]. Indeed, studies using the *in situ* rat brain perfusion technique observed increased radiolabeled lipophilic statins (Lovastatin and Simvastatin) in rat cerebral cortical brain tissue, indicating that lipophilic statins can cross the BBB [64]. However, this effect on the BBB was not observed with the radiolabeled hydrophilic statin, Pravastatin [64]. Therefore, one potential mechanism of action for statin-associated neuroprotection could be the ability of statins to cross the BBB.

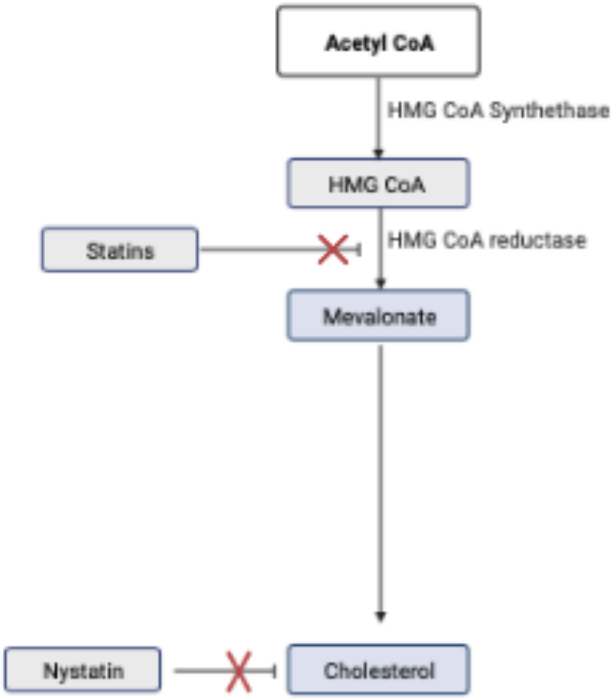

Figure 6: Statins inhibition of cholesterol biosynthesis. Statins inhibit the mevalonate pathway by inhibiting the enzyme HMG-CoA reductase, thereby blocking the conversion of HMG CoA to Mevalonate. Nystatin serves as a cholesterol sequestering agent, thus influencing cholesterol availability.

The impact of lipophilicity of statins on neuroprotection was observed in both large retrospective cohort studies and cross-sectional studies, which found an association between the lipophilic statins (e.g., Lovastatin, Simvastatin, Atorvastatin) and decreased incidence of dementia [65, 67]. Interestingly, race and sex may play a role in lipophilic statin-associated decreased dementia. Studies using lipophilic statins found an association with decreased AD risk in Caucasian and Hispanic men and women, along with Black women [62, 68]. In contrast, statins have not been associated with a reduction of AD risk in Black men, regardless of lipophilicity [62]. More studies need to be conducted to examine the influence of sex and race on statin neuroprotection, as statins could have different effects on dementia risk in diverse patient populations. Generally, statin concentrations ranged between 100 nM and 1 μM in studies showing

a neuroprotective benefit [64]. Although statins have been linked with decreased dementia, statins have also been associated with reversible memory loss.

Table 1: Statins and the Blood-Brain-Barrier (BBB) permeability	
BBB Permeable Statins	BBB Non-Permeable Statins
Lovastatin	Pravastatin
Simvastatin	Rosuvastatin
Atorvastatin	Fluvastatin
Cerivastatin	

Table 1. Statins that cross the blood-brain-barrier (BBB) include Lovastatin, Simvastatin, Atorvastatin, and Cerivastatin while statins that do not cross the BBB are Pravastatin, Rosuvastatin, Fluvastatin. Therefore, BBB crossing statins may have increased clinical efficacy for the treatment of neurodegenerative disorders.

These differing effects of statins on cognitive function may be related to the concentration of the statin [64]. During the safety and tolerability testing of statins, statins were associated with reversible cognitive impairment [65]. These short-term reversible cognitive impairments could be observed shortly after the initiation of statin therapy or after an increase in the dosage of a statin [65]. The mechanism for statin's adverse effects on cognition is under active investigation. In addition to the statin dose, the characteristics of the statin may play a role. Since reports of cognitive dysfunction were associated with Atorvastatin and Simvastatin [65], the lipophilicity of the statins and their ability to cross the BBB may play a role. Therefore, further research needs to be conducted to examine the impact of statins on multiple variables, such as drug dose and patient population characteristics (e.g. race, sex, pre-existing conditions, and drug metabolism abilities) [65]. This is of utmost concern as 32 million Americans are prescribed statins [65].

It is important to note that although, cholesterol is a required intermediate in sex steroid synthesis, statins do not impact androgen production and serum androgen levels [59]. This was proven in a population-based, cross-sectional epidemiologic study which was carried out among 5,506 men and women between ages 30 to 79 who had been diagnosed with cardiovascular diseases [59]. Each participant was treated with statins which included atorvastatin, Fluvastatin, lovastatin, pravastatin, rosuvastatin, and simvastatin. Before and after the study, testosterone, sex hormone–binding globulin (SHBG), and dehydroepiandrosterone sulfate (DHEAS) levels were measured, and results showed that therapeutic concentrations of statins do not impact androgen synthesis and androgen serum levels [59]. It has been suggested that the mechanisms by which prostatic tissue maintains tissue androgen levels may include metabolism of androgens in the adrenal glands of adrenal or the *de novo* synthesis from cholesterol [59].

CHAPTER 2

THE ROLE OF LIPID RAFTS IN ANDROGEN'S NEUROTOXIC EFFECTS

2.1 SPECIFIC AIMS

We ***hypothesize*** that cholesterol-rich caveolar lipid rafts are necessary for androgens to induce OS generation in neurons via the mAR in the plasma membrane.

SPECIFIC AIMS 1: Determine the impact of the lipid rafts on the mAR-mediated OS signaling in neurons.

SPECIFIC AIMS 2: Examine the role of lipid rafts on localization of the mAR to caveolar lipid rafts in neurons.

2.2 MATERIALS AND METHODS

Reagents:

Testosterone (A6950-000) was obtained from Steraloids. Goat anti-Rabbit (sc-2004), androgen receptor C-19 (sc-7305) were obtained from Santa Cruz and Caveolin-1 (3267) antibodies were obtained from Cell Signaling. Beta Actin (GTX629630) was obtained from GeneTex and Spectrin Antibody (MAB1622) was purchased from Millipore. DMSO was purchased from VWR. RPMI 1640 and Penicillin-streptomycin (PS) were purchased from VWR. Fetal Bovine Serum (FBS) and phosphate buffer solution (PBS) were obtained from Corning. Charcoal-stripped Fetal Bovine Serum (CS-FBS) was purchased from Atlanta Biologicals. Pierce BCA Protein Assay Kit (23225), tris-buffered saline (TBS, BP2471), and Tween-20 (BP337), Nonidet P-40 lysis buffer were purchased from Thermo Fisher Scientific. SuperSignal West Pico/Femto chemiluminescent substrates, dithiothreitol (DTT) were obtained from Thermo Fisher Scientific. Tris, Any KD polyacrylamide gel, Tris-glycine buffer, and PVDF membranes were purchased from BioRad.

Testosterone was made using an ethanol vehicle (final concentration of ethanol < 0.001%). Since little to no albumin is present in healthy brains [69], hormones in the brain are not protein bound and considered free. In order to compensate for the albumin (2.1 g/dl) in the CS-FBS, we used the Vermeulen calculation to determine the appropriate hormone dosage to attain physiological brain hormone levels [70, 71]. Therefore, in CS-FBS 100 nM of testosterone is equivalent to 8 nM calculated free testosterone, which is consistent with LC-MS/MS brain testosterone levels in male rats (5-24 nM) [72-75].

Nystatin was used to sequester cholesterol in the lipid rafts. Nystatin is a polyene antifungal antibiotic, which also acts as a cholesterol sequestering agent. It functions by impacting cellular

function by sequestering cholesterol [76]. In our experiments, it can be seen that nystatin only serves to sequester cholesterol, without changing the concentration of cholesterol in the lipid rafts (**Figure 7**) ($F_{3, 8} = 0.010$, $p > 0.05$). This influences cholesterol's ability to interact and exert its effects on other membrane components. Cholesterol sequestration alters composition of the plasma membrane lipid rafts which is a bio signaling hub that houses many receptors, thus impacting the function of such receptors [77, 78].

In vitro cell culture:

The immortalized neuronal cell line (N27) was derived from female fetal rat mesencephalic tissue. The N27 cell line is positive for tyrosine hydroxylase (TH+) expression, an enzyme marker for dopamine neurons [79-82]. This cell line is also positive for the membrane androgen receptor, estrogen receptor alpha, and estrogen receptor beta [83]. N27 cells were cultured and maintained at 37℃ in 5% CO_2. Media used was RPMI 1640 medium supplemented with 10% FBS and 1% PS. N27 cells were only used within passages 13-19 to avoid changes in morphology.

Experimental Design:

N27 cells were plated onto 96-well and 6-well plates with RPMI 1640 media supplemented with 10% FBS and 1% penicillin/streptomycin and incubated to proliferate for 24 hours at 37°C, 5% CO_2. Prior to treatment for experimentation, the media was switched to RPMI 1640 with CS-FBS to avoid confounding effects due to the presence of hormones in the serum. Cells were either exposed to vehicle (media alone) or H_2O_2 as an oxidative stressor. Specifically, at 80% confluency the cells were treated with 18uM-30uM H_2O_2 to induce 20% cell loss and achieve the OS threshold necessary to see androgens negative effects on cells. Oxidative stress cells and control cells were exposed to the following treatments: 50uM of Nystatin (1hr), Nystatin and testosterone (24hr), positive control

(testosterone 24hr), negative control (media alone). Maintaining cells in charcoal-stripped FBS does not negatively impact cell viability, as the media still contain salt, glucose, amino acids, and other nutrients [83-85]. Following treatments, cells were analyzed for cell viability or protein expression of various markers. Our treatment paradigm is illustrated in (**Figure 8).**

Cell Viability Assay:

Cell viability was determined by MTT assay. Media was aspirated from all wells, replenished with 100 uL of RPMI-1640 phenol red-free medium, and supplemented with 10% CS-FBS, 1% PS, and 1% l-glutamine. This was followed by the addition of 20 uL of 5 mg/mL of MTT solution to each well. Experimental plates were then covered in foil to block additional light and incubated at 37 ºC with 5% CO_2 for 3 h. Following incubation, plates were read at an absorbance of 595 nm. The colorimetric intensity is directly proportional to the number of viable cells in each well. Readings from different treatment groups were then normalized to the control group to determine cell viability. Three different experiments were conducted on three separate plates; each n is a mean of eight wells per treatment group on one plate.

Cell lysate and Homogenization:

After treatments were carried out as stated in the experimental design, cells were placed on ice, washed with PBS, and lysed using Nonidet P-40 lysis buffer with a cocktail of dithiothreitol (1 μM), EDTA (1 mM), phosphatase and protease inhibitors (1:100). The lysates were homogenized and centrifuged at 12,753 rpm at 4°C for 20 minutes. The supernatant was then collected, and protein concentrations were measured using the Pierce bicinchoninic acid protein assay kit (BCA kit), according to the manufacturer's instructions.

Western Blot:

Equal amounts of (30 ug protein) denatured whole cell lysates were loaded into a Bio-Rad Any KD polyacrylamide gel, electrophoresed in a Tris-glycine buffer. Proteins were separated by SDS-PAGE at room temperature at 25 mA. Next, proteins were transferred for 2-3 hours at 80 V onto a polyvinylidene difluoride (PVDF) membrane at 4°C. Membranes were washed 3 times for 10 minutes each under agitation with Tris-buffered saline with Tween 20 (TBST) and then, membranes were blocked for 1 hour with 5% nonfat milk in TBS-Tween at room temperature. Following blocking, the membranes were incubated with specific primary antibodies (AR C-19 1:1000 to examine the membrane androgen receptor, Spectrin 1:1000 to examine caspase-3 activity, Beta Actin 1: 2000 for protein normalization, and caveolin-1 1: 1,000 to examine lipid rafts) in TBS-Tween with 1% nonfat milk overnight in 4℃. Afterward, the membranes were washed every 10-minutes for 30 minutes and then incubated with secondary antibodies (1:5000 dilution for goat anti-rabbit and goat anti-mouse) in TBS-Tween with 1% nonfat milk for 30 minutes at room temperature. After washing membranes three times for 30 minutes, protein bands on the membrane were visualized using an enhanced chemiluminescence detection assay (Thermo Fisher Scientific). Protein band intensities were imaged using GeneSys software corresponding with the Syngene G-Box Chemi XRQ system. Protein band densities were quantified by NIH Image J densitometer software and normalized to Beta Actin for whole cell lysates.

Human Cases:

Associations between cholesterol (hyperlipidemia), testosterone, and cognitive status in men and women over the age of 50 years who participated in the Texas Alzheimer's Research

Care and Consortium (TARCC) were quantified [86]. Non-fasting peripheral blood samples were collected at the time of interview, as detailed in our previous publication [86]. Total testosterone plasma levels were separated into two groups based upon mean distribution/sex: low testosterone (< 2.6 ng/ml for men; < 1.2 ng/ml for women) and normal testosterone (≥ 2.6 ng/ml for men and ≥ 1.2 ng/ml for women). Cognitive function was assessed using the Clinical Dementia Rating Sum of Boxes (CDRSUM), which is widely used to stage severity of cognitive impairment over time [87].

Statistical Analysis:

Analysis was performed using IBM SPSS Statistics version 21 software. A 1-way ANOVA was used to analyze the factors of OS and treatment. Tukey's LSD was used for post-hoc analysis. Data were expressed as mean ± SEM. Significance was set at $p \leq 0.05$.

2.3 RESULTS

Testosterone, under high cholesterol conditions, is associated with cognitive decline in men greater than 50 years of age

In order to examine the impact of hyperlipidemia and testosterone levels on cognitive function, individuals who self-reported hyperlipidemia status were examined for cognitive decline in THE TARCC. Neither hyperlipidemia status ($F_{2, 2179} = 0.573$, $p > 0.05$) nor sex ($F_{1, 2179} = 0.337$, $p > 0.05$) impacted cognitive function. However, when individuals were grouped based on sex and their circulating plasma testosterone levels where low testosterone (women is <1.2 ng/ml and men < 2.6 ng/ml) and normal testosterone (women >1.2 ng/ml and men > 2.6 ng/ml), an interaction

between sex and hyperlipidemia status on cognitive function was observed ($F_{1, 640} = 3.076$, $p = 0.08$) (**Figure 9**).

Sequestering Cholesterol blocks testosterone's negative effects in an oxidative stress environment

In order to examine the impact of lipid rafts on testosterone's negative effects in an OS environment, we exposed the cells to the oxidative stressor, H_2O_2, to decrease cell viability in N27 cells by 20-30% ($F_{5,12} = 51.441$, $p \leq 0.05$). An OS burden of 20-30% cell loss is necessary to observe testosterone's neurotoxic effects instead of testosterone's neuroprotective effects [6]. Post-hoc analysis showed that testosterone exacerbated H_2O_2 induced cell loss and a significance was observed in this group compared to the other groups. Since the membrane androgen receptor (i.e. AR45, a 45 kDa MW androgen receptor variant) resides within a cholesterol-rich caveolin-1 lipid raft [51], we used nystatin to deplete cholesterol. We observed that when cells are pretreated with nystatin to deplete cholesterol that testosterone in an OS environment was unable to exacerbate H2O2-induced cell loss (**Figure 10**). These results support the role of testosterone acting through a membrane androgen receptor localized within a cholesterol-rich lipid raft.

Sequestering Cholesterol decreases caveolin-1 lipid rafts and membrane androgen receptors

To determine the role of caveolin-1 lipid rafts and its associated membrane androgen receptors on OS and testosterone's ability to worsen H_2O_2-induced OS, we exposed cells to nystatin, a cholesterol-sequestering agent. OS (H_2O_2) did not impact caveolin-1 expression in N27 cells. In Post-hoc analysis, no significance effect on caveolin was shown when cells were exposed to OS, thus suggesting that OS does not impact caveolin-1 expression. There was an overall effect

of treatment (nystatin) on caveolin-1 expression ($F_{5,24} = 4.350$, $p \leq 0.05$). These results show that nystatin decreased caveolin-1 lipid rafts, regardless of OS or hormone treatment (**Figure 11**).

In addition to nystatin impacting lipid rafts, we examined the effects of nystatin on the expression of the membrane androgen receptor that is localized to lipid rafts. OS (H_2O_2) had no effect on membrane androgen receptor expression in N27 cells, which is consistent with our previous publications[51, 57]. In Post-hoc analysis, no significance effect on androgen receptor expression was shown when cells were exposed to OS, thus suggesting that OS does not impact androgen receptor expression. There was an overall effect of nystatin on membrane androgen receptor expression ($F_{5,24} = 4.350$, $p \leq 0.05$). These results indicate that nystatin decreases membrane androgen receptors that reside within caveolin-1 lipid rafts (**Figure 12**).

Sequestering Cholesterol decreases apoptosis in oxidative stressed N27 cells exposed to testosterone

To determine the impact of sequestering cholesterol on testosterone-induced cell loss, which occurs via caspase-3 apoptosis[83], cells were treated with nystatin to sequester cholesterol. OS (H_2O_2) significantly increased caspase-3 mediated apoptosis in N27 cells ($F_{5,12} = 17.036$, $p \leq 0.05$). Post-hoc analysis showed that testosterone exacerbated H_2O_2 induced apoptosis. However, this effect of testosterone in an OS environment was blocked by decreasing lipid rafts and membrane androgen receptors by sequestering the cholesterol in the cells with nystatin (**Figure 13**). When the full-length Spectrin was also normalized with actin, no effect of testosterone was seen ($F_{5,12} = 2.850$, $p>0.05$).

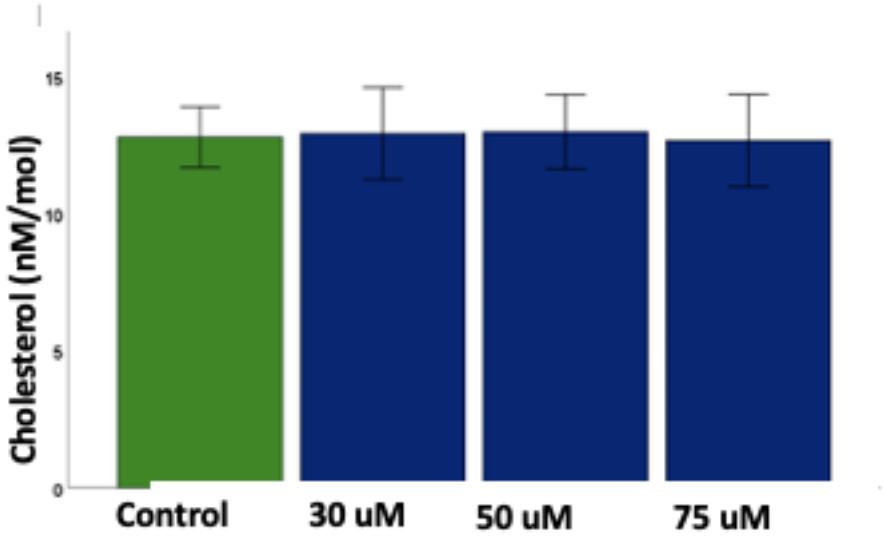

Figure 7: Cholesterol Sequestering and Cholesterol Concentration. Treatment with nystatin does not decrease cholesterol concentration regardless of the concentration. The n/group = 3

Figure 8: **Treatment Timeline for N27 Cells.** N27 cells were plated with FBS-media for 24 hours. After 24 hours, at 80% confluency, cells were treated with and without H_2O_2 (1 hour) to induce 20% cell loss, the OS threshold necessary to see androgens negative effects. Following H2O2 treatment, cells were treated with and without nystatin (1 hour) in addition to H_2O_2. After nystatin treatment, cells were exposed to testosterone or vehicle control for 24 hours. All treatments were done using CS-FBS that does not contain hormones to avoid confounds.

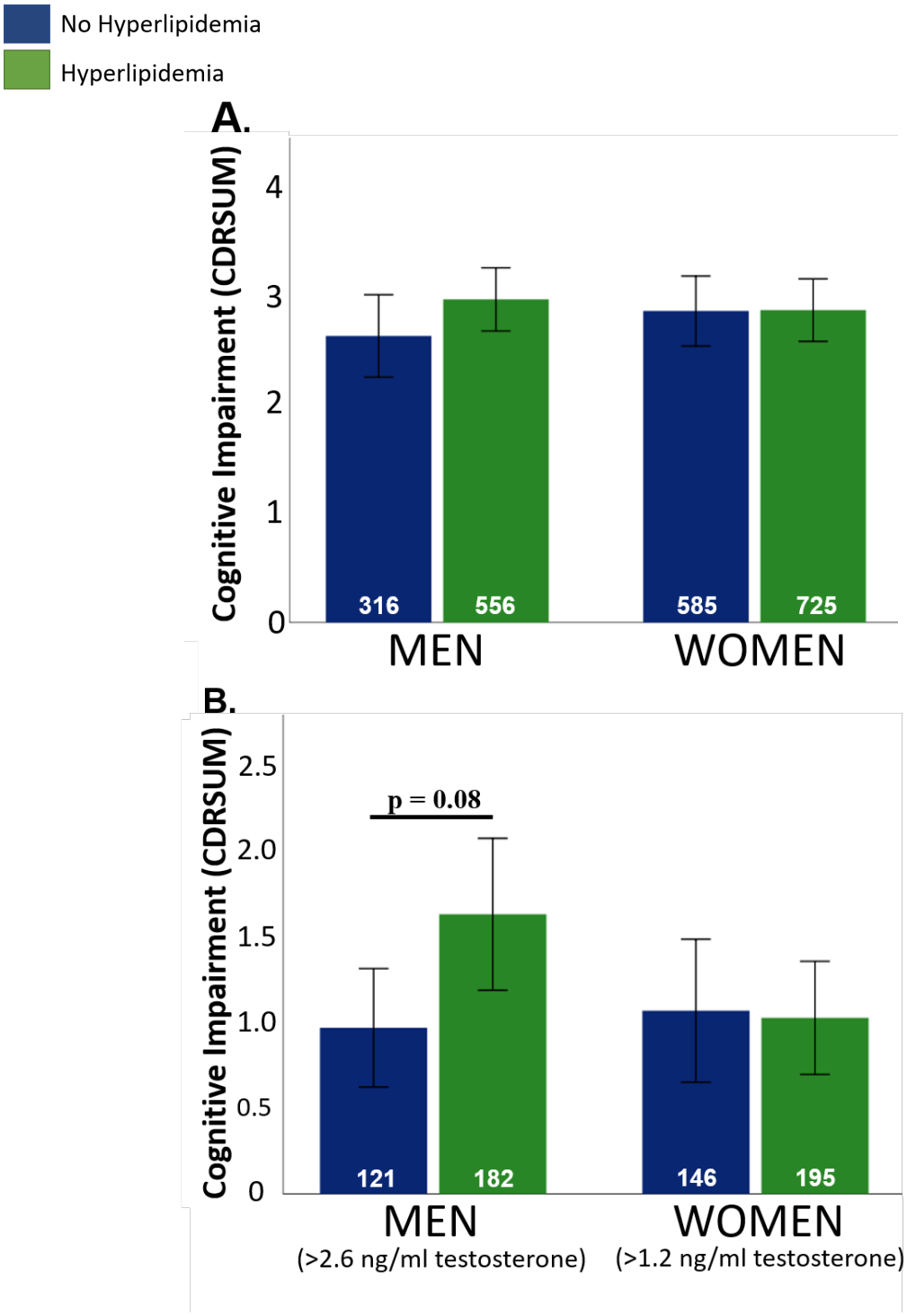

Figure 9: **Hyperlipidemia, sex, testosterone, and dementia**. No associations between cholesterol (hyperlipidemia) status and cognitive impairment in men and women of the age of 50 years old (A). Cholesterol is associated with dementia in men with elevated testosterone. This association is not present in women, regardless of cholesterol or testosterone status (B). The n/group is indicated on the bars.

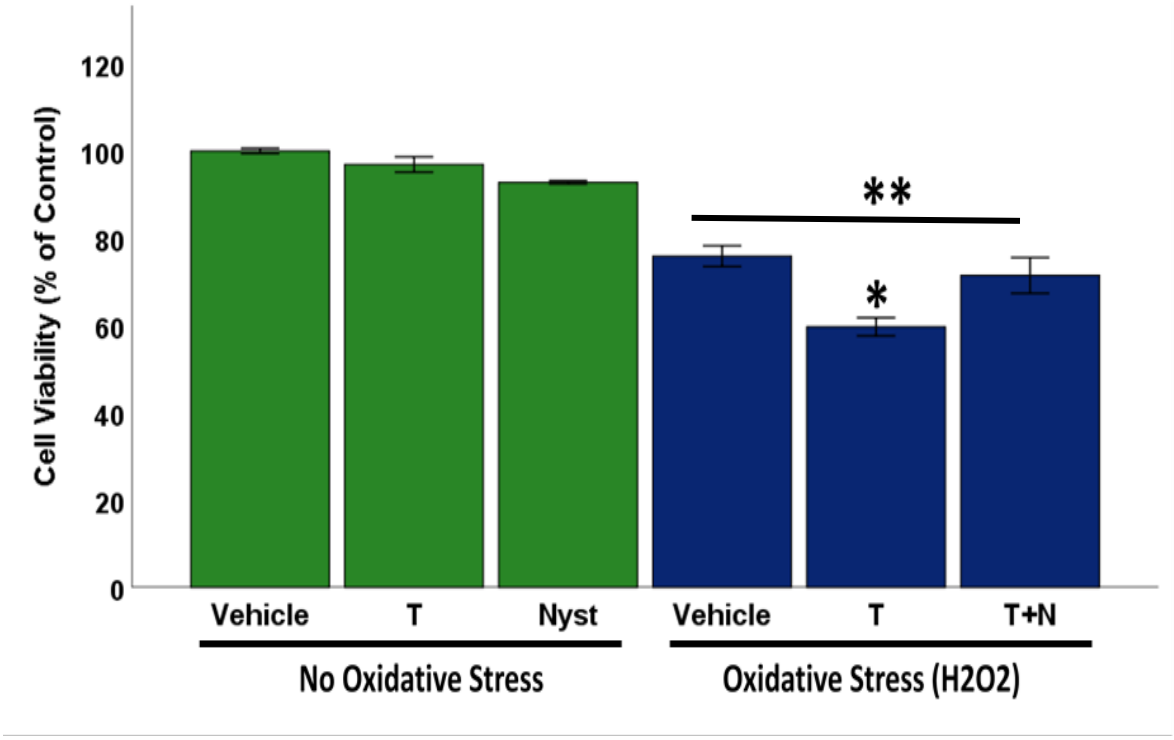

Figure 10: Testosterone, oxidative stress, cholesterol depletion, cell viability. Testosterone, alone, has no negative effects. In an oxidative stress environment testosterone exacerbates H_2O_2- induced cell loss in N27 cells. Cholesterol depletion via nystatin blocks testosterone's negative effects and increases cell survival. ** oxidative stress vs. no oxidative stress; * vs other treatments. $P \leq 0.05$. The n/group = 3

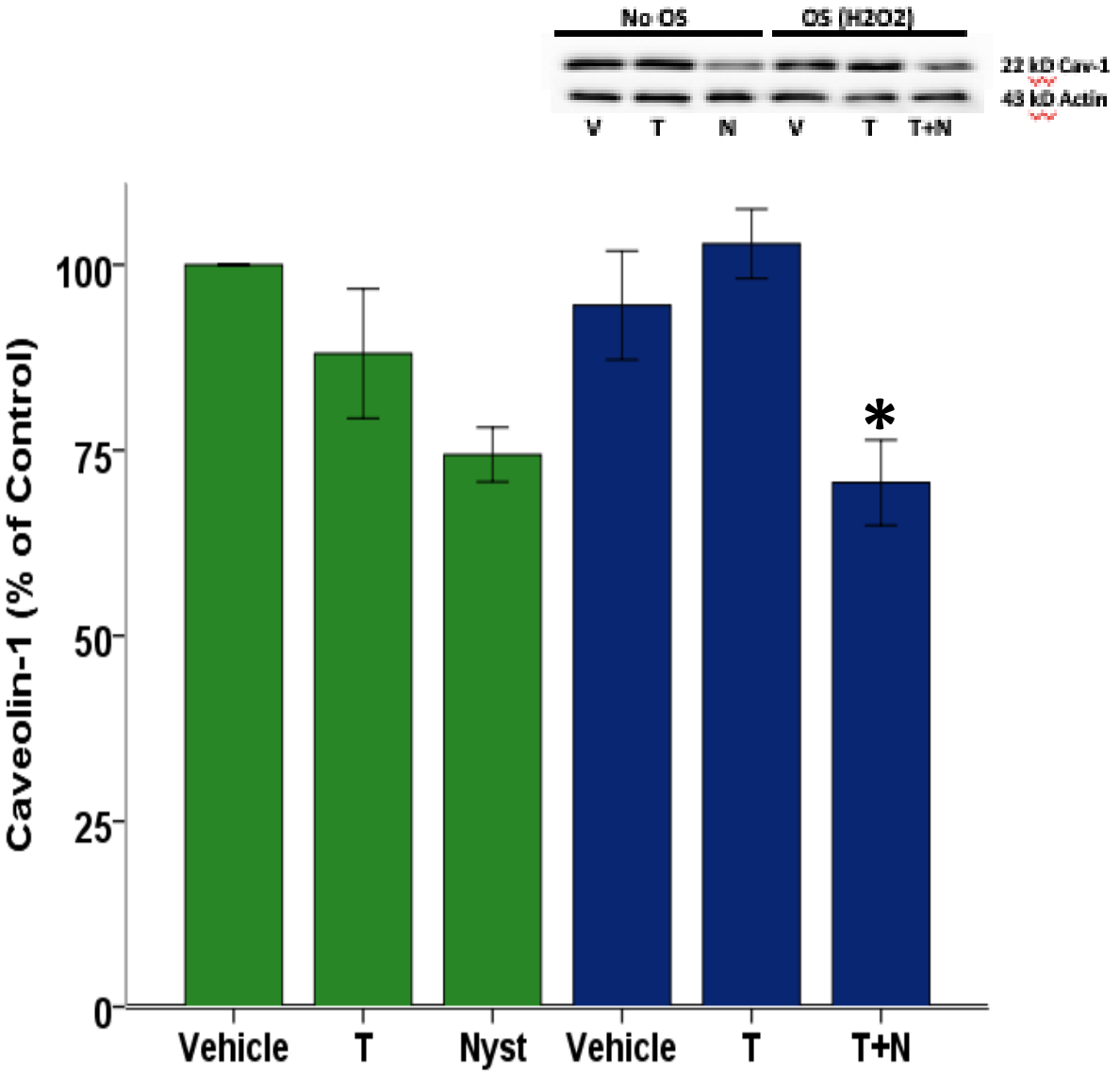

Figure 11: Testosterone, oxidative stress, cholesterol depletion, caveolin-1 lipid rafts. Neither oxidative stress nor testosterone alters caveolin-1 lipid raft expression. Cholesterol depletion by nystatin decreased caveolin-1 expression. * vs other treatments (vehicle and T) in the groups (no oxidative stress and oxidative stress). $P \leq 0.05$. The n/group = 5

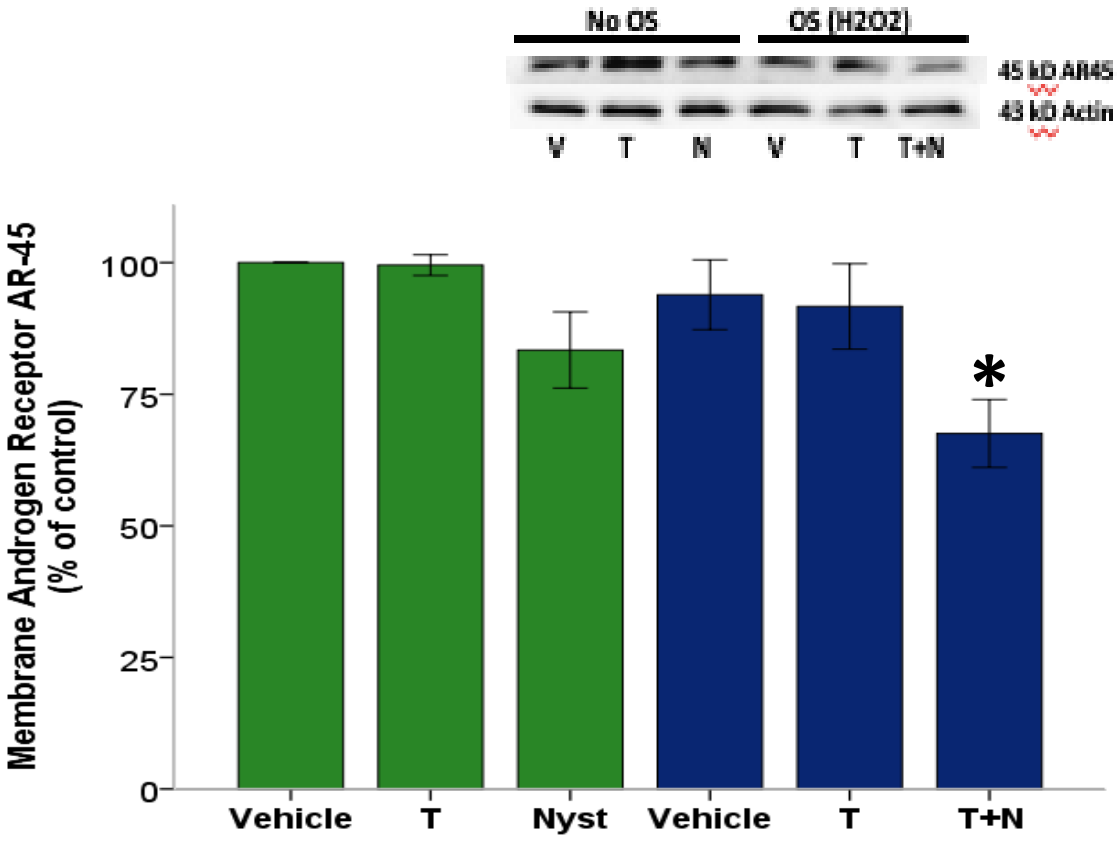

Figure 12: Testosterone, oxidative stress, cholesterol depletion, membrane androgen receptor (AR45). Neither oxidative stress nor testosterone alters membrane androgen receptor expression. Cholesterol depletion by nystatin decreased membrane androgen receptor expression. * vs other treatments (vehicle and T) in the groups (no oxidative stress and oxidative stress). $P \leq 0.05$. The n/group = 5

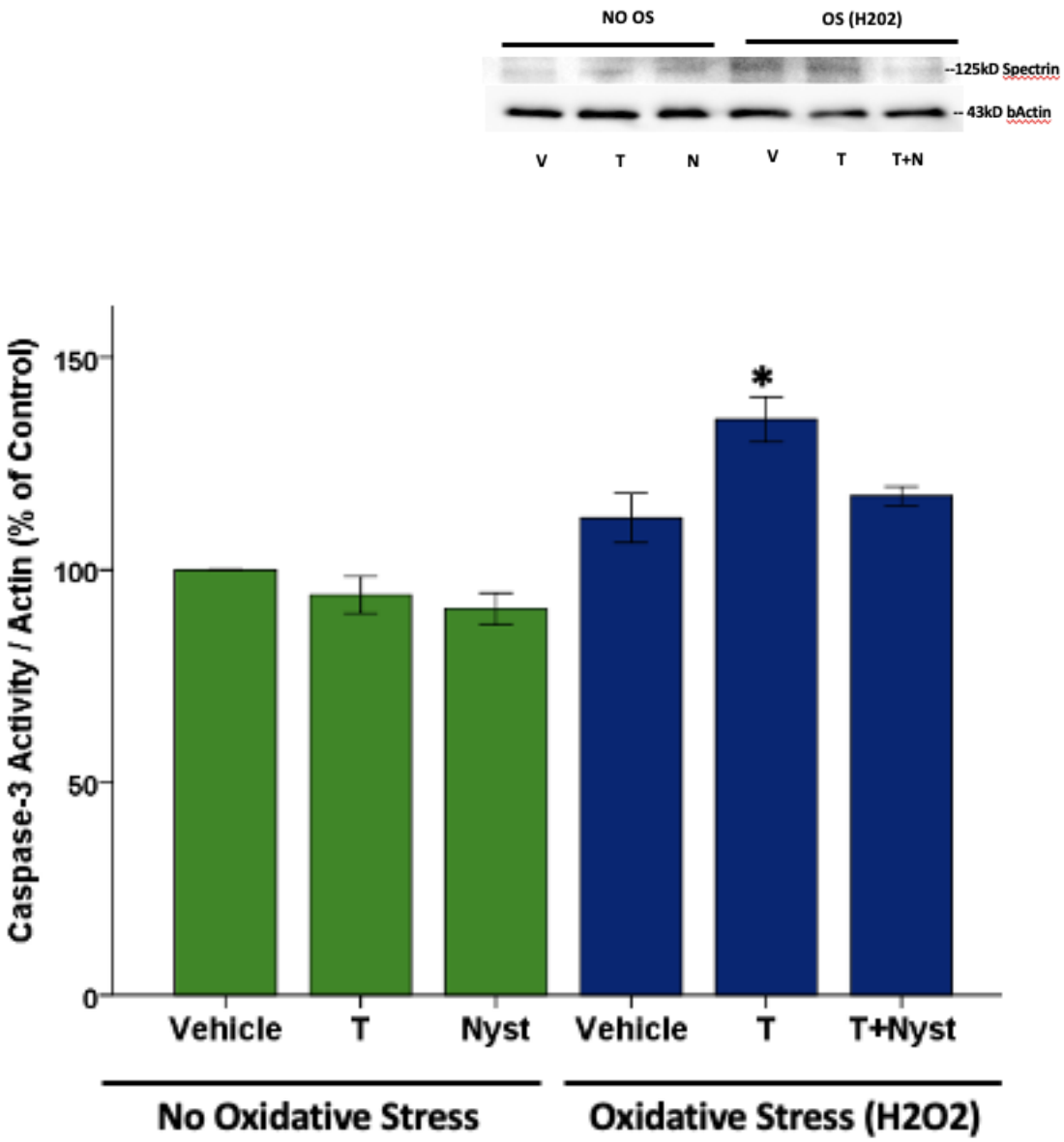

Figure 13: Testosterone, oxidative stress, cholesterol depletion, caspase-3 activity. Caspase-3 activity is not increased in the absence of oxidative stress. In the presence of oxidative stress, testosterone exacerbates oxidative stress induced caspase-3 activity. Depletion of cholesterol by nystatin blocks testosterone-induced caspase-3 activity in the OS environment.* vs other treatments (Vehicle and T) in oxidative stress. $P \leq 0.05$. The n/group = 5

CHAPTER 3

DISCUSSION AND CONCLUSIONS

The prevalence and incidence of VaD increases significantly after age 65 [17]. However, the incidence of VaD is higher in men regardless of age [88]. Sex hormones, such as androgens, have been implicated in this disproportion [88]. Androgens are steroids that are essential for male health and development [89]. In an OS environment, androgens can worsen cognitive impairment in neurodegenerative diseases in a dose-dependent manner [90]. Androgen's actions on the brain are complex, as they have been shown to have both detrimental and neuroprotective effects, based on different factors such as age, dose, and disease state [90, 91]. VaD impacts different parts of the brain including the hippocampus (memory), hypothalamus, and the frontal lobe (executive function) in the cerebrum [92], and all these brain areas have androgen receptors that can respond to androgens.

The major findings of this study are 1) cognitive impairment is associated with hyperlipidemia in aged men with greater than the mean testosterone levels (2.6 ng/ml circulating testosterone), 2) cholesterol sequestering blocks testosterone exacerbation of OS induced apoptotic cell loss, and 3) cholesterol sequestering decreases caveolin-1 lipid rafts, resulting in decreased membrane androgen receptor expression. These findings indicate that localization of the membrane androgen receptor to cholesterol-rich lipid rafts is necessary for testosterone's neurotoxic effects in an OS environment. Furthermore, the association between cognitive impairment, hyperlipidemia (or high cholesterol), and testosterone in aged men could be due to increased expression of lipid rafts containing membrane androgen receptors. Our previous studies

have shown that testosterone is associated with cognitive impairment in aged men with elevated OS [86], and that the membrane androgen receptor is expressed in hippocampal post-mortem tissue obtained from donors diagnosed with AD [6].

This is the first study to show that lipid rafts are necessary for testosterone's neurotoxic effects in an OS environment. Our previous studies have shown that degrading the membrane androgen receptor was sufficient to block testosterone's activation of a cell signaling cascade that can exacerbate OS signaling through the NADPH oxidase signaling cascade [57]. However, inhibition of androgen receptors may not be clinically appropriate as androgens also have neuroprotective effects [91], and the loss of these neuroprotective actions of testosterone could negatively impact health. Therefore, the purpose of these experiments was to determine how to interfere with androgen's neurotoxic effects in an OS environment without impacting overall androgen signaling. Based on our data showing that aged men with hyperlipidemia and elevated androgens had increased cognitive impairment, we focused on the membrane androgen receptor that is localized to cholesterol-rich lipid rafts in the brain [93].

In men above the age of 50 years, we found that the relationship between high cholesterol and cognitive function in high OS environment is driven by testosterone. Although prior studies have shown that hyperlipidemia is a potential risk factor for dementia [94], this study focuses on the effect of high levels of testosterone on cognition in the presence of high cholesterol and OS. Our results show that there are no associations between cholesterol levels and cognitive impairment in men and women 50 years old and greater with normal testosterone levels. However high cholesterol is associated with cognitive impairment in men over 50 years of age with circulating testosterone levels greater than the mean (e.g., >2.6 ng/ml circulating testosterone). This association was not noticed in women with testosterone levels greater than the mean (e.g., >

1.2 ng/ml circulating testosterone). Our results show that decreasing membrane androgen receptors by decreasing cholesterol-rich lipid rafts blocked the neurotoxic effects of androgens in an OS environment. These results suggest that cholesterol depletion by statins may have a beneficial effect in individuals that exhibit elevated OS and androgens. A few epidemiological studies have examined the efficacy of statins in treating AD [95]. While some studies have shown success for statins to decrease the risk of AD [96], others have not [97, 98]. Our results suggest that these equivocal findings in the different epidemiological studies may be because sex was used as a covariate and not as an independent variable. Epidemiological studies may be more successful in showing a decrease in the risk of AD after statin use if sex and gonadal hormone status are independently examined.

This study has provided evidence that cholesterol is essential for membrane androgen receptor cell signaling. Nystatin, a cholesterol sequestering agent [76], was used to determine whether sequestering cholesterol in lipid rafts can alter cell signaling through the membrane androgen receptor and improve cell survival. Nystatin sequestered the cholesterol-rich lipid rafts and blocked the negative effects of testosterone on cell viability in an OS environment, indicating that the cholesterol-rich lipid rafts are necessary for membrane androgen receptors to mediate androgen's neurotoxic effects. As a result of the function and structure relationship that is observed between the cell viability of neuronal cells and the sequestering of cholesterol-rich lipid rafts, as evidenced by the reduction in expression of caveolin-1 and membrane androgen receptor, it can be concluded that testosterone's negative effects are dependent on the membrane androgen receptor being localized to caveolin-1 lipid rafts.

Cholesterol is involved in the steroidogenesis of steroid hormones including androgens [99]. Since statins can lower plasma cholesterol levels [100, 101], statins impact on

steroidogenesis has been examined. Prior studies have found that statins do not impact androgen production and serum androgen levels [59]. It has been suggested that the mechanisms by which prostatic tissue maintains tissue androgen levels may include metabolism of androgens in the adrenal glands of adrenal or *de novo* synthesis from cholesterol [59].

Based on the results of our studies, testosterone can increase the risk of dementia in men with hyperlipidemia. Our results suggest that cholesterol-lowering agents, such as statins, may be a useful therapeutic agent for older men with cognitive disorders such as VaD that are impacted by high levels of cholesterol and elevated androgens.

CHAPTER 4

LIMITATIONS AND FUTURE STUDIES

Our study has many strengths, which include using *in vitro* and human models to study the effects of cholesterol on androgen's neurotoxic effects in high OS environment. There are some limitations in this study that include the fact that N27 cells are derived from female rat mesencephalic tissues, and we did not include a cell line from a male rat. However, we have previously published that testosterone in an OS environment negatively impacts cell viability through the membrane androgen receptor, regardless of the sex (XX vs XY) of the cells [6].

Another limitation is that under *in vivo* conditions, cells interact through a network of various cell types. However, *in vitro* models fail to capture the inherent complexity of organ systems and biochemical processes that take place during metabolism, thus influencing results. Because *in vitro* studies involve isolating cell lines, thus depriving them from the interaction that they have with other cell types and thus influencing results, our future studies will be transferring our *in vitro* model results to *in vivo* models.

Our future clinical studies will include examining the expression of caveolin-1 lipid rafts and membrane androgen receptors in post-mortem tissue from donors with dementia and hyperlipidemia with and without cholesterol reducing medications (e.g., statins). This will allow us to determine if men with hyperlipidemia are at an increased vulnerability due to higher circulating androgens and increased membrane androgens receptor expression in caveolin-1 lipid rafts that can result in deleterious membrane androgen receptor signaling.

CHAPTER 5

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