

THE EFFECTS OF OXIDATIVE STRESS AND TESTOSTERONE ON DOPAMINE
NEURON VIABILITY: IMPLICATIONS FOR PARKINSON'S DISEASE
DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
Health Science Center at Fort Worth
in Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

By

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Fort Worth, Texas

September 2015

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Rebecca L. Cunningham for this opportunity, allowing me to use her lab for my doctoral studies. She is an amazing person that has encouraged me to explore research ideas and network opportunities that have lead to my growth as a student and individual. Rebecca has been one of the most influential individuals throughout this endeavor and for that, I thank you. I look forward to continuing the relationship.

I would like to thank my committee members, Drs. Meharvan Singh, Derek Schreihofner, Harlan Jones, Robert Luedtke, and Hassan Alizadeh for their encouragement and guidance throughout this process. They have encouraged me to explore problems and influences from research literature, which has allowed me to expand my understanding of this material. Their guidance is greatly appreciated.

As a first generation student, my parents have always supported higher education. With that said, I would like to thank my parents, Samuel and Jeanette Holmes for encouraging education. Thank you dad for buying me a chemical lab set as an adolescent to fuel my science curiosity. Thank you mom for always encouraging me to be self-sufficient and goal-oriented. I am truly thankful for them supporting my venturing endeavors and giving me a solid foundation to achieve my goals. You have always been my major supports and for that, I love you.

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CHAPTER I

INTRODUCTION

PARKINSON'S DISEASE HISTORY

In 1817, James Parkinson was the first to identify PD as shaking palsy [1]. Parkinson identified shaking palsy by his observations of patients with involuntary tremor marked by reduced muscle power [2, 3]. In addition, Parkinson's essay identified a unilateral onset of motor symptoms including tremor, rigidity, postural and gait difficulties [3]. In contrast, another physician, Jean-Martin Charcot, discovered through his observations that patients were not weak but slow to execute movement [2]. Further, Charcot observations lead him to reject shaking palsy and accept Parkinson's disease (PD) [1, 2].

In 1912, Fritz Lewy pathologically characterized PD to have intracellular inclusions [1]. Konstantin Tretiakoff identified these inclusions as *corps de Lewy* meaning Lewy Bodies [4]. The composition of Lewy Bodies was not understood until Spillantini et al., 1997 determined these inclusions to be a complex of alpha synuclein and ubiquitin [1, 5]. Currently, alpha synuclein is a prominent marker for identifying Lewy Bodies in PD. It wasn't until Lewy's death that *corps de Lewy* became the basis for PD diagnosis post-mortem [1].

More pathological findings recognized neuronal loss in the substantia nigra pars compacta (SNc) [6, 7]. The SNc is a component of the basal ganglia motor circuitry, a cluster of

nuclei involved in the initiation and execution of movement [8-10]. Furthermore, Foix and Nicolesco discovered that the neurons in the SNc specific to this neuronal loss are the neuromelanin-producing dopamine neurons [1, 11]. These dopamine neurons terminate in the striatum forming the nigrostriatal pathway. Dopamine terminals in the striatum regulate corticostriatal communication [12]. Thus, dopamine is a neurotransmitter that regulates the cerebral cortex activation in facilitating movement [12]. Due to other speculation of what could be causing PD, Avid Carlsson and his colleagues discovered that the loss of dopamine is solely responsible for PD [1, 13, 14]. Hornykiewicz and others confirmed this loss of dopamine in PD [1, 15].

Due to these pathological findings in the 20th century, a primary interest was to replace dopamine to alleviate PD motor symptoms [1, 16]. As a result, levodopa (L-Dopa), a dopamine precursor that is permeable to the blood brain barrier, was used to increase dopamine in the brain [6]. L-Dopa is converted to dopamine by DOPA decarboxylase [17, 18]. Currently, this drug is often used in combination therapy to prevent its conversion in the periphery and increase its presence in the brain [19]. Although there is no cure for PD, other therapies can alleviate symptoms and adverse reactions that also target other neurotransmitters of serotonin, acetylcholine and norepinephrine relevant to the basal ganglia circuitry [1, 20]. Even though there are other neurotransmitters involved, the major focus of PD pathology encompasses dopamine neurons and its neurotransmission.

PARKINSON'S DISEASE

Today, PD is the second most common neurodegenerative disorder in the United States that annually affects approximately 60,000 people [1, 21]. Of this population, PD affects twice as

many men than women (Table 1) [22]. It is estimated that approximately 4% of people living with PD are diagnosed before the age of 50 [23]. The incidence of PD increases exponentially by the age of 60 [24, 25]. PD, categorized as being familial and sporadic, is predominantly sporadic by diagnosis [21]. The diagnosis of PD is difficult but is clinically identified by the presence of motor symptoms such as bradykinesia, tremor, muscular rigidity, and postural instability [1]. These motor symptoms lead to the loss of independence in the aging population [6]. People with PD experience a progressive decline in motor and cognitive functions with an increased mortality rate [6]. This loss of independence can result in a national economic cost of 8 billion dollars to the United States in 2010 [26]. This economic burden is projected to increase to over 18 billion by 2050 as depicted in Figure 1, along with the elderly population [26].

The exact cause of PD remains elusive. Scientists have speculated on the idea that toxins could be the cause of PD. In particular, the environmental toxin, MPTP selectively degenerates dopaminergic neurons in human and experimental models [27-29]. Even though this toxin produces PD-like symptoms, it is not likely to be responsible for dopamine's neurodegeneration in PD [28]. Thus, MPTP exposure and toxicity is rare, but it is commonly used to study PD neurodegeneration [1, 27, 30, 31].

Although the cause of PD is not known, it is known that other conditions can cause Parkinsonism. For example, ischemic stroke can lead to Parkinsonism [32-34], a condition characterized by the presence of two cardinal motor symptoms identified in PD [22]. An ischemic stroke is an interruption of the cerebral blood flow that deprives the brain of oxygen causing necrotic tissue [35, 36]. Due to the damage and depending on the infarct volume, a stroke could impair motor functions as a consequence of neuronal death [34]. Thus, an ischemic stroke can increase the risk of neurodegeneration associated with movement disorder,

Parkinson's disease [34]. Similar to PD, men have a higher risk than pre-menopausal women for ischemic stroke [37]. Since stroke increases oxidative stress, a key feature of PD, it could increase the risk for PD.

Since the cause of PD is unknown, PD is considered an idiopathic progressive neurodegenerative disease of dopaminergic neurons in the SNc [6, 25]. Pathologically PD is characterized by the molecular signaling of oxidative stress, inflammation, alpha synuclein and apoptosis [38-40]. Dopamine is susceptible to oxidative stress via autoxidation and its metabolism [41]. Dopamine has an electron rich moiety that allows it to readily oxidize creating reactive oxygen species in the cellular environment [41]. In addition, the monoamine oxidase enzyme metabolizes dopamine; this mechanism induces reactive oxygen species that could contribute to oxidative stress [42, 43]. Moreover, dopamine neurons produce neuromelanin, a dark pigment identified in the SNc, which interacts with metals such as iron to form reactive oxygen species thus promoting oxidative stress [28, 44-46]. Our focus is to examine the sex bias in dopamine neurodegeneration implicated in PD.

OXIDATIVE STRESS

Oxidative stress increases with age [47-49]. Interestingly, the oxidative stress theory of aging states that the accumulation of oxidative stress damage results in age-related functional loss [25, 50]. Thus, aging is a risk factor for oxidative stress and neurodegeneration [25, 51, 52]. Oxidative stress defined by reactive oxygen species and antioxidant mechanisms may underlie dopaminergic neurodegeneration [46]. Oxidative stress and its damage are evident in postmortem and PD models [53-55]. However, oxidative stress is essential for cellular signaling and energy production to maintain biological functions and cellular homeostasis [56].

A. REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are highly reactive molecules because they have one or more unpaired electrons rendering them unstable [57]. ROS consists of superoxide (O_2^-) and hydroxyl radicals (OH^\cdot), as well as the reduced metabolites of oxygen such as hydrogen peroxide (H_2O_2) [58, 59]. These oxygen species are produced continuously within the cells as a result of the intracellular metabolism, mitochondrial electron transport chain and enzymatic byproducts [39, 60]. Thus, ROS are necessary biological processes of development, neuronal signaling and memory [61]. However, ROS can be toxic to the cellular environment causing DNA damage and dysfunctional proteins [62]. Particularly, hydrogen peroxide is a weak oxidant but a stable molecule that is permeable to the cellular membrane [63]. Hydrogen peroxide can directly release iron, inactivate enzymes, and oxidize DNA and lipids [64]. Also, hydrogen peroxide can interact with a reduced metal, iron, to produce highly toxic hydroxyl radicals via the Fenton reaction (Figure 2) [24, 65]. Moreover, the mitochondria functions as a major source of ROS [66, 67]. In fact, superoxide and hydrogen peroxide are formed via leakage of electrons along the mitochondrial electron transport chain [67]. In addition, factors that stimulate respiration increase the potential of reactive species via the mitochondria [63, 68]. Thus, the mitochondrial electron transport chain plays a direct and indirect role in ROS production [69, 70].

B. ANITOXIDANTS

ROS is converted by antioxidants into non-toxic compounds [62]. Antioxidants donate protons to ROS, which are electronegative molecules, neutralizing the charge [62]. Major antioxidants identified in the cellular environment are superoxide dismutase and glutathione that

act as enzymatic scavengers to counteract reactive species and their precursors [59]. Glutathione is the most abundant antioxidant in the brain [71]. Thus, glutathione is the major thiol source and antioxidant that maintains the cellular redox status [62]. Glutathione is synthesized in the cytosol by a rate-limiting enzyme, γ -glutamylcysteine synthetase [62]. In addition, glutathione is oxidized via glutathione peroxidase to glutathione disulfide and reduced via glutathione reductase to glutathione [67]. Glutathione actions involve reducing hydrogen peroxide via its thiol group and acting as a co-substrate to inactivate hydrogen peroxide (Figure 3) [72]. In fact, two molecules of glutathione are required to remove one hydrogen peroxide from the intracellular environment (Figure 4) [64]. Glutathione is an important reducing agent that maintains enzymatic activity [73]. Since, glutathione is the prominent antioxidant in reducing oxidative stress, it can be used as an indirect measurement for oxidative stress. For example, a decrease in GSH represents a change in antioxidant defenses promoting oxidative stress [72].

C. OXIDATIVE STRESS LEVELS

The balance between levels of antioxidants to reactive oxygen species determines the level of oxidative stress (Figure 5) [56, 64]. In the aging brain, there is a reduced capacity for antioxidants and a decrease in radical scavenging [49, 74, 75]. When the antioxidants are insufficient to keep reactive oxygen species below a toxic threshold, oxidative stress increases [72]. Interestingly, alterations in oxidants and antioxidants are identified in PD [71, 73, 76, 77]. Reductions of glutathione in the substantia nigra suggest that oxidative stress is specific to PD and not a secondary effect in neurodegeneration [78, 79]. Oxidative stress induced increases in oxidative markers represent a decreased efficiency in the removal of damaged molecules and

thus oxidative damage [72]. In fact, there are high levels of oxidative stress and oxidative damage in PD substantia nigra [80, 81].

High levels of ROS have detrimental effects on cellular dynamics [61]. As a result, ROS can functionally modify lipids, proteins and DNA molecules [66, 82-84]. Oxidatively damaged lipids result in the loss of membrane fluidity, reduced membrane potential and increased membrane permeability to calcium [67]. Calcium is an intracellular messenger that can participate in cellular metabolism, survival and death [85, 86]. In addition, calcium accumulation is associated with high levels of oxidative stress, thus calcium potentiates oxidative damage [63, 86, 87]. Oxidative damage can activate and inactivate cellular proteins [67]. Oxidative repair mechanisms are critical to maintaining cellular homeostasis [88]. However, ROS damages DNA and the products of oxidatively damaged proteins contribute to the inactivation of DNA repair mechanisms [67, 89]. Also, ROS alters mitochondria respiration and changes the mitochondria permeability transition pore [39, 90, 91]. Opening of the permeability transition pore causes the mitochondria to depolarize, and release matrix solutes and substances into the cytoplasm [63, 92, 93]. Thus, high levels of ROS are closely associated with mitochondrial dysfunction involved in PD [94].

D. MITOCHONDRIA

The mitochondria are the cellular powerhouses [69]. Thus, they produce energy through its respiratory chain and participate in calcium metabolism [69, 70]. The mitochondria function as a source of ROS due to its electron transport chain, which generates superoxide that can be converted into hydrogen peroxide [78, 95-99]. In addition, the mitochondria are a target of ROS, because ROS can increase calcium mobilization, which interacts with the mitochondria [24]. The

mitochondria are dynamic organelles that can detect and respond to changes in the cellular environment [100]. The high levels of ROS can induce the release of calcium from intracellular endoplasmic reticulum stores [101, 102]. To maintain cellular calcium homeostasis, the mitochondria senses the rise in intracellular concentrations and functions as a buffer for cytosolic calcium [67, 103]. Calcium is transported into the mitochondria through uniporters acting as channels that open in response to the rise of cytoplasmic calcium concentrations [104, 105].

Disturbances in maintaining intracellular calcium homeostasis can lead to irreversible cell injury [106, 107]. Thus, calcium can mediate mitochondrial damage via inducing executioner enzymes and further generating ROS [47]. Furthermore, calcium increases the mitochondria permeability transition pore and uncouples respiration mediating mitochondrial dysfunction [63]. The high levels of ROS can increase oxidatively modified molecules and cellular dysfunctions that induce different signaling mechanisms of inflammation and apoptosis [41, 102].

INFLAMMATION

The pathogenic mechanisms responsible for PD are controversial, but there is strong evidence that oxidative stress and inflammation play a role in dopamine neurodegeneration. In fact, oxidative stress and inflammation are primary hallmarks identified in PD [108]. The chronic signaling of inflammation has been implicated in neurodegeneration and may play a critical role in the pathogenesis of PD [21, 109, 110]. Inflammation, a response to oxidative stress, is characterized by the production of cytokines, transcription factors, enzymes, prostaglandins, and oxidative stress [111]. Even though oxidative stress induces inflammation, inflammation can also increase oxidative stress to create a vicious cycle mediating cellular toxicity [9, 41].

A. INFLAMMATORY MEDIATORS

Transcription factors, such as Nuclear Factor kappa B (NFkB), can regulate neuronal responses by activating a variety of stimuli [112, 113]. NFkB is involved in the regulation of chronic diseases through its inflammatory signaling [114]. NFkB located in the cytoplasm resides as a heterodimer composed of p50 and p65 subunits, is bound to an inhibitor protein, Ikb, which renders it inactive [115-117]. NFkB is an oxidative stress responsive transcription factor with inducible activity present in most cells [115, 118, 119]. During the activation of NFkB via oxidative stress, Ikb is dissociated and the heterodimer is translocated to the nucleus, where it can bind to cognate DNA sequences [115, 120]. The substantia nigra dopaminergic nuclei are positive for inactive cytoplasmic NFkB [121]. Active Nuclear NFkB are identified in PD patients suggests that NFkB translocation is essential for its activation and pathophysiology in PD [121]. A potent NFkB inhibitor, CAPE protects dopaminergic neurons from Interferon-gamma/Lipopolysaccharide (IFN- γ /LPS) insults [122]. The nuclear expression and activity of transcription factor NFkB identifies it as an important factor in dopamine neurodegeneration.

As a transcription factor, NFkB can upregulate the transcription of target protein, inflammatory cyclooxygenase- 2 (COX2) [115]. Thus, nuclear NFkB and cytoplasmic COX2 protein are expressed in the same cerebral cortex neurons [115]. This identifies that nuclear NFkB could regulate the protein expression of COX2. COX2 has two NFkB binding sites in its promoter region to stimulate its transcription [115]. In fact, NFkB has a strong binding affinity for the COX2 distal promoter NFkB1 binding site [115]. COX2, constitutively expressed in neurons, participates in prostaglandin synthesis and neuronal damage [123, 124]. The expression and activity of COX2 is inducible in response to pro-inflammatory stimuli [125]. The expression of COX2 is increased within the SNc in PD [126, 127]. As an inflammatory enzyme, COX2 has

two enzymatic activities as a cyclooxygenase and peroxidase (Figure 6) [123]. COX2 is a cyclooxygenase that induces the synthesis of prostaglandins. Secondly, COX2, in prostaglandin synthesis, acts as a peroxidase releasing free radicals into the cytoplasm further contributing to oxidative stress [123]. Thus, the peroxidase activity of COX2 contributes to dopamine oxidation in the substantia nigra [126, 128]. Ibuprofen, a nonselective inhibitor of COX2, acts to inhibit its activity. In fact, ibuprofen increases dopamine survival against glutamate toxicity in dopaminergic neurons [129].

Since COX2 participates in dopamine oxidation, COX2 is closely associated with alpha synuclein formation and stabilization of synuclein oligomeric species [126-128]. Interestingly, alpha synuclein oligomeric intermediates are major toxic elements to neurons [130]. The formation of alpha synuclein aggregates is associated with oxidative stress [24]. The expression of COX2 has been co-localized with alpha synuclein oligomers in catecholamine neurons [127]. Alpha synuclein, a secondary hallmark to oxidative stress and inflammation, aggregates into Lewy bodies [131, 132]. In fact, alpha synuclein aggregates contributes to neuroinflammation and neurodegeneration [133].

Alpha synuclein, a small intrinsically unfolded cytoplasmic protein expressed in the brain is predominantly expressed in neurons and presynaptic terminals [5, 134, 135]. Alpha synuclein is understood to play a role in vesicular trafficking [132]. In fact, increased alpha synuclein accumulation and decreased ubiquitin function are evident in PD. Ubiquitin is a part of a proteasome system that degrades and removes damaged or misfolded proteins [136-138]. An increased level of oxidative stress causes the ubiquitin system to dysfunction and increases the likelihood of misfolded and aggregated proteins [78, 139]. Thus in oxidative stress, alpha synuclein aggregates and contributes to neuronal death potentially through mitochondria

dysfunction, impairment of ubiquitination and vesicular trafficking, and endoplasmic reticulum stress [132].

APOPTOSIS

Apoptosis is a fundamental process in living organisms that maintains the balance of cellular replication and optimizes cellular organization through selectively removing dysfunctional cells without causing further damage to the cellular environment [140]. Postmortem PD studies identify that dopaminergic neurons undergo apoptotic cell death [141, 142]. Oxidative stress triggers apoptosis potentially through the chronic signaling of inflammation [143]. In fact, the nuclear expression of NFkB is localized in dopaminergic neurons undergoing apoptosis [121]. Apoptosis, a programmed cell death, is characterized by nucleus shrinkage, condensation and fragmentation of chromatin, and DNA degradation [48, 144].

This process of apoptosis can be initiated through a mitochondrial-signaling pathway, since the mitochondria are affected by ROS-induced calcium signaling. The mitochondria intake of calcium competes for the cytochrome c binding site, resulting in cytochrome c being released into the cytoplasm to initiate executioner enzymatic signaling of caspases [47, 48]. Activated forms of caspase 9 are reported in dopaminergic neurons in PD [145]. Thus, initiator caspase 9 cleaves executioner caspase 3 that activates apoptosis [146]. Caspase 3 is highly expressed in dopaminergic neurons in PD [147]. The activation of caspase 3 is shown to precede apoptosis, signifying it is not a consequence of apoptotic signaling [147]. Executioner caspase 3, through the intrinsic mitochondria pathway, has been shown to increase apoptosis in response to oxidative stress [148]. Also, caspase 3 signals for apoptosis through the condensing of nuclear

chromatin [140]. Thus, the deleterious effects of ROS disrupt the mitochondria function via oxidative injury resulting in a programmed cell death of apoptosis.

ANDROGEN RECEPTOR

Androgen receptors are members of the steroid receptor family of transcription factors [149, 150]. Thus, androgens primary action is to regulate gene transcription [149]. The mRNA expression of the androgen receptor is localized in the midbrain and other regions in the central nervous system [149, 151]. This mRNA expression suggests that the androgen receptors could be expressed throughout the brain. Testosterone and its metabolite, dihydrotestosterone, are androgens that have strong binding affinity for the androgen receptor [152, 153].

A. GENOMIC ANDROGEN RECEPTOR

Classically, the androgen receptors reside in the cytosol as a single receptor with 4 domains consisting of an N-terminal regulation domain, DNA-binding domain, small-hinge region, a ligand-binding domain, and heat shock protein 90 (Hsp90) [152, 154, 155]. The N-terminus domain mediates the transcriptional activity of the androgen receptor [149, 152, 156]. Androgens, such as testosterone, enter cells and bind to the ligand-binding domain of the cytoplasmic androgen receptor [149, 157]. The binding of the ligand induces a conformational change releasing the Hsp90 and phosphorylating the receptor [149, 155, 157] This androgen receptor complex translocates to the nucleus and dimerizes [149, 157]. The dimerized receptor binds to a specific DNA sequence, the androgen response elements (AREs) recognized by the DNA-binding domain and regulates gene transcription (Figure 7) [149, 153]. The androgen receptor is sensitive to the antagonist, flutamide, and flutamide's active metabolites

hydroxyflutamide and cyproterone acetate [149, 158]. Flutamide is a pure classical androgen receptor antagonist that blocks androgens from binding to the receptor [149, 159].

B. NON-GENOMIC ANDROGEN RECEPTOR

Non-genomic androgen receptors are localized to extranuclear sites, which indicate androgens have a second mode of action independent of genomic DNA interactions [149, 160, 161]. The actions at the extranuclear sites are rapid and involve a putative membrane associated androgen receptor on the plasma membrane [149]. This non-genomic pathway does not involve transcription and translation, but stimulates an increase in intracellular calcium and activates protein kinases, which trigger signaling cascades of oxidative stress and inflammation [149, 152, 162]. This putative membrane androgen receptor may be associated with g-proteins and phospholipase c (Figure 7) [163]. The membrane androgen receptor is sensitive to g-protein coupled receptor antagonists but not the classical androgen receptor antagonists [149, 162, 164].

TESTOSTERONE

The androgen testosterone is the major male sex hormone that is conserved among all vertebrates signifying its importance in evolution and development [149, 165]. Testosterone participates in neuronal development, neuroprotection and neurotoxicity in the central nervous system [48, 166]. Testosterone is a sex steroid hormone that regulates male behavior and libido [167-170]. Testosterone is expressed in both sexes identifying that it is essential for motivation and libido [171-173]. As the major male sex hormone, circulating levels of testosterone are ten times greater in males than females [163, 165].

Like all steroids, testosterone is synthesized from cholesterol (Figure 8) [165, 174]. Testosterone is metabolized into two active steroids, dihydrotestosterone and estradiol by 5-alpha reductase and aromatase, respectively [175, 176]. In addition, dihydrotestosterone can be metabolized into 3 α -diol and 3 β -diol by hydroxysteroid dehydrogenase enzymes, which are identified as positive and negative modulators of GABA-A receptors (Figure 9) [175, 177, 178].

A. AGE RELATED CHANGES IN TESTOSTERONE

Men can experience an age-related decline in testosterone, wherein there is a greater loss of free testosterone than total testosterone [163, 179]. This loss of free testosterone could be due to multiple factors, such as the age-associated increase in sex hormone-binding globulin that binds free testosterone and even medication binds testosterone [180-183]. Therefore, total testosterone levels are still within the normal range in aging men, even though free testosterone is decreased [163, 184]. Moreover, decreased levels of circulating free testosterone can be accompanied with symptoms of decreased muscle mass, bone density, libido, erectile dysfunction, and an increase in depression, irritability, and fatigue [185, 186]. Even though there is an age-related decline in male testosterone, testosterone is still higher in males than females [187].

B. TESTOSTERONE REPLACEMENT THERAPY

Testosterone supplements are commercially advertised for men that experience erectile dysfunction, decreased libido, low energy and depression [173]. The use of testosterone has increased ten-fold since 2000 [185]. Testosterone supplements and hormone replacement therapy are recommended to treat men diagnosed with hypogonadism that experience consecutively low

morning testosterone and erectile dysfunction [185, 188]. Current contraindications for testosterone are cardiovascular disease, prostate cancer and sleep apnea.

There is a public health issue, which highlights the use of testosterone therapy without prior testosterone measurement [185, 189]. Further, there is a greater risk for prescribing testosterone in older men than younger men. For instance, older men could have pre-existing conditions of heart failure, and testosterone therapy could make these conditions worse or cause adverse reactions of cardiovascular disease and advance prostate cancer [185, 190, 191]. Thus, testosterone therapy should not be an option for older men with pre-existing conditions of cardiovascular disease, prostate cancer and sleep apnea [191-193].

C. TESTOSTERONE IN NEURODEGENERATION

Testosterone could play a neuroprotective role in men, exerting a preconditioning effect in cells. Preconditioning is a naturally adaptive process that provides protection in target tissues such as the brain [21, 194, 195]. This protection is characterized by exposure to marginal insults that allow cells to resist severe damage [21, 194, 195]. In fact, testosterone can induce minimal levels of oxidative stress; non-toxic to neuronal cells, suggesting that testosterone can act as a preconditioning stimulus. However, with the age-related decline in testosterone, this preconditioning effect could be lost.

The decline in testosterone levels is variable with age [181, 185, 196-198]. However, chronically ill men experience a greater decline in testosterone [181, 185, 196-198]. In fact, acute and chronic conditions, such as PD, men have lower levels of total testosterone [199]. Moffat et al., 2004 discovered that testosterone deficiency is linked to increased neurodegeneration in men

[170, 200]. In addition, the deficiency of testosterone is correlated with increasing oxidative stress levels, thus a risk for neurodegeneration [201, 202].

D. TESTOSTERONE SIGNALING

Testosterone can act as a neuroprotectant and neurotoxic molecule. Pike et al., 2008 identifies that androgens, such as testosterone, inhibit amyloid β accumulations through pathways of estrogen and estrogen-independent signaling [166]. This recognizes that testosterone's neuroprotective effect could be mediated through testosterone and its active metabolites, estrogen and dihydrotestosterone [166]. Testosterone can act as a neurotoxic molecule that creates an oxidative environment inducing inflammation and apoptosis. For instance, testosterone in leukocytes induces COX2 inflammatory signaling exerting negative effects of cell death [203]. Moreover, studies show that sex steroids can regulate the neurotransmission and fate of dopamine neurons in the substantia nigra [204, 205]. Cunningham et al. 2009 shows the chronic signaling of testosterone can induce apoptosis in N27 dopaminergic neurons [48]

CELLULAR MODEL

Midbrain neuronal cell lines are major sources of catecholamines [152, 206]. These cell lines include brain structures of the substantia nigra, ventral tegmental area and red nucleus that are associated with motor control, cognition, emotion and motivational control [152, 207-210]. Midbrain cell lines are simple and relevant models used to understand the molecular signaling relative to several pathological conditions, such as stroke, PD, and schizophrenia [152, 207].

Also, these cell lines allow us to understand steroid hormone signaling associated with catecholamine function and dysfunction [152].

One such cell line is the N27 cell line. This immortalized N27 cell line is derived from the rat midbrain, and has been used by several research laboratories to study acute and chronic conditions associated with stroke and PD (Figure 10) [152, 211, 212]. This neuronal cell line is positive for neuron specific enolase, dopamine, and tyrosine hydroxylase activity [48, 152, 213-215]. In addition, N27 cells do not express D1 receptors, consistent with the mature substantia nigra [48, 213]. Also, this cell line is positive for the estrogen receptor alpha and beta mRNA but lacks the androgen receptor mRNA [152].

Even though there is a lack of androgen receptor mRNA, testosterone was associated with the increased activity of protein kinase C delta, supporting the hypothesis of a putative membrane androgen receptor that has yet to be cloned [48, 164, 216]. Despite no androgen receptor mRNA, Cunningham et al. 2009 used the rabbit polyclonal androgen receptor (C19) antibody and found the androgen receptor protein expression in N27 cells via Western blot analysis (Figure 11) [48]. Consistently, the androgen receptor is expressed in the SNc dopaminergic neurons in the rat substantia nigra [48, 217]. Even though the androgen receptor is expressed in this cell line, the classical androgen receptor antagonist, flutamide, had no effect on the damaging effects of testosterone, further supporting the signaling of a membrane androgen receptor [218].

SPECIFIC AIMS

The long-term goal of this study was to determine testosterone effects in oxidative stress induced signaling pathways involved in dopamine neurodegeneration implicated in PD. Our

objective was to determine the effects of testosterone signaling in oxidative stress, and understand how testosterone may further promote inflammation, alpha synuclein and apoptosis in oxidative stress conditions. Our central hypothesis was under oxidative stress conditions, testosterone would decrease dopamine neuronal cell viability through the exacerbation of oxidative stress induced inflammation (Figure 12). Thus, I will 1.) Examine the effects of testosterone on dopamine neuronal cell viability and 2.) Examine the effects of testosterone on inflammation, alpha synuclein and apoptosis in oxidatively stressed dopamine neurons.

SIGNIFICANCE

PD, the second most common neurodegenerative disease, is the most common movement disorder affecting the aging population [26]. Aging males have a higher incidence of PD than aging females [22]. This suggests that the androgen, testosterone, may play a role in mediating the pathological features in PD in men or estrogen may play a neuroprotective role. Therefore, examining the role of androgens under conditions of oxidative stress, a key feature of PD, could help identify the underlying cellular mechanisms mediating the sex bias in PD and result in targeted therapeutics.

SUMMARY

Pathological features of oxidative stress, inflammation and apoptosis are identifiable markers in PD. Interestingly, testosterone and oxidative stress, oxidative stress and NFkB, NFkB and COX2, and COX2 and alpha synuclein have been associated with each other in the scientific literature. However, these pathological features have not been associated together in a signaling

mechanism. Thus, we have taken the initiative to associate these factors implicated in PD to identify the signaling pathways of inflammation and apoptosis in dopamine neurodegeneration.

Microglia are understood to act as intermediates in mediating neuronal signaling. However, using this cellular model of N27 dopaminergic neuronal cells, I have identified the signaling cascades that can initiate and mediate neuron-neuron interactions (Figure 13). In addition, I have used this cell line to identify the sex bias involved in cellular mechanisms implicated in dopamine neurodegeneration associated with PD. Oxidative stress is a key feature in dopamine neurodegeneration that mediates the loss of dopamine, and increased inflammation and cell death. Since males have a two-fold incidence of PD, I have examined the effects of testosterone in oxidative stress conditions, to understand its role in dopamine's demise.

INCIDENCE OF PARKINSON'S DISEASE IN MALES

Risk factor	PD
Age (1-y increment)	1.10 (1.04–1.16)
Male gender	2.13 (1.11–4.11)

ILSA = Italian Longitudinal Study on Aging.

Table 1 **Incidence of Parkinson's disease in males.** Shows the average annual age and sex specific incidence rates of Parkinsonism and Parkinson's disease (PD). This study shows that men have a two fold incidence for PD [22].

THE MEDICAL BURDEN OF PARKINSON'S DISEASE IN THE UNITED STATES

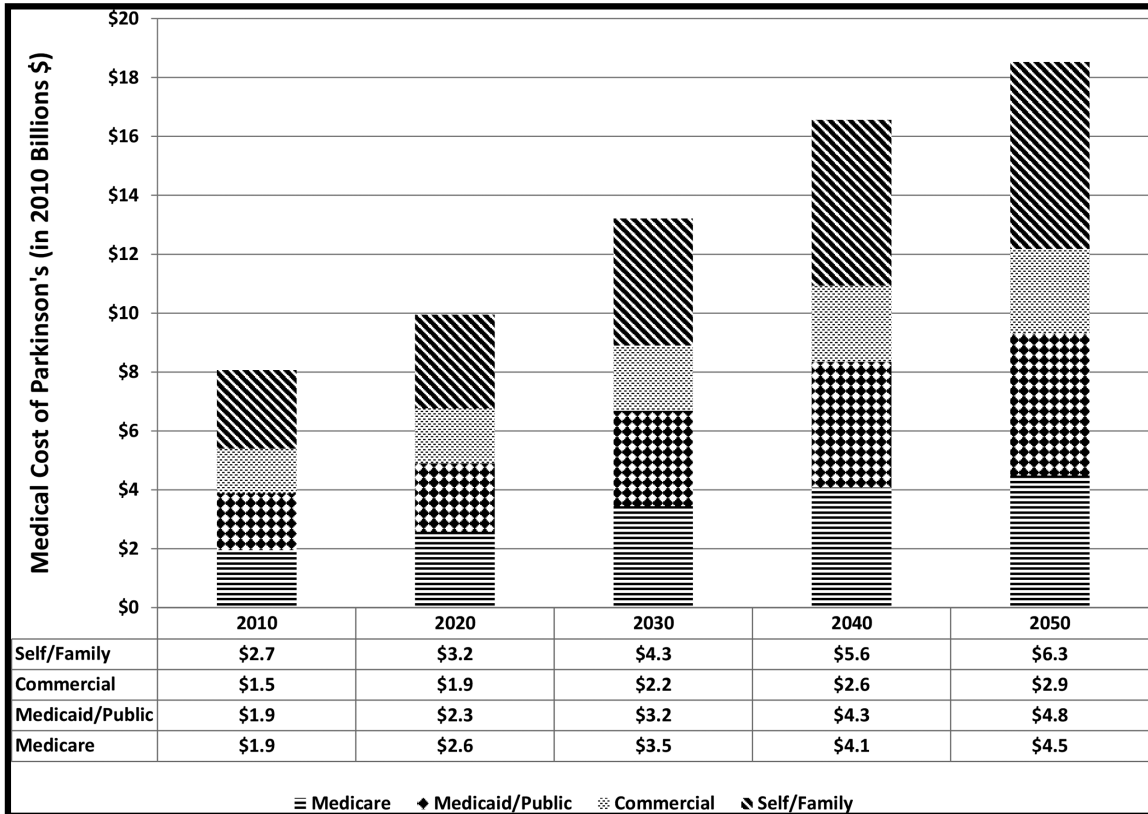


Figure 1. **The medical burden of Parkinson's disease in the United States** identifies the cost estimates of Parkinson's disease by over time. The cost of Parkinson's is estimated to increase exponentially over the next 40 years [26].

THE FENTON REACTION



Figure 2. **The Fenton reaction.** Fenton reaction is the process between reduced iron, Fe^{+2} and hydrogen peroxide, H_2O_2 . This reaction produces oxidized iron Fe^{+3} and hydroxyl radicals, making it an important factor in oxidative damage [64]. Adapted from Kohen et al., 2002.

GLUTATHIONE NEURTALIZES HYDROGEN PEROXIDE

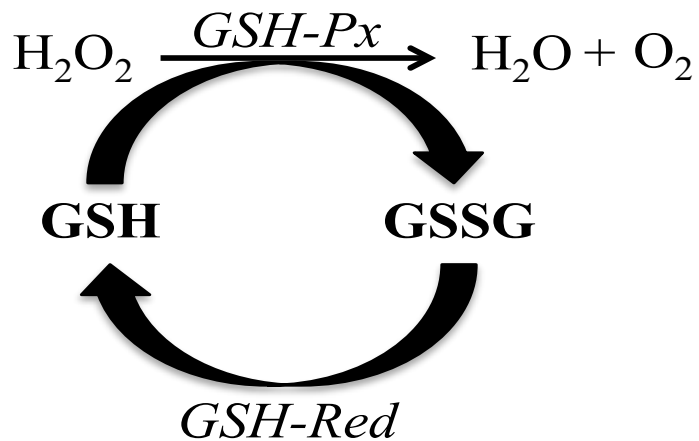


Figure 3. **Glutathione neurtalizes hydrogen peroxide.** In the process of scavenging H_2O_2 , GSH acts as a co-substrate with GSH-Px to remove H_2O_2 from the cellular environment. As a result, GSH is oxidized to GSSG and reconverted to GSH by GSH-Red. H_2O_2 : hydrogen peroxide, H_2O : water, O_2 : molecular oxygen, GSH-Px: glutathione peroxidase, GSSG: glutathione disulfide, GSH-Red: glutathione reductase, GSH: glutathione [67]. Adapted from Simonian et al., 1996.

TWO MOLECULES OF GLUTATHIONE FOR ONE HYDROGEN PEROXIDE

MOLECULE

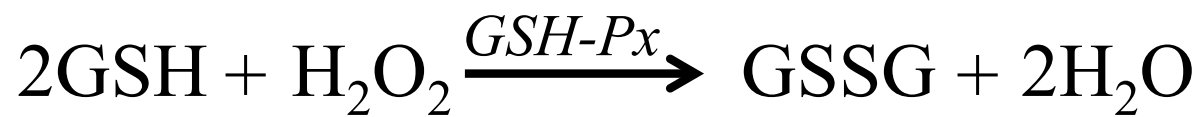
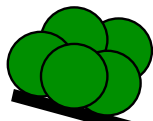


Figure 4. **Two molecules of glutathione for one hydrogen peroxide molecule.** The removal of hydrogen peroxide: H_2O_2 consumes two molecules of GSH: glutathione, producing GSSG: glutathione disulfide, and two molecules of H_2O : water [64]. Adapted from Kohen et al., 2002.

Balance of Oxidative Stress

Antioxidants



Reactive Oxygen Species

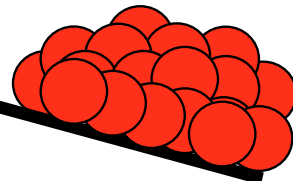


Figure 5. **Balance of oxidative stress.** Oxidative stress is the measurement between antioxidants and reactive oxygen species. With insufficient antioxidants, reactive oxygen species tip the scale creating high oxidative stress conditions [56, 64]. Adapted from Kohen et al., 2002 and Finkel 2003.

COX2 ENZYMATIC ACTIVITIES

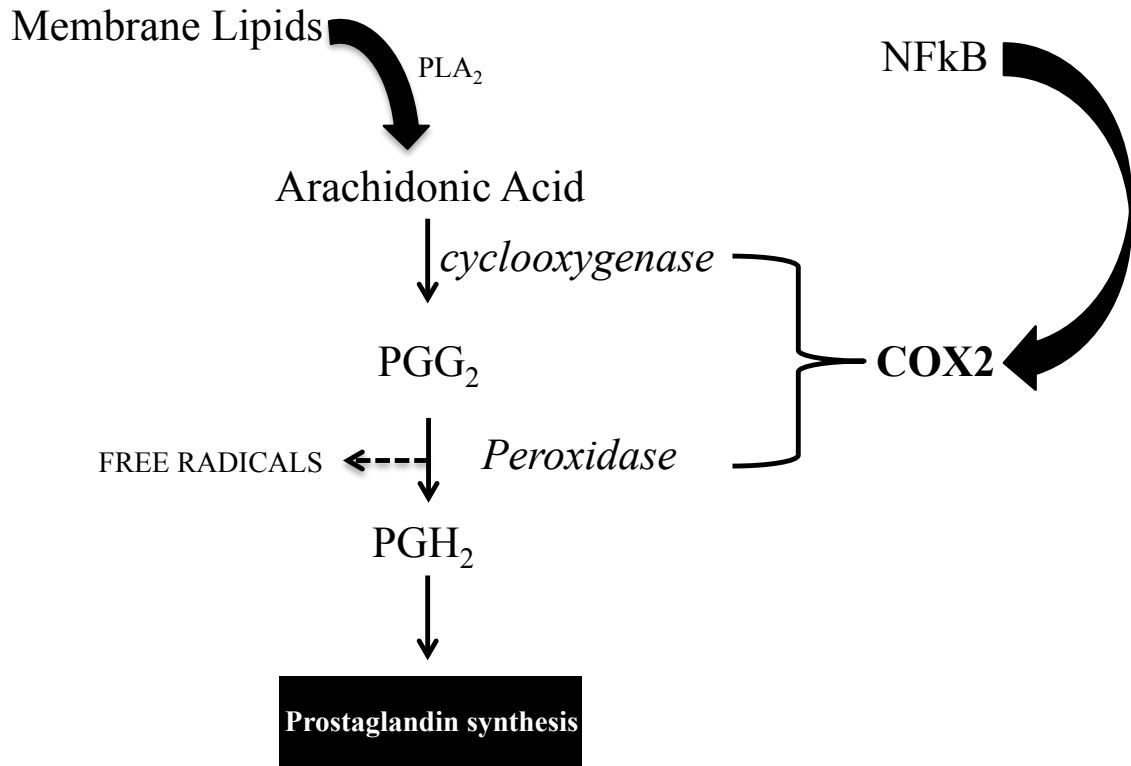


Figure 6. **COX2 enzymatic activities.** Consilvio et al., 2004 shows that COX2 acts as a cyclooxygenase and peroxidase in the production of prostaglandins. As a peroxidase, COX2 releases free radicals into the cytoplasm. Also, this figure shows that NFkB regulates COX2 [123]. Adapted from Consilvio et al., 2004.

ANDROGEN RECEPTOR MECHANISMS

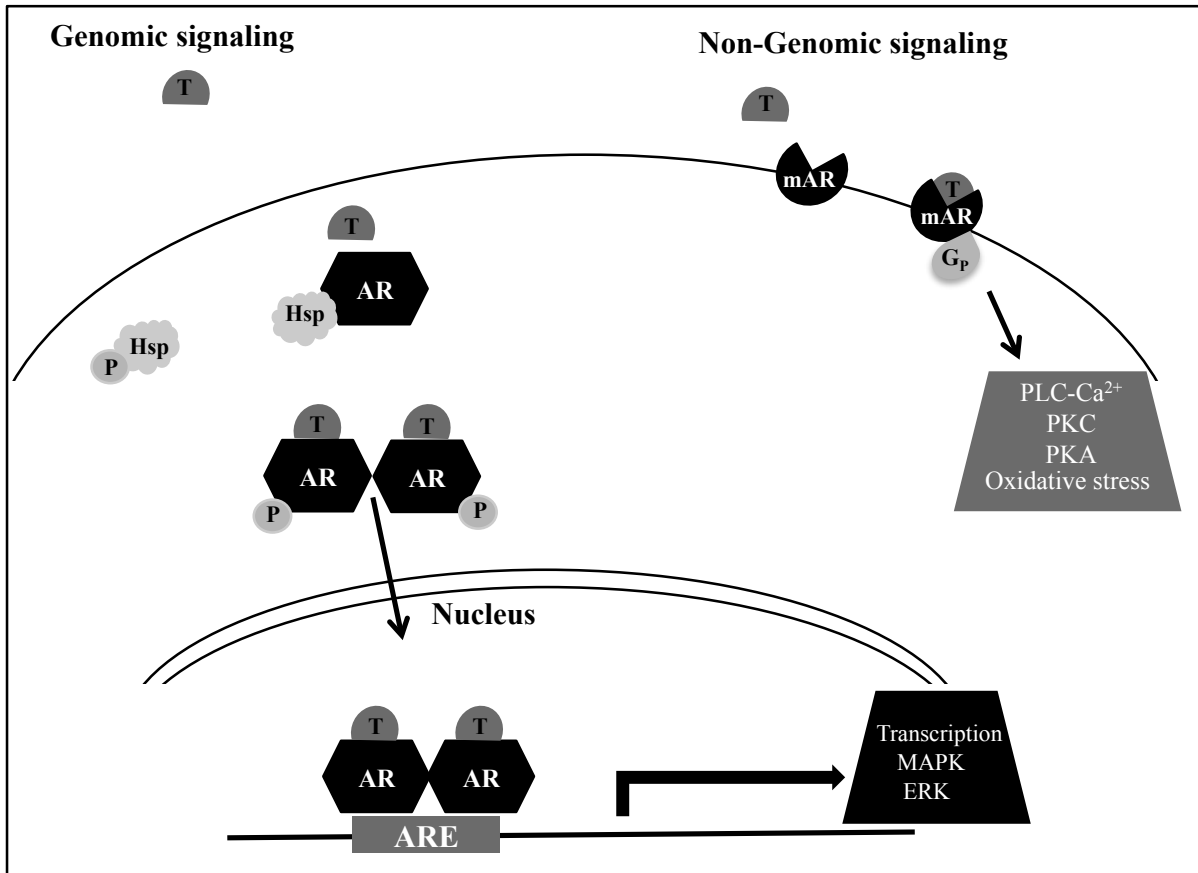


Figure 7. **Androgen receptor mechanisms.** Genomic and non-genomic signaling of testosterone. In genomic signaling, testosterone binds to the inactive androgen receptor, releasing the Hsp (heat shock protein) and phosphorylating the receptor resulting in dimerization and activation. The receptor translocates to the nucleus and binds to ARE (androgen response elements) upregulating gene transcription that regulates downstream signaling of MAPK and ERK. Non-genomic androgen receptor, testosterone binds on the plasma membrane to the mAR (membrane androgen receptor) interacting with G_p (G-proteins) to increase calcium mobilization, induce oxidative stress and activate protein kinases [149, 152, 216, 219-221]. Adapted from Gatson et al., 2006; Walker 2009; Li et al., 2009; Su et al., 2012; Cunningham et al. 2012; Dent et al., 2012.

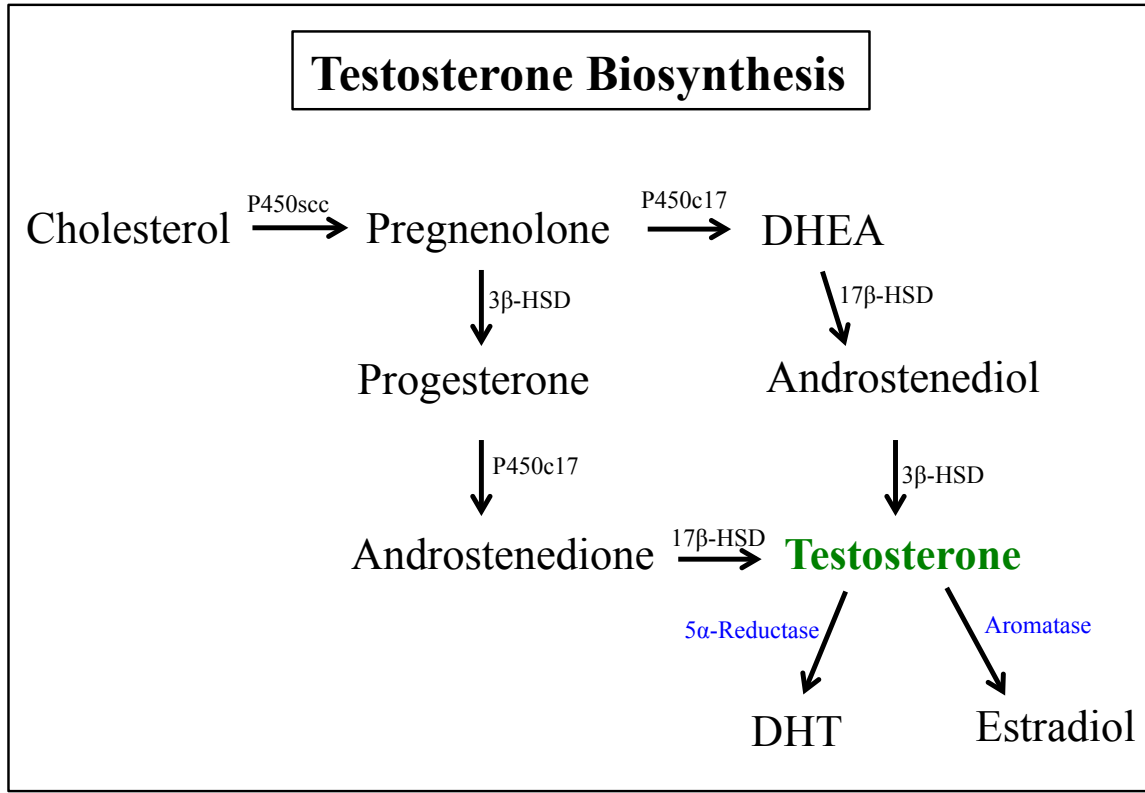


Figure 8. **Testosterone biosynthesis.** The biosynthesis of testosterone from cholesterol. Testosterone is synthesized and released into the bloodstream. P450scc: p450 side chain cleavage; 3β-HSD: 3beta-hydroxysteroid dehydrogenase; 17β-HSD: 17β- hydroxysteroid dehydrogenase [165, 174]. Adapted from Durdiakova et al., 2011.

DIHYDROTESTOSTERONE IS METABOLIZED INTO ACTIVE METABOLICS

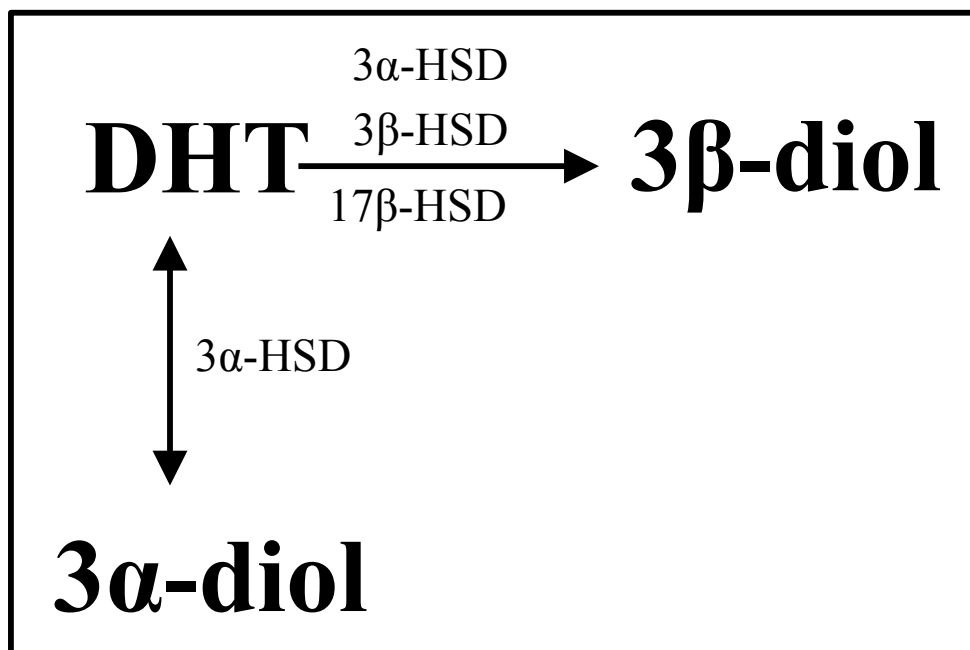


Figure 9. **Dihydrotestosterone is metabolized into active metabolites.** Dihydrotestosterone (DHT) is reduced by hydroxysteroid dehydrogenase enzymes into 3 α -diol and 3 β -diol. 3 β -diol is the more favorable diol produced. In addition, 3 α -diol is reconverted to DHT by 3 α -HSD. 3 α -hydroxysteroid dehydrogenase: 3 α -HSD; 3 β -HSD: 3 β -hydroxysteroid dehydrogenase; 17 β -HSD: 17 β -hydroxysteroid dehydrogenase. [175]. Adapted from Robert Handa et al., 2008.

IMMORTALIZED N27 DOPAMINERGIC NEURONAL CELL LINE

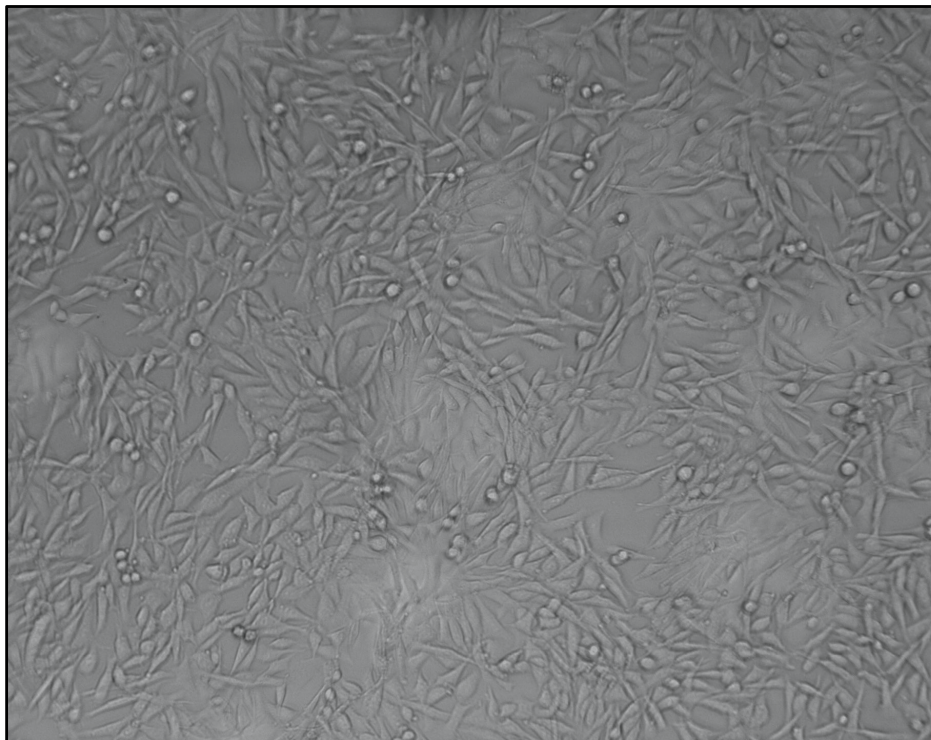


Figure 10. **Cellular model - immortalized N27 dopaminergic neuronal cell line.** N27 dopaminergic neuronal cells have long budding processes.

N27 CELLS EXPRESS THE ANDROGEN RECEPTOR



Figure 11. **N27 cells express the androgen receptor.** N27 dopaminergic cells express the androgen receptor protein [48].

CENTRAL HYPOTHESIS

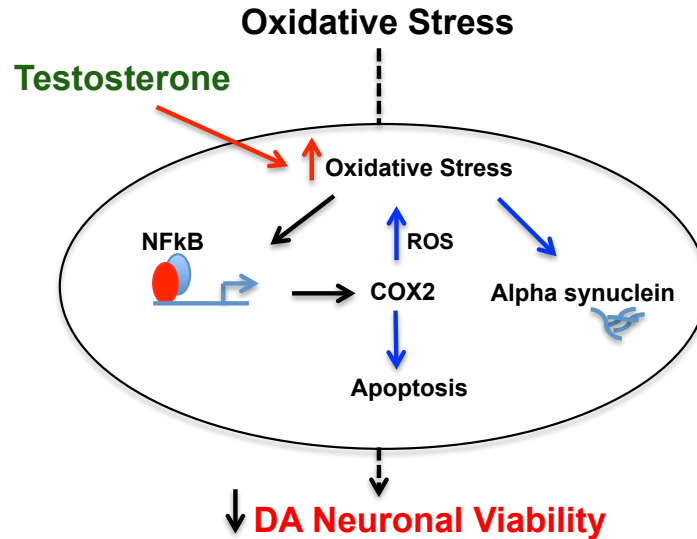


Figure 12. **Central Hypothesis.** Under oxidative stress conditions, testosterone will exacerbate oxidative stress-induced signaling to increase the nuclear localization of NFkB targeting COX2 protein expression. This increase in COX2 contributes to oxidative stress via reactive oxygen species (ROS) in the cellular environment increasing alpha synuclein accumulation and apoptosis responsible for the decrease of dopamine neuronal viability

NEURONAL INTERACTIONS MEDIATE NEURONAL SIGNALING

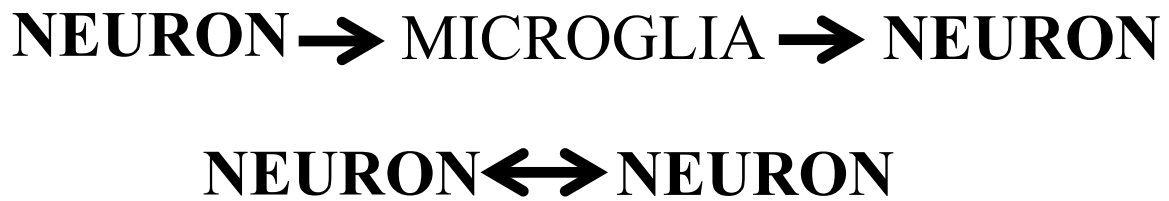


Figure 13. **Neuronal interactions mediate neuronal signaling.** Microglia are key intermediates in promoting dopamine's injury. However, neurons can mediate their own demise. Neuronal signaling promotes dopamine cell injury [108]. Adapted from Stanley 2012.

CHAPTER II

OXIDATIVE STRESS DEFINES THE NEUROPROTECTIVE OR NEUROTOXIC PROPERTIES OF ANDROGENS IN AN IMMORTALIZED FEMALE RAT DOPAMINERGIC NEURONAL CELL LINE

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ABSTRACT

Males have a higher risk for developing Parkinson's disease (PD) and Parkinsonism following ischemic stroke than females. While estrogens have been shown to play a neuroprotective role in PD, there is little information on androgens' actions on dopamine neurons. In this study, we examined the effects of androgens under conditions of oxidative stress to determine if androgens play a neuroprotective or neurotoxic role in dopamine neuronal function. Mitochondrial function, cell viability, intracellular calcium levels, and mitochondrial calcium influx were examined in response to androgens, under both non-oxidative and oxidative stress conditions. Briefly, N27 dopaminergic cells were exposed to the oxidative stressor, hydrogen peroxide, and physiologically relevant levels of testosterone or dihydrotestosterone, applied either before or after oxidative stress exposure. Androgens, alone, increased mitochondrial function via a calcium-dependent mechanism. Androgen pretreatment protected cells from oxidative stress-induced cell death. However, treatment with androgens after the oxidative insult increased cell death, and these effects were, in part, mediated by calcium influx into the mitochondria. Interestingly, the negative effects of androgens were not blocked by either androgen or estrogen receptor antagonists. Instead, a putative membrane associated androgen receptor was implicated. Overall, our results indicate that androgens are neuroprotective when oxidative stress levels are minimal, but when oxidative stress levels are elevated, androgens exacerbate oxidative stress damage.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by Lewy body formations and motor disturbances, such as bradykinesia, rigidity, resting tremor, and postural instability [222]. The loss of dopamine neurons within the substantia nigra pars compacta is one of the primary pathological features of PD [223-227] and has been clearly linked with the motor disturbances associated with PD [228, 229]. Dopamine neurons are inherently vulnerable to oxidative stress due to the process of dopamine metabolism, which can generate reactive oxygenated species (ROS) and free radicals [230]. Oxidative stress, in turn, has been described as a key factor in the pathogenesis of PD [231-233].

Two clinically known risk factors for PD are aging and gender [230, 234-237]. Oxidative stress increases with age [238], and PD incidence increases exponentially in individuals above the age of 65 [239, 240]. Further, aged men have a greater prevalence of PD than aged women [230, 234-237]. Interestingly, men also have a higher incidence of parkinsonism after ischemic stroke than women [20, 241], a neurodegenerative condition in which oxidative stress is strongly implicated as an important factor in the progression of cell death [242, 243]. Therefore, these studies indicate a potential role for androgens in oxidative stress-mediated neurodegeneration.

The role of androgens, the major male sex hormone, in the higher incidence of PD is unclear. Although, androgen levels decrease with age [244], androgen levels continue to remain higher in aged males than females [245]. Some studies support a neuroprotective role for androgens, in which androgens can protect against oxidative stress damage [246, 247]. A possible mechanism for androgen-induced neuroprotection is

preconditioning, since androgens, alone, can moderately increase oxidative stress and apoptosis [48]. However, other studies indicate that androgens may not always be neuroprotective. For example, in a pre-existing oxidative stress environment androgens can further exacerbate oxidative stress damage [49]. These results suggest that the level of oxidative stress determines if androgens play a positive or negative role in neuronal function. We propose that below a certain oxidative stress threshold, androgens can be neuroprotective, but once a certain oxidative stress threshold is reached, such as occurs with PD and ischemic stroke, androgens can become damage promoting.

The oxidative stress-associated organelles, mitochondria, serve a variety of metabolic functions within the cell, including a critical role in calcium buffering [248]. From a calcium homeostasis point of view, mitochondria can modulate the amplitude and frequency of calcium transients [249, 250], resulting in spontaneous and sustained depolarization of the mitochondria [251]. Increased intracellular calcium accumulations have been implicated in the generation of increased oxidative stress [252] and the initiation of cell death in PD [253-256] and ischemic stroke [257, 258]. These studies indicate a role for calcium in mediating neuronal vulnerability.

Given the role of oxidative stress in regulating dopaminergic cell fate and the conflicting studies regarding androgens and oxidative stress-mediated neurodegenerative disorders, we examined in the present study if oxidative stress acts as a molecular switch to define androgen actions on dopamine neurons.

MATERIAL AND METHODS

Reagents- Testosterone propionate, dihydrotestosterone (DHT), flutamide, testosterone-BSA, tert-butyl-hydrogen peroxide, ICI 182,780, tetramethylrhodamine methyl ester (TMRM) were from Sigma (St. Louis, MO). Ruthenium 360 (Ru360) and Calcein AM were from Calbiochem (Darmstadt, Germany). The esterified, membrane-permeant derivative 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzo furan-5-oxy]-2-(2'-amino-5'-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid, acetoxymethyl ester Fura-2 AM, RPMI 1640 medium, fetal bovine serum (FBS), charcoal-stripped FBS, penicillin-streptomycin, and phosphate buffered saline (PBS) were purchased from Invitrogen; Molecular Probes (Carlsbad, CA). Testosterone and DHT were dissolved in ethanol with a final concentration <0.0001% ethanol

Cell Culture- The rat immortalized mesencephalic dopaminergic neuronal cell line 1RB3AN₂₇ (N27) was used. This cell line expresses androgen receptor protein [48]. The N27 cell line, which is derived from 12-day-old female rat fetal mesencephalic tissue (unpublished data), is composed of immortalized cells positive for tyrosine hydroxylase [213]. To confirm the gender origin of the cells used in our study, we used quantitative real-time PCR method to amplify *Sry* (sex-determining region Y), a male-specific gene expressed in human and rat embryos [259]. qRT-PCR with rat *Sry* primers (Taqman Gene Expression Assay #Rn04224592_u1) detected no expression of this gene in the N27 cells, confirming their female origin. N27 cells were cultured at 5% CO₂ at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) and subcultured weekly. All experiments were performed between passages 12-19 and at approximately 80% confluency in RPMI 1640

medium without serum or with charcoal-stripped FBS to avoid confounders due to the presence of hormones in the serum [48].

Treatment Paradigm- For mitochondria membrane potential experiments, N27 cells were incubated for 30 min with 10 nM TMRM (mitochondrial membrane potential indicator) [260-262] in serum- and phenol red-free RPMI 1640 prior to exposure to physiologically relevant levels of androgens for 2 hours [263-265]. In some experiments, a 30 min pre-incubation with the mitochondrial uniporter inhibitor, ruthenium 360 (Ru360, 1 μ M), was included. Inhibition of the mitochondrial uniporter blocks calcium influx across the inner membrane of the mitochondria [92]. For cell viability experiments, cells, incubated in charcoal-stripped FBS, were exposed to hydrogen peroxide (100-250 μ M) for 12-14 hours to induce 20-30% cell loss followed by a 24-hour exposure to androgens, unless stated otherwise. Since subsequent testosterone exposure does not alter cell viability in experiments that have less than 20% oxidative stress-induced cell loss, only experiments in which hydrogen peroxide induced 20-30% cell loss were examined. Cells were exposed to the various inhibitors 30 minutes prior to androgen exposure. Testosterone, DHT, and flutamide were made from a stock solution in ethanol (final concentration of ethanol <0.001%). Vehicle control treated cells were exposed to <0.001% ethanol.

Mitochondrial Membrane Potential Imaging- Images were collected by a Zeiss confocal microscope with a 63X oil objective. TMRM was excited at 543 nm and the fluorescence detected beyond a 560 nm long pass barrier filter. Each scan contained approximately 300 mitochondria. Scans were randomly selected at the two hour time point and Z-series images of TMRM-incubated cells were obtained [262]. At the

conclusion of the experiment, the mitochondrial protonophore p-trifluoromethoxy-phenylhydrazone (FCCP) (10 μ M) was used to uncouple the mitochondrial proton gradient across the inner mitochondrial membrane and eliminate membrane potentials [266]. To avoid the confounding contribution of background fluorescence, only data sets in which FCCP abolished the membrane potential were used for analysis. The level of fluorescence in mitochondria per scan (n=3 scans) was analyzed with an NIH Image J program plug-in. This program is based on a modified form of the Nernst equation, which uses the fluorescence measured in individual mitochondria to calculate mitochondrial membrane potentials as follows:

$$V_{mit} = -60 \text{ mV} \log[F_{mit} / F_{cyto}]$$

Where V_{mit} is the mitochondrial membrane potential; F_{mit} is the intramitochondrial fluorescence; and F_{cyto} is the cytosolic fluorescence [267]. After analysis, Z-series images were converted to Z-projections to show the population response. The membrane potential can be used as a measure of mitochondrial function, since it is also influenced by ATP demand, respiratory chain capacity, proton conductance, substrate availability, and mitochondrial calcium sequestration [268].

Intracellular Calcium Imaging- Cells were plated on 25mm micro circle cover glasses at a density of 45,000 cells in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Cells were incubated at 37°C, 5% CO₂. At 80% confluency, cells were washed twice with Hanks' balanced salt solution (HBSS). Fura-2 AM was used to determine intracellular free calcium concentration. ($[Ca^{++}]_i$) The ratio of fluorescent intensity at 340 nm excitation (fura-2 AM calcium-bound complex) to 380 nm excitation (calcium-free fura-2 AM) was

used as a measure of intracellular calcium concentration. The emission wavelength was 520 nm. Cells were loaded with 5 μ M of fura-2 AM for 30 min at 37°C, 5% CO₂. The cells were washed twice with HBSS to remove excess Fura-2 AM. Fresh HBSS media was added to the cells, and the basal 340/380 nm ratio (R) was measured. Following basal calcium measures, the cells were treated with 100 nM testosterone or vehicle and the 340 and 380 nm measurements were obtained. At the end of the ten minute experiment the maximum 340/380 nm ratio was obtained by the addition of 2 μ M ionomycin (Santa Cruz) to the media to stimulate intracellular calcium release and the minimum 340/380 nm ratio was determined following the addition of 500 mM ethylene glycol tetraacetic acid (EGTA). Intracellular calcium concentration was estimated from the following equation: $[Ca^{++}]_i = bK_d (R-R_{min})/(R_{max}-R)$, in which K_d is the dissociation constant of fura-2 AM (224 nm under normal conditions), R is any given 340/380 ratio value of fluorescence measured for calcium-free and calcium-bound fura-2 AM, R_{min} is the 340/380 ratio in the absence of calcium when the cells are exposed to EGTA, R_{max} is the 340/380 ratio when the cells are exposed to ionomycin, and b is the ratio of the fluorescence measured at 380 nm in calcium-depleted and calcium saturated [269]. Images were captured using NIS-Elements AR 3.0 software, on a Nikon Eclipse TE2000-S microscope, with a 40X (NA .60) objective and a Photometrics Cascade camera. Thirteen cells were analyzed in the testosterone condition, and seven cells were examined in the vehicle condition. All experiments were carried out at room temperature and measured within 15 minutes.

Cell Viability – Cells were plated in triplicate into a 96-well black tissue culture plate at a density of 4×10^4 cells/well per experiment. Three independent replications,

consisting of three separate plates, were performed for a final n=3/treatment group. Controls were normalized to 100%. For analysis of viability, media was aspirated and replaced with the fluorescent membrane-permeant cell marker, calcein AM (2 mM) in PBS. Cells were incubated at 37 °C for 30 minutes. Fluorescence was measured in a fluorescent plate reader with the temperature setting at 37 °C and the excitation and emission wavelengths at 485 nm and 530 nm, respectively. The intensity of the fluorescence is directly proportional to the number of live cells.

Statistical Analysis- Analysis was computed by the IBM SPSS Statistics version 21 software. All data were expressed as mean \pm SEM. Significance (P values \leq 0.05) for mitochondrial membrane potentials and cell viability was determined by ANOVA followed by the Fisher's LSD post hoc test. Paired t-tests were used to determine statistically significant differences between treatment groups in the intracellular calcium analysis experiments.

RESULTS

Testosterone and DHT alter mitochondrial function in N27 cells — In a recent study, we showed that androgens, possibly through the mitochondria, play a role in dopaminergic function [48]. In the current study, mitochondrial function was examined indirectly by measuring mitochondrial membrane potentials. Specifically, cells were incubated in the mitochondrial membrane potential indicator (TMRM, 10nM) 30 min prior to testosterone (10nM or 100nM) or DHT (10 nM) for 2 hours. Figure 14A and B shows fluorescent micrographs of dopaminergic N27 cell mitochondria following testosterone and DHT exposure. At 2h, testosterone and DHT significantly increased

mitochondrial membrane potential activation (derived using a Nernst-based equation), as evidenced by the increased fluorescence, compared to vehicle controls ($F_{3,11} = 25.987$, $p < 0.05$). These results suggest an underlying androgen-dependent mechanism on mitochondria membrane potential. We have previously shown that androgens, alone, can moderately increase cell death (10%) in N27 cells after 48 hours of exposure [48], but androgens do not have a detrimental effect on cell viability after 24 hours of exposure (Figure 14C) or in conditions that hydrogen peroxide induces less than 20% cell death (data not shown).

The timing of androgen exposure relative to the induction of oxidative stress determines the neuroprotective or neurotoxic properties of androgens on neuronal function — In order to determine the role of oxidative stress on androgen-mediated neuronal function, N27 cells were exposed to 100 nM testosterone 1) before (2hr) the application of the oxidative stressor, hydrogen peroxide, 2) 12-14 hrs after the induction of oxidative stress with hydrogen peroxide, or 3) at the same time as when oxidative stress was induced (i.e., co-application of testosterone and hydrogen peroxide). Figures 15-16 depict cell viability measurements. In Figure 15A, ANOVA reveals a significant overall effect of treatment groups ($F_{3,92} = 203.471$, $p < 0.05$), time ($F_{5,92} = 11.505$, $p < 0.05$), and an interaction between time and treatment groups ($F_{15,92} = 23.014$, $p < 0.05$). In Figure 15B ANOVA shows a significant overall effect of treatment groups ($F_{4,14} = 36.158$, $p < 0.05$). In Figure 16A & B there was a significant effect of treatment groups ($F_{3,11} = 9.640$, $p < 0.05$) and ($F_{5,17} = 4.513$, $p < 0.05$), respectively. These results indicate that when cells are pretreated with testosterone (100 nM) for 2 hours prior to oxidative stress, testosterone is neuroprotective against subsequent oxidative stress. Conversely, when

cells are pretreated for 12-14 hours with hydrogen peroxide to induce 20-30% cell loss and then exposed to testosterone for 24-hour, testosterone becomes neurotoxic, as evidenced by a concentration-dependent increase in oxidative stress induced cell death (Figure 15). However, if cells are simultaneously exposed to hydrogen peroxide and testosterone, testosterone does not alter cell viability, indicating that a cellular response to hydrogen peroxide is required for androgens' negative effects on cellular function. Hydrogen peroxide at concentrations that induce 20-30% cell loss in N27 cells increase ROS generation, and this increase in ROS generation can be blocked by antioxidants [211]. To determine if hydrogen peroxide-induced oxidative stress mediates androgens deleterious effects on cell viability, the antioxidant, N-acetyl-cysteine (Nac, 1mM), was applied to cells at the same time as hydrogen peroxide, 12-14 hours prior to testosterone (100 nM). In the presence of the oxidant species scavenger, Nac [270, 271], testosterone did not compromise cell viability. These results indicate the involvement of ROS in androgens' negative effects on cell viability (Figure 16).

The neurotoxic properties of testosterone are androgen- and estrogen receptor independent in N27 cells – The role of androgen and estrogen receptors were examined in the testosterone post-hydrogen peroxide treatment in Figure 17. ANOVA reveals a significant effect of treatment groups ($F_{11,35} = 35.699$, $p < 0.05$). Similar to previous results, testosterone (100 nM) following hydrogen peroxide exposure significantly decreased cell viability. This effect was neither blocked by the androgen receptor antagonist, flutamide (500 nM), nor the estrogen α/β receptor antagonist, ICI 162,780 (1 μM), suggesting that testosterone's neurotoxic effects on cell viability, under

conditions of oxidative stress, are independent of testosterone's cognate receptors in N27 cells.

Intracellular calcium mediates testosterone's negative effects in an oxidative stress environment - Previous studies have shown the ability of testosterone to increase intracellular calcium levels in catecholaminergic SH-SY5Y cells [272]. To determine if testosterone, alone, increases intracellular calcium in N27 cells, non-oxidative stressed, "normal" cells were bathed in a calcium-free buffer and incubated with the fluorescent calcium sensor dye, FURA-2 AM, to measure intracellular free calcium concentration ($[Ca^{++}]_i$). In the calcium-free buffer, baseline $[Ca^{++}]_i$ was 4.5 nM and testosterone significantly increased $[Ca^{++}]_i$ to 250 nM in response to 100 nM testosterone, well below the ~500 nM $[Ca^{++}]_i$ considered cytotoxic [273]. These results suggest that testosterone induces the release of calcium from intracellular calcium stores (paired t (167) = 9.205, $p < 0.05$) (Figure 18A).

Testosterone-induced increase of mitochondrial membrane potential is mediated via calcium influx into the mitochondria — To ascertain if androgens, alone, induce mitochondrial activation through a calcium mediated mechanism, cells were pretreated with the mitochondrial calcium uniporter inhibitor, ruthenium 360, which blocks calcium influx into the mitochondria. TMRM-incubated cells were treated with testosterone (100 nM), with or without ruthenium 360 (1 μ M) for 2 hours. Ruthenium 360 blocked the testosterone-induced increase of mitochondrial membrane potential ($F_{3,11} = 19.032$, $p < 0.05$) (Figure 18B). To determine if mitochondrial calcium influx mediates testosterone's neurotoxic effects in an oxidative stress environment, ruthenium 360 was applied 30 minutes prior to testosterone exposure in hydrogen peroxide treated cells

(Figure 18C). Similar to previous results, testosterone (100 nM) following hydrogen peroxide exposure significantly decreased cell viability. The negative effect of testosterone on cell viability was blocked by the mitochondrial calcium uniporter inhibitor, ruthenium 360 ($F_{7,23} = 22.752$, $p < 0.05$), indicating that calcium influx into the mitochondria plays a role in testosterone's neurotoxic effects on cell viability in an oxidative stress environment.

Membrane-associated androgen receptor involvement in testosterone's neurotoxic effects in an oxidative stress environment—Putative membrane-associated androgen receptors (mAR) have been found in several cell types [162, 164, 216, 274, 275]. Generally, mAR is indicative of non-genomic signaling, as mAR is insensitive to androgen receptor antagonists, such as flutamide, and associated with increased intracellular calcium signaling [162]. To determine the involvement of the mAR in the neurotoxic and neuroprotective properties of testosterone in an oxidative stress environment, testosterone conjugated to BSA (TBSA) (500 nM) was used to bind putative mARs on the cell membrane [164]. In Figure 19A, ANOVA revealed a significant overall effect of treatment groups ($F_{3,11} = 25.683$, $p < 0.05$), in which, TBSA treatment following oxidative stress exposure significantly decreased cell viability. Further, ANOVA showed a significant effect of treatment ($F_{3,11} = 29.639$, $p < 0.05$), wherein TBSA two-hour pretreatment prior to a 6-hour oxidative stress exposure protected N27 cells from oxidative stress-induced cell death (Figure 19B). Therefore, these results implicate the involvement of mAR.

DISCUSSION

This study shows that the timing of androgens relative to the onset of oxidative stress defines the neuroprotective and neurotoxic properties of androgens in dopaminergic N27 cells. The key findings were that: 1) The effects of testosterone on N27 cell viability is dependent on the oxidant load of the cell (i.e., protective when administered prior to the pro-oxidant insult, hydrogen peroxide, but cytotoxic when administered following a period of hydrogen peroxide exposure, 2) The neurotoxic effects of testosterone are blocked by the mitochondrial calcium uniporter inhibitor, ruthenium 360, and 3) The neurotoxic effects of testosterone appear to be mediated by a receptor other than the classical androgen or estrogen receptors, and may be mediated by a putative membrane androgen receptor (mAR).

Testosterone has been shown to increase brain mitochondrial activity in the rat [276]. Similarly, we found that 2h androgen exposure significantly increased mitochondrial membrane potentials. This increase in mitochondrial function is mediated by calcium influx into the mitochondria. Previous studies have shown an association between androgens and calcium. In a catecholaminergic cell line (SH-SY5Y), nanomolar concentrations of testosterone increased intracellular calcium and calcium oscillations within the cytosol [272]. These calcium oscillations have been implicated in many cellular activities [277-279]. In this study we found that androgens increased cytosolic calcium levels in dopaminergic N27 cells. This increase in intracellular calcium is likely attributed to calcium release from intracellular calcium stores, since the buffer in which the cells were incubated did not contain calcium. The increase in cytosolic calcium resulting from exposure to testosterone under “normal” conditions is below the predicted

“cytotoxic” levels of calcium (~500 nM) that result in overt cell dysfunction and death [273]. Additionally, when calcium influx across the inner membrane of the mitochondria was blocked using the mitochondrial uniporter inhibitor, ruthenium 360, the androgen-induced increase in mitochondrial membrane potentials were inhibited. Similar effects of androgens on the mobilization of intracellular calcium has been demonstrated in other cells, including human prostate cancer cells [280], rat heart myocytes [281], dopaminergic neuroblastomas [272], and macrophages [282].

The role of androgens in neuroprotection and neurodegeneration is unclear. Some studies support a neuroprotective role for androgens, in which androgens can protect against oxidative stress damage [246, 247]. A possible mechanism for androgen-induced neuroprotection is preconditioning, in which androgens promote cellular adaptation against subsequent oxidative stress insults. In our previous findings, testosterone exposure for 48 hours induced a low level of apoptosis (10%) and moderately increased oxidative stress in N27 cells [48]. Furthermore, in the current study a 2h testosterone exposure was sufficient to increase mitochondrial activity, intracellular calcium release, and calcium influx into the mitochondria. These effects of androgens on oxidative stress, intracellular calcium, and mitochondrial activation may have “preconditioned” N27 cells to become more resistant to a subsequent oxidative insult, consistent with other studies that found calcium-mediated neuroprotection [283, 284]. This cellular preconditioning may be one of the underlying mechanisms involved in the neuroprotective effects of testosterone against oxidative stress damage, as observed in this study and others [246, 247].

It is possible that low levels of testosterone may predispose individuals to neurodegenerative disorders, since low testosterone has been linked with oxidative stress-associated neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and ischemic stroke [170, 199, 285-287]. A possible mechanism could be a reduction in testosterone-induced preconditioning, leading to greater vulnerability of specific neuronal systems to oxidative stress. However, once oxidative stress reaches levels at or above the "threshold" that can induce cellular damage, testosterone switches to a damage-promoting hormone by exacerbating oxidative stress-induced damage. As evidenced in this study, if testosterone is applied after a period of increased oxidative load that induces at least 20% cell death, testosterone exacerbates oxidative stress-related damage in a concentration-dependent manner. However, in low oxidative stress conditions that induce less than 20% cell death, testosterone does not impact cell viability. These results indicate that the presence of oxidative stress may act as a molecular switch, converting the neuroprotective effects of testosterone to that which is neurotoxic. Supporting this hypothesis that oxidative stress was a key determinant of testosterone's effect on cell viability, we found that the free radical scavenger, N-acetyl cysteine, and the mitochondrial calcium uniporter inhibitor prevented the cytotoxic effects of testosterone. A possible mechanism is that N-acetyl cysteine and the mitochondrial calcium uniporter inhibitor reset the cellular oxidative stress system, so that the effects of subsequent testosterone exposure may be more similar to effects observed in the testosterone pretreatment and concurrent oxidative stress condition, respectively.

This action of oxidative stress as a molecular switch for androgen actions is especially relevant to the debate of treating the elderly with androgens. Recently, testosterone replacement therapy (TRT) has been proposed as a neuroprotective therapeutic in aged men to prevent or treat age-related disorders [204, 285, 288]. However, results have been equivocal. These equivocal results were of sufficient concern to prompt the NIH and the Institute of Medicine (IOM) to question the value of TRT in elderly men. They concluded that further research is required to determine whether TRT is, in fact, a viable treatment for age-related disorders [289]. According to our data, we propose that TRT would be beneficial as a preventative strategy. However, TRT use as a treatment in oxidative stress conditions (i.e. Parkinson's disease, ischemic stroke) may exacerbate neuronal damage or in the case of ischemic stroke increase the risk for parkinsonism in men. Further, the physiological state of the patient with respect to oxidative stress levels should be examined prior to TRT to ensure that individuals with high levels of oxidative stress are not exposed, as TRT could be damage promoting in this group of individuals.

Mechanisms underlying testosterone's neurotoxic effects could include increased free radical generation and intracellular calcium. Testosterone, alone, increased ROS in N27 cells [48], and the free radical scavenger, N-acetyl cysteine, blocked the deleterious effects of testosterone on cell viability, indicating the involvement of ROS. These results are in agreement with previous studies investigating the contribution of sex differences in mitochondrial dysfunction where male rats had higher levels of peroxide production, lower mitochondrial glutathione (GSH), and increased oxidative damage to mitochondrial DNA compared to females [290, 291]. Further, human males have higher urinary

excretion of 8-hydroxyguanosine (8-OH-dG, a measure of oxidative damage to DNA) than females [292]. Together, these observations reinforce the hypothesis of androgen involvement in mitochondrial dysfunction and oxidative stress damage.

Another possible mechanism underlying androgen's neurotoxic effects is intracellular calcium. Interestingly, calcium accumulation can be an oxidative stressor per se [293]. Further, mitochondrial calcium overload has been implicated in the generation of ROS and in the release of several pro-apoptotic proteins [60], which may play an important role in the neurotoxic properties of androgens. In addition to free radical generation, excitotoxicity through calcium overload has been implicated in dopaminergic neuronal death [294]. In the current study, testosterone increased intracellular calcium concentrations via calcium release from intracellular calcium stores. In an oxidative stress environment, this increase in intracellular calcium can lead to calcium overload and neurotoxicity. Indeed, our results indicate that calcium influx into the mitochondria is one of the contributing factors for testosterone-induced cell loss in an oxidative stress environment. Inhibition of mitochondrial calcium influx blocked testosterone's damage promoting effects on N27 cells, consistent with other studies that showed neuroprotection in response to mitochondrial calcium inhibition [91, 295-297]. Therefore, the observation that aged men have a greater prevalence of Parkinson's disease [230, 234-237] and increased incidence of parkinsonism after ischemic stroke than aged women [20, 241], may be related to 1) the loss of androgen mediated preconditioning, resulting in increased oxidative stress and 2) androgen-induced calcium overload in the mitochondria leading to further increases in oxidative stress to induce dopaminergic neurotoxicity.

Interestingly, it does not appear that the classical androgen and estrogen receptors mediate androgen's neurotoxic effects in an oxidative stress environment, as this effect was insensitive to androgen and estrogen receptor antagonists. As an alternative, we proposed the potential involvement of a putative membrane-associated androgen receptor (mAR). Generally, mAR is indicative of non-genomic signaling, as mAR is insensitive to androgen receptor antagonists and associated with increased intracellular calcium signaling [162, 275, 298]. Consistent with mAR actions, the neurotoxic properties of testosterone in this study were insensitive to the androgen receptor antagonist, flutamide, and involved increased intracellular signaling. Furthermore, similar to what was observed with testosterone, testosterone-conjugated to BSA (TBSA), a mAR ligand, significantly decreased cell viability in an oxidative stress environment, but in a pre-treatment paradigm protected cells from oxidative stress-induced cell loss. Further research in this area needs to be conducted to determine if manipulation of this non-genomic pathway can provide more effective therapies for oxidative stress-mediated neurodegenerative conditions.

In conclusion, our results indicate that androgens are neuroprotective against subsequent oxidative stress-induced damage, possibly through a preconditioning mechanism. However, in a pre-existing high oxidative stress environment androgens can become neurotoxic and exacerbate oxidative stress damage. Our results offer a mechanism that reconciles the differing effects of androgens in an oxidative stress environment, such as the *in vivo* studies in male rodents from our laboratory and other laboratories, in which 1) androgens protected against oxidative stress-related insults, such as middle cerebral artery occlusion (MCAO), b-amyloid, and 3-nitropropionic acid [299-

302] and 2) androgens exacerbated damage resulting from oxidative stress-related insults of MCAO and 6-hydroxydopamine [49, 303, 304]. Further, this study provides further support for the possible role of androgenic mechanisms underlying the greater prevalence of Parkinson's disease [230, 234-237] and increased incidence of parkinsonism after ischemic stroke in men compared to women [20, 241].

ACKNOWLEDGEMENTS

We would like to thank Dr. Lora Talley-Watts, Dr. Andrea Giuffrida, Hai-Ying Ma, Nataliya Rybalchenko, Rosa Lemus, Dr. Victoria E. Centonze, Dr. James L. Roberts, and Dr. James Lechleiter for their technical assistance.

ANDROGENS ALTER MITOCHONDRIAL FUNCTION

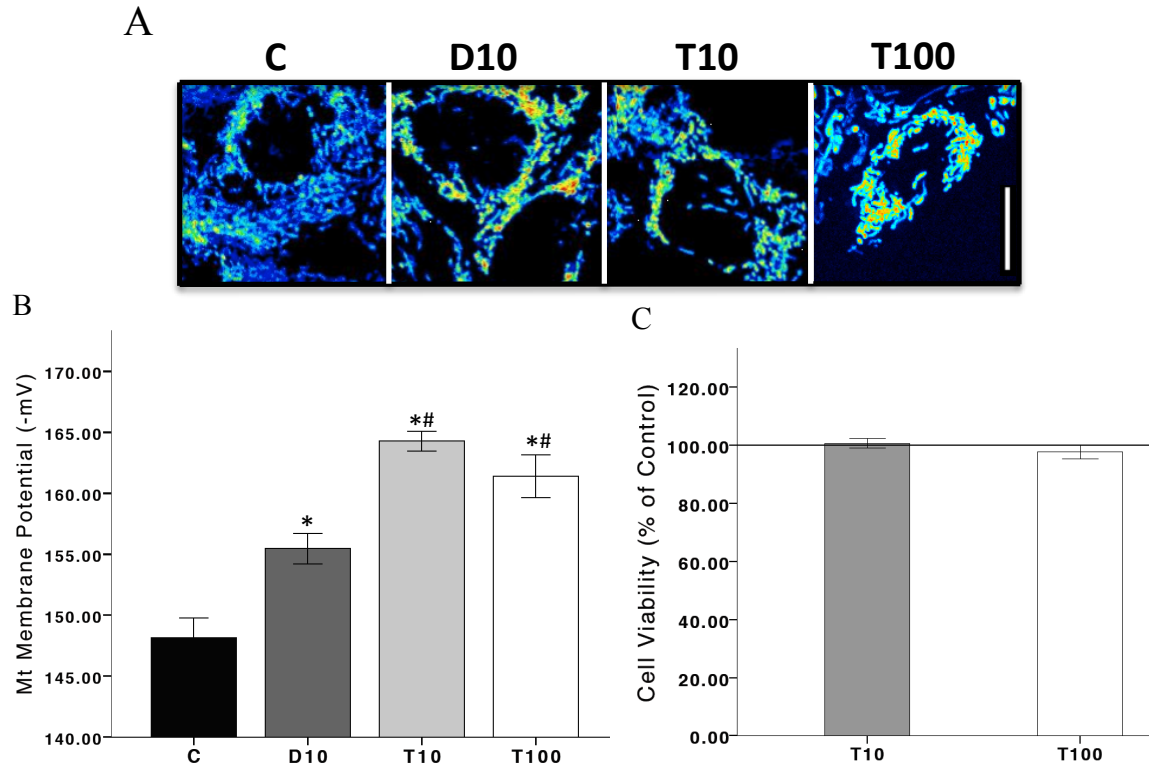


Figure 14. **Androgens alter mitochondrial function.** Mitochondrial membrane potentials were assayed with the fluorescent dye TMRM. Increased fluorescence is indicative of increased mitochondria membrane potentials. N27 cells were exposed to either testosterone (10 nM and 100 nM) or dihydrotestosterone (10 nM) for 2h (A, B). After 2h of exposure, testosterone and dihydrotestosterone significantly increased TMRM fluorescence in N27 cells compared to vehicle control. Testosterone significantly increased TMRM fluorescence compared to dihydrotestosterone. Testosterone exposure for 24h did not influence cell viability (C). T10: 10 nM testosterone; T100: 100 nM testosterone; D10: 10 nM dihydrotestosterone; C: vehicle control. ANOVA followed by LSD post hoc test. Scale bar = 10 μ m. * and # indicates significance compared to C and D10, respectively. Horizontal line demarcates controls normalized to 100%.

TIMING OF HYDROGEN PEROXIDE AND TESTOSTERONE.

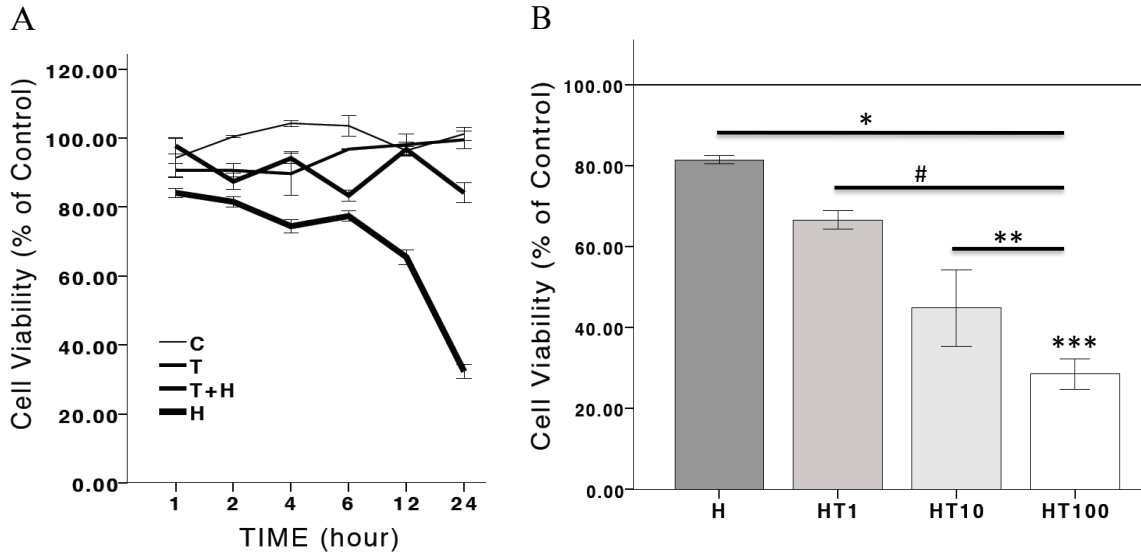


Figure 15. **Timing of hydrogen peroxide and testosterone.** Cell viability is presented as a percent of control. Hydrogen peroxide significantly decreased cell viability. 2h testosterone pre-treatment significantly blocked hydrogen peroxide-induced cell death (A). Hydrogen peroxide significantly decreased cell viability. 24h testosterone post-treatment significantly increased hydrogen peroxide-induced cell death in a dose-dependent manner (B). H: hydrogen peroxide; T: 100 nM testosterone; T+H: 100 nM testosterone pre-treatment; HT: testosterone 100 nM post-treatment; HT1: testosterone 1 nM post-treatment; HT10: testosterone 10 nM post-treatment; HT100: testosterone 100 nM post-treatment; C: vehicle control. ANOVA followed by LSD post hoc test. Significance symbols: * compared to C, # compared to H, ** compared to HT1, *** compared to HT10. Horizontal line demarcates controls normalized to 100%.

OXIDATIVE STRESS AND ANDROGEN INTERACTION

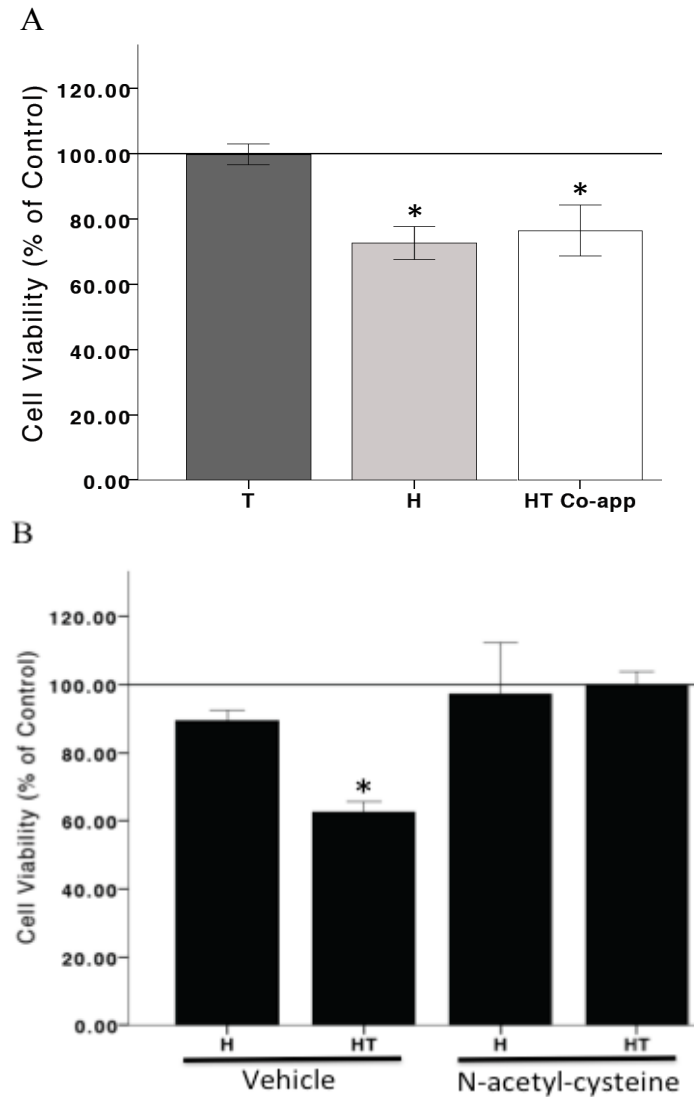


Figure 16. **Oxidative stress and androgen interaction.** Hydrogen peroxide significantly increased cell death. Testosterone does not alter hydrogen peroxide induced cell death when co-applied with hydrogen peroxide (A). The antioxidant, N-acetyl-cysteine (1 mM), blocked testosterone post-treatment and hydrogen peroxide-induced cell death (B). C: vehicle control; H: hydrogen peroxide; HT: testosterone 100 nM post-treatment. ANOVA followed by LSD post hoc test. Significance symbols: * compared to C. Horizontal line demarcates controls normalized to 100%.

**THE DELETERIOUS EFFECTS OF TESTOSTERONE ARE NOT MEDIATED
BY ANDROGEN OR ESTROGEN RECEPTORS**

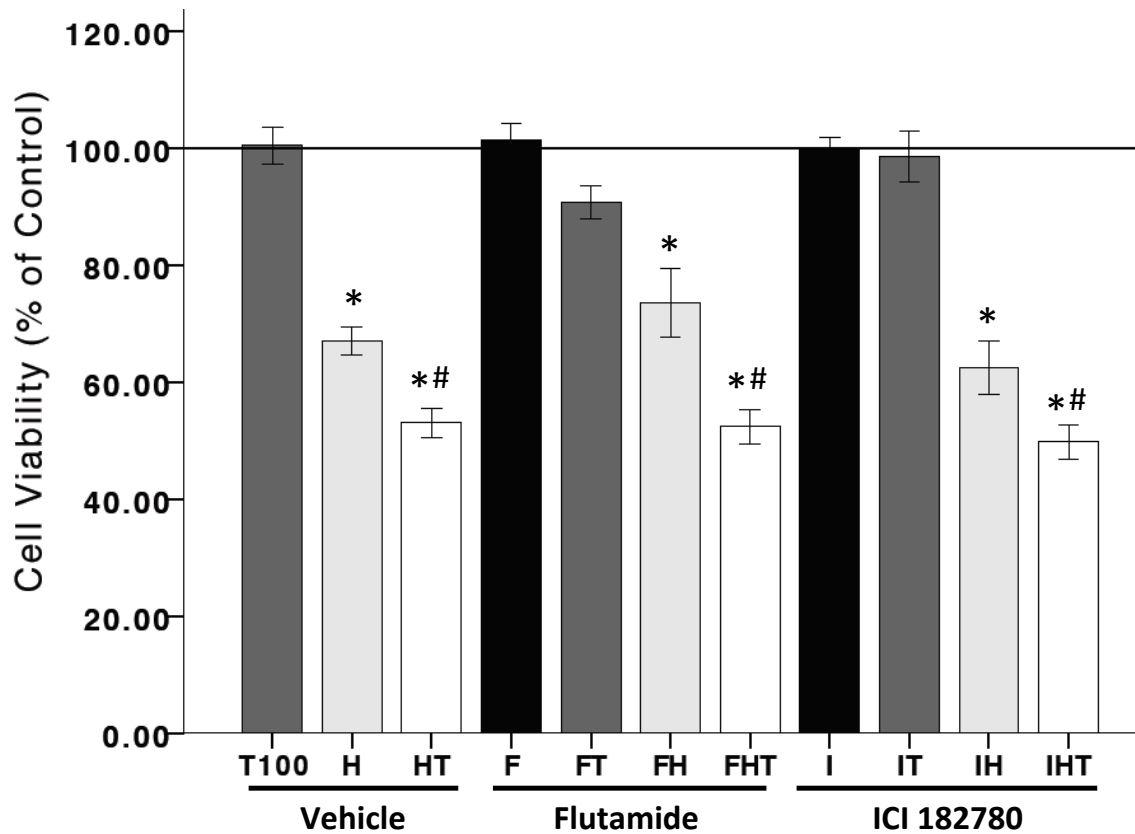


Figure 17. **The deleterious effects of testosterone are not mediated by androgen or estrogen receptors.** Neither the androgen receptor nor estrogen a/b receptors mediate testosterone post-treatment negative effects on cell viability, as evidenced by the lack of effect in response to the androgen receptor antagonist, flutamide (500 nM), and the estrogen a/b receptor antagonist, ICI 182,780 (1 uM). H: hydrogen peroxide; T: 100 nM testosterone; HT: testosterone post-treatment; C: vehicle control. ANOVA followed by LSD post hoc test. Significance symbols: * compared to C, # compared to H. Horizontal line demarcates controls normalized to 100%.

TESTOSTERONE AND INTRACELLULAR CALCIUM

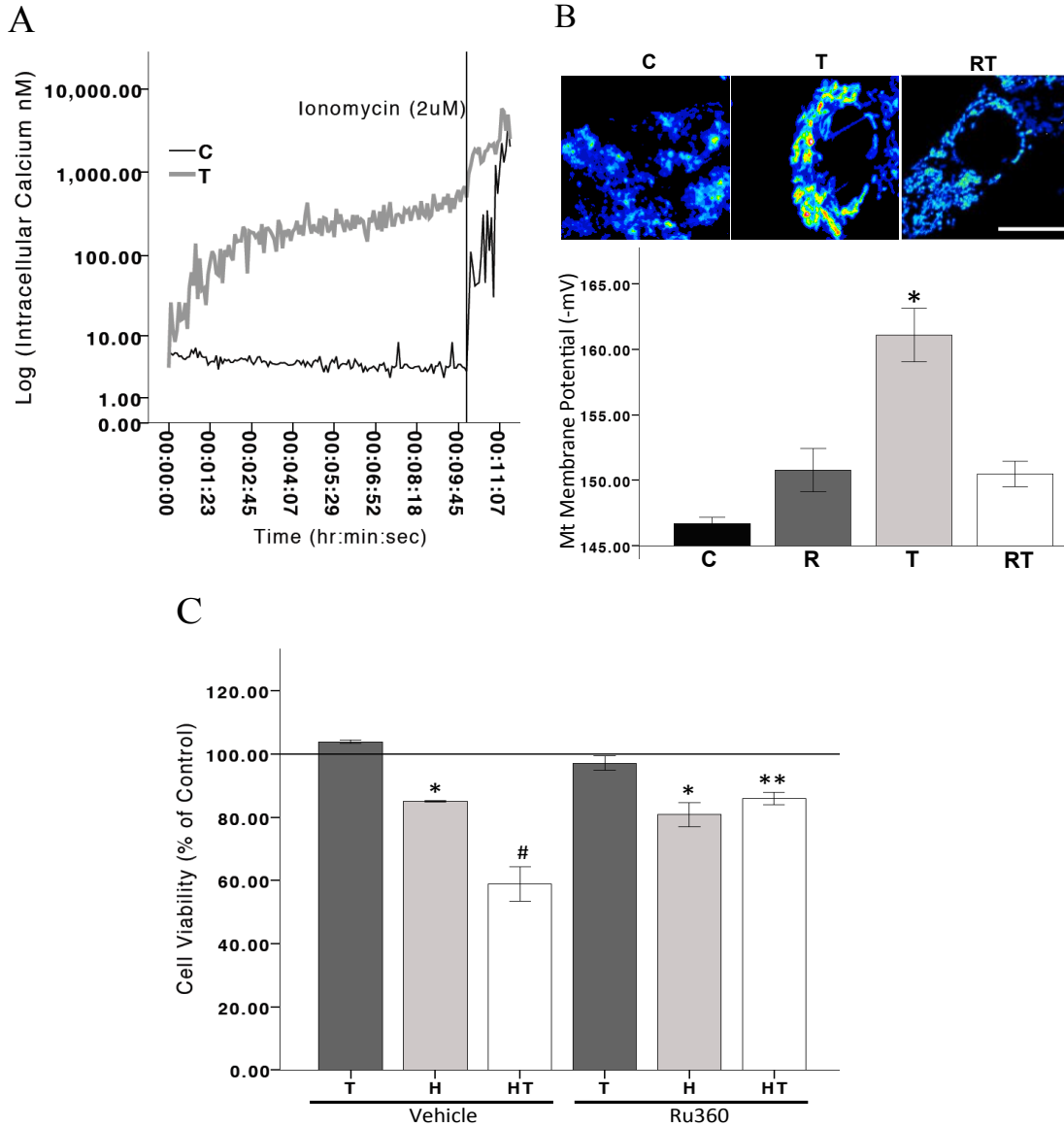


Figure 18. **Testosterone and intracellular calcium.** Intracellular calcium ($[Ca^{++}]_i$) levels were measured to determine if androgens induce calcium release from intracellular stores. FURA-2AM loaded N27 cells were bathed in a calcium-free HBSS buffer and basal $[Ca^{++}]_i$ were determined until stable. One minute after stabilization, cells were treated with vehicle control or testosterone. In the calcium-free buffer, baseline calcium levels were 4.5 nM and testosterone significantly increased $[Ca^{++}]_i$ to 250 nM. The

addition of 2 μ M ionomycin to the media was used to stimulate maximum intracellular calcium release. Paired t-test was used for analysis (A). Mitochondrial function in response to ruthenium 360 (1 μ M), a mitochondrial calcium uniporter inhibitor, was used to determine if the androgen-induced effects on mitochondrial function were mediated through calcium influx into the mitochondria. N27 cells were exposed to either testosterone or ruthenium 360 + testosterone for 2h. Ruthenium 360 significantly blocked the androgen-induced increase in membrane potentials (B). To determine the involvement of mitochondrial calcium influx in testosterone post-treatment of hydrogen peroxide induced cell death, ruthenium 360 was applied 30 min prior to testosterone exposure. Ruthenium 360 significantly blocked testosterone induced cell death in an oxidative stress environment (C). H: hydrogen peroxide; T: 100 nM testosterone; HT: testosterone post-treatment; R: ruthenium 360; RT: ruthenium 360 + testosterone; C: vehicle control. ANOVA followed by LSD post hoc test. Significance symbols: * compared to C, # compared to C, H, ** compared to HT. Scale bar = 10 μ M. Horizontal line demarcates controls normalized to 100%.

MEMBRANE-ASSOCIATED ANDROGEN RECEPTOR INVOLVEMENT

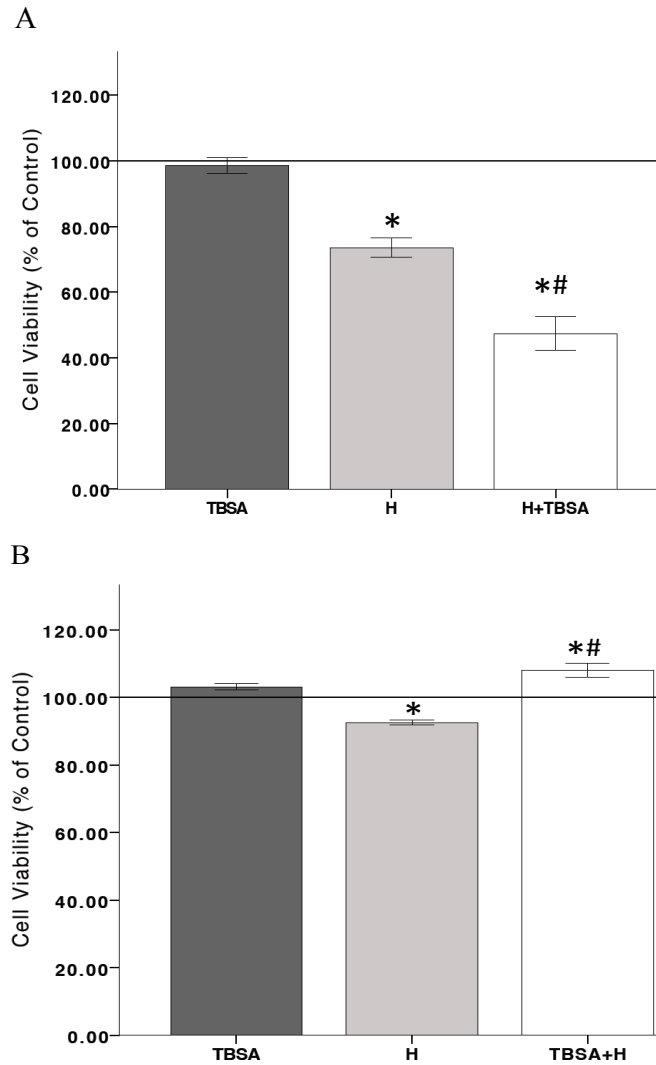


Figure 19. **Membrane-associated androgen receptor involvement.** To examine the role of membrane-associated androgen receptors, testosterone conjugated to BSA (TBSA) was used to bind only putative membrane receptors. TBSA alone does not affect cell viability. Hydrogen peroxide significantly decreased cell viability. 24h TBSA post-treatment significantly increased hydrogen peroxide-induced cell death (A). TBSA pre-treatment for 2h blocked cell death induced by a 6h exposure to hydrogen peroxide (B). H: hydrogen peroxide; TBSA: 500 nM testosterone-conjugated to BSA; H+TBSA: TBSA

post-treatment; TBSA+H: TBSA pre-treatment. C: vehicle control. ANOVA followed by LSD post hoc test. Significance symbols: * compared to C, # compared to H. Horizontal line demarcates controls normalized to 100%.

CHAPTER III

THE EFFECTS OF OXIDATIVE STRESS AND TESTOSTERONE ON PRO- INFLAMMATORY SIGNALING IN A FEMALE RAT DOPAMINERGIC NEURONAL CELL LINE

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Under review with Endocrinology

ABSTRACT

Parkinson's disease, a progressive neurodegenerative disorder characterized by distinctive motor dysfunctions, is associated with oxidative stress, inflammation and alpha synuclein. These pathological markers can contribute to the loss of dopamine neurons in the substantia nigra pars compacta. Interestingly, men have a two-fold increased prevalence for Parkinson's disease than women. While the mechanisms underlying this sex difference remain elusive, we propose that the primary male sex hormone, testosterone, is involved. Our previous studies show that testosterone through a putative membrane associated androgen receptor can increase oxidative stress induced neurotoxicity in dopamine neurons. Based on these results, this study examines the role of Nuclear Factor Kappa B (NFkB) and cyclooxygenase 2 (COX2), alpha synuclein, and apoptosis in the deleterious effects of androgens. Specifically, we hypothesize that under conditions of oxidative stress, testosterone will increase COX2 mediated apoptosis in dopamine neurons. To test our hypothesis, we exposed a N27 dopaminergic cell line to a sublethal concentration of the pro-oxidant, tert-butyl hydrogen peroxide followed by physiologically relevant concentrations of testosterone to assess oxidative stress, cell viability, COX2, alpha synuclein and apoptosis. Our data show that testosterone, under oxidative stress conditions, increased the expression of COX2, alpha synuclein and apoptosis in dopamine neurons. This data further supports the role of testosterone mediating the loss of dopamine viability under oxidative stress conditions, which may be a key mechanism contributing to the increased prevalence of Parkinson's disease in men compared to women.

INTRODUCTION

Oxidative stress and neuroinflammation are key mechanisms involved in the pathogenesis of Parkinson's disease (PD) [71, 305, 306]. PD is clinically identified by motor dysfunctions of bradykinesia, postural instability and rigidity, and this loss of motor dysfunction is associated with the substantial loss of dopamine neurons [307]. In addition, this loss of neurons has been linked to oxidative stress and neuroinflammation [5, 41, 308]. Oxidative stress, a biomarker recognized in PD, is characterized by high reactive oxygen species and low antioxidant mechanisms [53-55]. Antioxidants such as glutathione are responsible for scavenging reactive oxygen species to maintain cellular homeostasis [24, 309]. Moreover, oxidative stress is present in dopamine neurons due to the metabolism of dopamine [18]. Dopamine can be metabolized by an enzyme monoamine oxidase, which produces hydrogen peroxide inducing oxidative stress in a neuronal environment [18, 78]. Furthermore, dopamine can readily auto-oxidize creating reactive oxygen species in the cellular environment that could contribute to oxidative stress [18]. In addition to the susceptibility of dopamine neurons, oxidative stress can be a major contributor to cell death through the activation of neuroinflammation and apoptosis implicated in PD [18, 53].

The classical signaling of neuroinflammation is initiated by microglia and increased by neuronal signaling [78, 308, 310]. But neuroinflammation can be initiated by oxidatively stressed neurons through the activation of Nuclear Factor kappa B (NFkB) and cyclooxygenase 2 (COX2) [78]. As a pro-inflammatory mediator, transcription factor NFkB heterodimer is activated by high oxidative stress and translocated to the nucleus to upregulate inflammatory proteins, such as COX2 [115, 120]. COX2 contains binding

sites for NFkB in its promoter region, wherein NFkB can regulate the protein expression of COX2 [115]. COX2 can act as a peroxidase releasing free radicals into the cytoplasm, which can further contribute to oxidative stress generation [123]. This accumulation of free radicals in the cytoplasm stimulates the accumulation of alpha synuclein, a major component of Lewy Bodies expressed in PD [134, 311]. Hence, alpha synuclein associated Lewy Bodies are linked with the peroxidase activity of COX2 that could influence dopamine's oxidation [38, 127]. In addition to alpha synuclein, chronic oxidative stress generation can cause DNA damage and thus activating apoptosis [48, 211]. Apoptosis, programmed death of cells, is characterized by DNA fragmentation and chromatin condensation in neurons [121, 312, 313].

In addition to understanding the signaling mechanisms involved in PD, our previous study shows that sex hormones may be involved in dopamine neurodegeneration. Interestingly, men have a two-fold higher risk for PD than women, suggesting that androgens may play a role in PD [22]. Moreover, there is an age-related decline in androgens, such as testosterone, in men [8, 163]. However, testosterone is still higher in men than women, which may contribute to the high prevalence of PD in men [22, 309]. Previously, we found that in an oxidative stress environment, testosterone increases oxidative stress, leading to dopamine neurodegeneration [218]. These damaging effects of testosterone are associated with a putative membrane associated androgen receptor characterized by testosterone binding to a plasma membrane receptor and mediating the release of intracellular calcium [218, 314]. This rise in intracellular calcium can increase oxidative stress to induce neurotoxicity [315, 316]. Hence, testosterone through non-genomic signaling can increase oxidative stress and further exacerbate free

radical generation in oxidative stress environments, such as PD [218]. Since, oxidative stress has been linked with increased inflammation in dopamine neurons [5, 41]. We propose that testosterone via oxidative stress generation will activate the inflammatory signaling pathway in dopaminergic neurons. In this study, we will examine the effects of oxidative stress and testosterone via the membrane androgen receptor on the pro-inflammatory markers, NFkB and COX2, involved in dopamine cell viability.

MATERIALS AND METHODS

Reagents- Testosterone, tert-butyl hydrogen peroxide and β -actin were obtained from Sigma (Saint Louis, MO). Dihydrotestosterone 3-CMO: BSA (DHT-BSA) was obtained from Steraloids (Newport, RI). COX2 was obtained from Abcam (Cambridge, MA), and alpha synuclein was obtained from Cell Signaling (Danvers, MA), and Goat anti-Rabbit was obtained from Thermo Scientific (Rockford, IL). Ibuprofen was purchased from Calbiochem (San Diego, CA) and Caffeic acid phenethyl ester (CAPE) was purchased from Santa Cruz (Santa Cruz, CA). DMSO was purchased from VWR and phosphate buffer solution (PBS) from Corning (Manassas, VA). RPMI 1640 and Penicillin-streptomycin (PS) were purchased from Thermo Scientific (Logan, UT), Fetal Bovine Serum (FBS) from Life technologies (Grand Island, NY), Charcoal-stripped Fetal Bovine Serum (CS-FBS) from Atlanta Biologicals (Lawrenceville, GA), and Penicillin-streptomycin (PS) from Thermo Scientific (Logan, UT). Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma (Saint Louis, MO). Vybrant Apoptosis assay was obtained from Invitrogen; Molecular probes (Eugene, OR). Fluorescent thiol detection assay was purchased from Cell Technology (Fremont, CA). Super signal West

Pico chemiluminescent substrate was obtained from Thermo Scientific (Rockford, IL). Testosterone was made from a stock solution in ethanol (final concentration of ethanol < 0.001%), CAPE and NFkB inhibitors were made from a stock solution in DMSO (final concentration of DMSO < 0.001%). Vehicle control was less than 0.001% DMSO.

Cell Culture- The immortalized neuronal cell line 1RB3AN₂₇ (N27) was derived from fetal female rat mesencephalic tissue [48, 218]. The N27 cell line is positive for tyrosine hydroxylase and androgen receptor protein expression [48]. Cells were maintained at 37°C in 5% CO₂ and grown in RPMI 1640 medium supplemented with 10% FBS and 1% PS. The cells were sub-cultured weekly and all experiments were performed between passages 13-19, and at approximately 80% confluency in RPMI 1640 medium with Charcoal-stripped FBS to avoid confounders due to the presence of hormones in the serum [48].

Cell Viability- Cells were plated into 96-well tissue culture plates at an approximate density of 4.0×10^4 cells in each well. Cells were treated with 50 – 100 μ M tert-butyl hydrogen peroxide for 2 hours to induce approximately 20% cell loss, followed by 2 hours of 100nM testosterone or 500nM DHT-BSA based on previous studies [164, 218]. Inhibitor concentrations were determined by cell viability in our concentration response studies in N27 cells. Cells were exposed to inhibitors 1-2 hours prior to tert-butyl hydrogen peroxide. For analysis of cell viability, media was aspirated and replaced with phenol-red free RPMI 1640 medium with CS-FBS and 20 μ L of 5 mg/mL MTT for an

incubation period of 3-4 hours at 37°C. Absorbance was read at 595 nm. The colorimetric intensity is directly proportional to the number of live cells.

Oxidative stress- Cells were grown in 60mm x 15mm and 100mm cell culture plates. Cells were treated at 80% confluency with 50-100 μ M tert-butyl hydrogen peroxide for 24 hours to induce approximately 20% cell loss followed by the exposure to testosterone for 24 hours. For oxidative stress quantification, the level of reduced thiols in the cell lysates (1.0×10^5 cells/mL) was determined by using the Fluorescent Thiol detection kit, according to our previous published method, with at least three independent experiments [48]. Fluorescence was measured at 488 excitation and 525 nm emission wavelengths.

Cell lysates- Cells were treated at 80% confluency with 50-100 μ M tert-butyl hydrogen peroxide to induce approximately 20% cell loss, followed by the exposure to testosterone. Cells were washed with PBS and lysed using a cocktail containing NP40 lysis buffer and phosphatase inhibitors (1:100) on ice. The lysates were clarified by centrifugation at 4°C for 20 minutes at 9,300g. Protein concentrations were determined by using the Bradford Protein assay (Thermoscientific), according to the manufacturer's instructions.

Western Blot- Equal amounts of (20-40ug) of cell lysates were loaded and separated in BioRAD Any KD polyacrylamide gels and transferred onto a PVDF membrane. Membranes were washed with TBS-Tween and blocked for 30 minutes with 5% nonfat milk. The membranes were incubated with specific primary antibodies (COX2 1:500, Alpha synuclein 1:1000 and β actin 1:50000) for 2 hours – overnight in 4°C followed by

incubation of secondary antibodies at 1:2,000 for 45 minutes at room temperature. Protein bands were visualized with an enhanced chemiluminescence detection assay (Thermoscientific) for 2 minutes. Band densities were analyzed by NIH Image J densitometer software.

Apoptosis- Cells were plated and treated into 35mm x 10mm tissue culture plates at a density of 8.6×10^5 cells in each plate. Cells were treated at 80% confluency with 50-100 μ M tert-butyl hydrogen peroxide to induce approximately 20% cell loss, followed by the exposure to testosterone. The Vybrant apoptosis assay (Hoechst 33342) was used to quantify apoptosis, according to our previously published method [48]. This assay stains pro-apoptotic condensed chromatin. Results were quantified from at least three independent replications using NIS-elements software.

Statistical Analysis- Analysis was performed using IBM SPSS Statistics version 21 software. Data were expressed as mean \pm SEM. Significance ($p \leq 0.05$) was determined by ANOVA followed by Tukey's post hoc analysis for the assessment of group differences. At least three independent replications per experiment were performed for this study.

RESULTS

Testosterone is an oxidative stressor in N27 dopamine neurons- In our previous studies, testosterone increased oxidative stress and promoted neurotoxicity in oxidative stressed N27 cells [48, 218]. The current study examines oxidative stress

indirectly by measuring the levels of reduced thiol, glutathione. Glutathione, a common indicator for oxidative stress, identifies the reducing capacity in a cellular environment [163]. Figure 20 shows N27 dopaminergic cells treated with hydrogen peroxide for 24 hours followed by testosterone exposure for 24 hours. In Figure 20A, testosterone significantly decreased reduced thiols and thus increased oxidative stress, regardless of the oxidative stress environment ($F_{3, 28} = 853.459$, $p < 0.05$). Unlike Figure 20A, Figure 20B shows testosterone only decreases cell viability in cells pretreated with tert-butyl hydrogen peroxide ($F_{3, 11} = 87.758$, $p < 0.05$). These results suggest that testosterone can act as an oxidative stressor, but only negatively impacts dopamine cell viability in oxidative stress environments.

Testosterone and Oxidative Stress influence on NFkB signaling in N27 dopamine neurons- NFkB is an oxidative stress responsive transcription factor that can promote pro-inflammatory signaling [120]. CAPE, a potent inhibitor of NFkB activation has been shown to increase dopamine neuronal survival [317]. Interestingly, we show that CAPE blocked testosterone's negative effects on cell viability in an oxidative stress environment ($F_{4, 14} = 82.420$, $p < 0.05$, Figure 21A). In addition, CAPE decreased the protein expression of COX2 in dopamine neurons ($F_{3, 8} = 10.507$, $p < 0.05$, Figure 21B). These results indicate that testosterone can potentiate NFkB activity, resulting in decreased dopamine cell viability and increased COX2 expression.

Testosterone increases COX2 expression in N27 dopamine neurons- In response to oxidative stress, NFkB targets the transcription and expression of pro-inflammatory COX2 [112, 115]. Figure 22A shows that testosterone significantly increased COX2 protein expression in cells pretreated with tert-butyl hydrogen peroxide

($F_{3, 11} = 34.942$, $p < 0.05$). Prior studies show that a COX2 nonselective inhibitor, ibuprofen, can protect dopamine cells against neurotoxic insults of MPP⁺ and 6-OHDA on dopamine neurons [318, 319]. We show that ibuprofen significantly decreased testosterone's effect on the protein expression of COX2 in N27 dopamine neurons ($F_{3, 9} = 25.586$, $p < 0.05$, Figure 22B). Figure 22C shows that, ibuprofen significantly blocked testosterone's negative effects on cell viability in oxidative stressed treated cells ($F_{4, 14} = 26.663$, $p < 0.05$). Previously, we have shown that the negative effects of testosterone can be mediated through a putative membrane associated androgen receptor [218]. Indeed, the cell membrane impermeable reduced testosterone, dihydrotestosterone conjugated to BSA (DHT-BSA), also decreased cell viability in oxidative stress conditions (Figure 22D). These results further indicate the involvement of a putative membrane androgen receptor in the effects of testosterone on dopaminergic neurons. Interestingly, in Figure 22D inhibition of COX2 by ibuprofen significantly blocked the negative effects of DHT-BSA on cell viability in an oxidative stressed environment ($F_{4, 19} = 24.075$, $p < 0.05$). These results indicate that testosterone in an oxidative stress environment can exacerbate pro-inflammatory COX2 signaling through the membrane associated androgen receptor.

Ibuprofen increases survival in N27 dopamine neurons – Ibuprofen has been shown to lower the risk of PD in clinical trials and increase dopaminergic viability possibly by decreasing the pro-inflammatory activity of COX2 [318, 320] Thus, in Figure 23, we examine the association between COX2 activity and oxidative stress signaling of apoptosis. Testosterone alone does not lead to apoptosis in dopamine cells. However, in our studies, testosterone can exacerbate hydrogen peroxide induced apoptosis. Interestingly, the inhibition of COX2 by ibuprofen can block testosterone's negative

effects on apoptosis in an oxidative stress environment (Figure 23A, B). Further supporting the role of COX2 activity and apoptosis, Ibuprofen significantly decreased apoptosis in cells treated with both oxidative stress and testosterone ($F_{4, 26} = 113.551$, $p < 0.05$, Figure 23B). Ibuprofen significantly increased the reduced thiols, decreasing oxidative stress in dopamine neurons ($F_{4, 66} = 79.002$, $p < 0.05$, Figure 23C). Therefore, these results indicate that COX2 is involved in testosterone-induced apoptosis in oxidative stress environments.

Testosterone increases alpha synuclein expression in N27 dopamine neurons-

In addition to its pro-inflammatory effects; COX2 can act as an oxidative stressor to further promote free radical generation [112, 123]. This increase in oxidative stress can initiate the accumulation of alpha synuclein, the major component of Lewy bodies in PD [127, 321]. Alpha synuclein accumulation is a prominent marker in PD [322]. In Figure 24A we show that in oxidative stress conditions, testosterone significantly increased alpha synuclein protein expression ($F_{3,7} = 152.069$, $p < 0.05$). Ibuprofen significantly decreased testosterone's effect on the protein expression of alpha synuclein in dopamine neurons, indicating the involvement of COX2 in alpha synuclein accumulation ($F_{3,8} = 21.703$, $p < 0.05$, Figure 24B). These results show that testosterone in an oxidative stress environment can induce alpha synuclein accumulation; a key component of Lewy Bodies, through a COX2 mediated mechanism.

The effects of Ibuprofen on Testosterone- In our studies, ibuprofen has been targeting the additive effect of testosterone in oxidative stress conditions. Previously, we have identified testosterone alone as an oxidative stressor. Thus, we examined whether ibuprofen affects testosterone induced oxidative stress signaling. Interestingly, ibuprofen

significantly increased the reduced thiols and thus decreased oxidative stress ($F_{3,23} = 12.205$, $p < 0.05$, Figure 25). These results show that COX2 is mediating the oxidative actions of testosterone in dopamine neurons.

DISCUSSION

The high prevalence of PD in men indicates a sex bias that suggests the involvement of testosterone in dopamine neurodegeneration. Testosterone identified as an oxidative stressor can promote apoptotic cell death [167, 203, 218, 323, 324]. Thus, in an oxidative stress environment, testosterone signaling can be detrimental. Our previous publication showed that testosterone decreases dopamine cell viability through the exacerbation of oxidative stress [218]. Oxidative stress commonly associated with the degeneration of dopamine neurons, is a biomarker in PD [325, 326]. Further, the indication of oxidative stress marked by the decrease in reduced thiols is identified in PD brains [327]. In this study, we show 1) A decrease in reduced thiols and cell viability as a result of oxidative stress signaling in dopamine neurons, which can be exacerbated by the presence of testosterone and 2) Testosterone-induced oxidative stress decreases cell viability through the signaling of pro-inflammatory mediators (NFkB and COX2).

As a transcription factor NFkB binds to the COX2 promoter region regulating COX2 protein expression [115]. Since NFkB targets COX2, we found the protein expression of COX2 to be decreased with an inhibitor of NFkB. High COX2 expression has been identified in postmortem PD brains [126, 328]. In comparison, we show that testosterone can exacerbate COX2 expression in an oxidative stress environment. The non-selective COX2 inhibitor, ibuprofen, decreased the protein expression of COX2 in

oxidative stressed dopamine neurons. Furthermore, COX2 activity as a peroxidase contributes to oxidative stress through the production of free radicals in the cytoplasm. Since, ibuprofen increased cell viability in midbrain neurons [319] we decided to look at the effect of COX2 signaling in dopamine neurons. In our study, ibuprofen increased the survival of N27 dopamine neurons by reducing oxidative stress. Oxidative stress is a signaling mechanism that contributes to dopamine oxidation and alpha synuclein accumulation. Because testosterone is an oxidative stressor, it could contribute to alpha synuclein accumulation. Interestingly, we show that testosterone via oxidative stress contributes to the accumulation of alpha synuclein. Alpha synuclein aggregates are found in patients with Lewy Body dementia [329, 330]. The inhibition of COX2 decreased the accumulation of alpha synuclein in oxidative stressed dopamine neurons, identifying that COX2 plays a vital role in testosterone mediated Lewy Bodies.

Further, the exacerbation of oxidative stress induces cellular damage beyond repair, stimulating executioner proteins of apoptosis [48]. Thus, COX2 activity contributes to oxidative stress signaling that increases apoptotic cell death. Indeed, our study shows that the inhibition of COX2 decreased testosterone-induced apoptosis in N27 dopamine neurons. This study suggests that COX2 plays a major role in the signaling of oxidative stress and testosterone. In summary, our results indicate that testosterone may mediate the sex biased observed in PD by acting as an oxidative stressor to exacerbate inflammation leading to apoptosis.

Recently, we identified that testosterone's neurotoxicity can be mediated through a non-genomic mechanism via a putative membrane androgen receptor [218]. Our previous studies showed that the membrane androgen receptor-testosterone complex is

characterized by the release of intracellular calcium stores upon the binding of testosterone [218]. Thus indicating that testosterone mediates calcium signaling, which can lead to high oxidative stress via calcium neurotoxicity [167, 315, 316]. Consistent with our previous study, the membrane androgen receptor mediated testosterone's negative effects on cell viability and oxidative stress. Interestingly, our data suggests that hydrogen peroxide and testosterone increase oxidative stress by different mechanisms. For example, hydrogen peroxide easily diffuses through the cell membrane having a direct effect on cell viability and DNA damage that is irreversible [316, 331]. In comparison, we show that ibuprofen can block testosterone's effects on cell viability, oxidative stress and alpha synuclein accumulation, whereas Ibuprofen had no effects on hydrogen peroxide induced cell damage.

In conclusion, Figure 26 identifies the signaling of oxidative stress and testosterone on neuroinflammatory markers in dopamine neurons. Our studies identify that testosterone through a putative membrane associated androgen receptor increases oxidative stress. However, in the presence of oxidative stress induced by hydrogen peroxide, testosterone exacerbates oxidative stress generation, as evident by decreased reduced thiols (GSH) and thereby decreases cell viability. This decrease in cell viability is mediated by inflammatory signaling via NFkB-COX2 pathway, which increases alpha synuclein Lewy bodies and apoptosis. Therefore, this pathway may be a future therapeutic target for decreasing PD risk and progression in men.

ACKNOWLEDGEMENTS

We would like to thank Nataliya Rybalchenko and Rizwan Nazarali for their technical assistance.

TESTOSTERONE IS AN OXIDATIVE STRESSOR IN N27 DOPAMINE

NEURONS

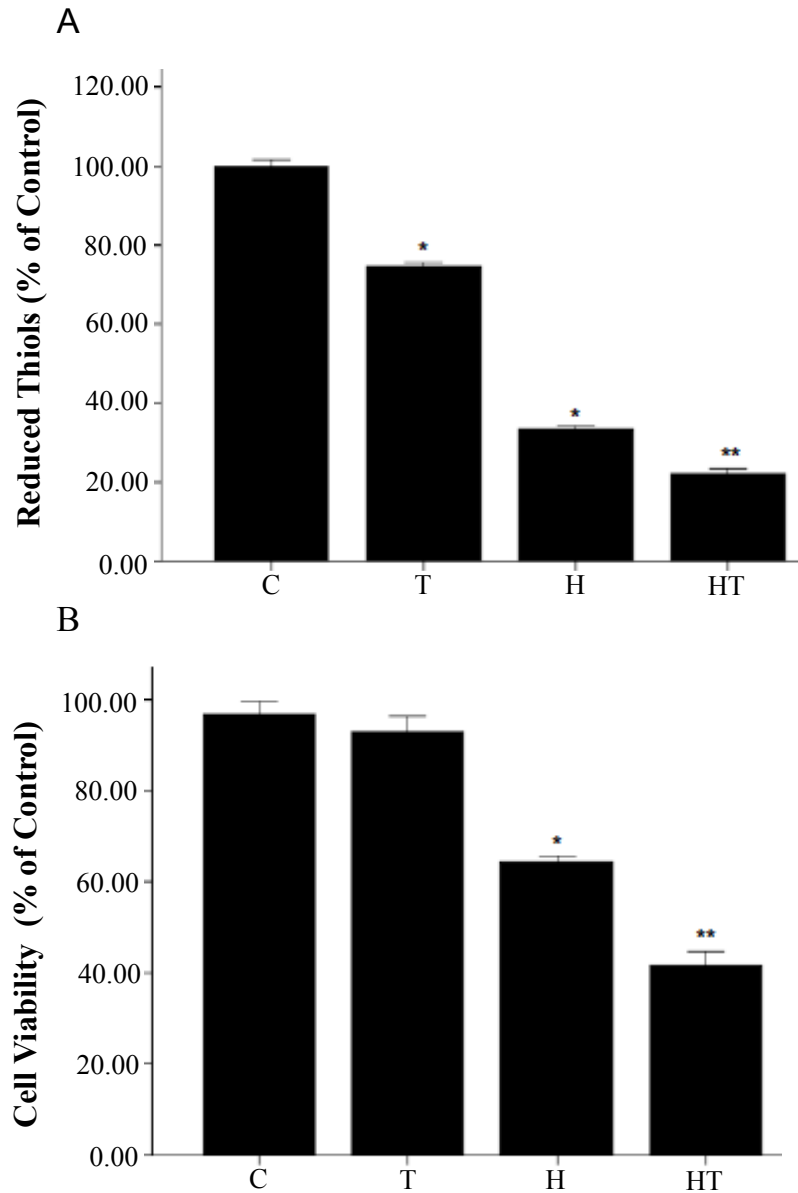


Figure 20. **Testosterone is an oxidative stressor in N27 dopamine neurons.** Oxidative stress is an indirect measure of reduced thiols. A) Testosterone significantly decreased the levels of reduced thiols. Testosterone significantly decreased the levels of reduced thiols in cells pretreated with tert- butyl hydrogen peroxide. B) Testosterone has no effect

on cell viability. Tert-butyl hydrogen peroxide (H) significantly decreased cell viability. Testosterone only significantly decreased cell viability in cell pretreated with tert-butyl hydrogen peroxide. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, ** compared to H. n=3 per treatment group, at least three independent replications were performed.

TESTOSTERONE AND OXIDATIVE STRESS INFLUENCE ON NFkB

SIGNALING IN N27 DOPAMINE NEURONS

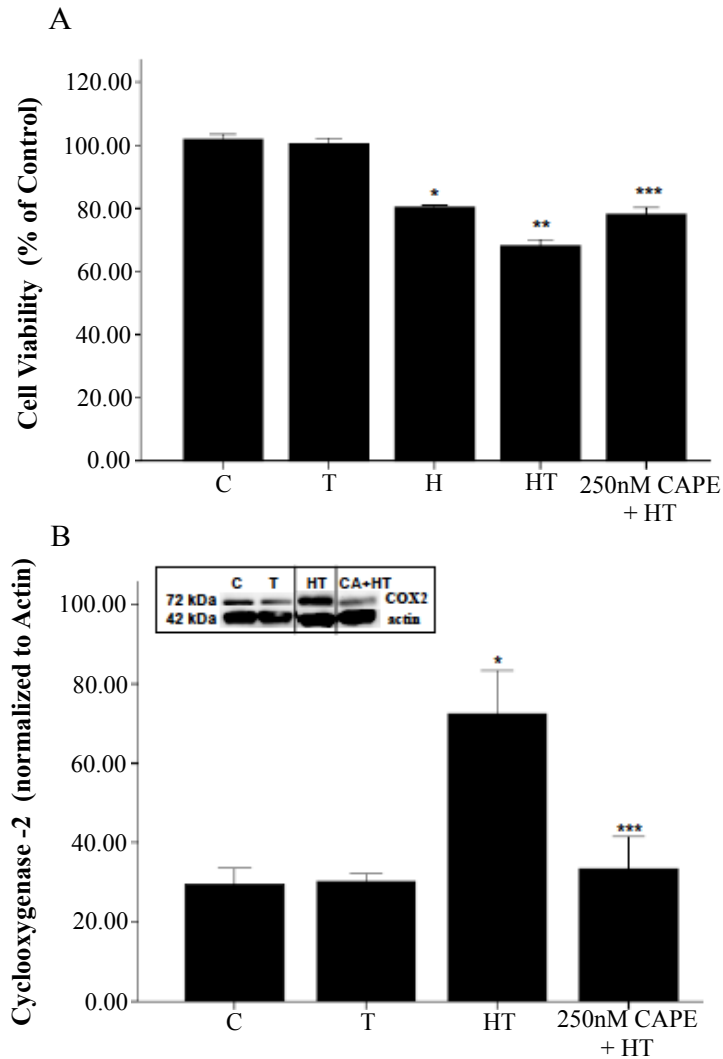


Figure 21. **Testosterone and Oxidative Stress influence on NFkB signaling in N27 dopamine neurons.** A) CAPE increases dopamine neuronal cell viability. B) CAPE decreases COX2 protein expression. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone; CA: CAPE. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, ** compared to H, ***

compared to HT. Line demarcates breaks in membrane. n=3 per treatment group, at least three independent replications were performed.

TESTOSTERONE INCREASES COX2 EXPRESSION IN N27 DOPAMINE

NEURONS

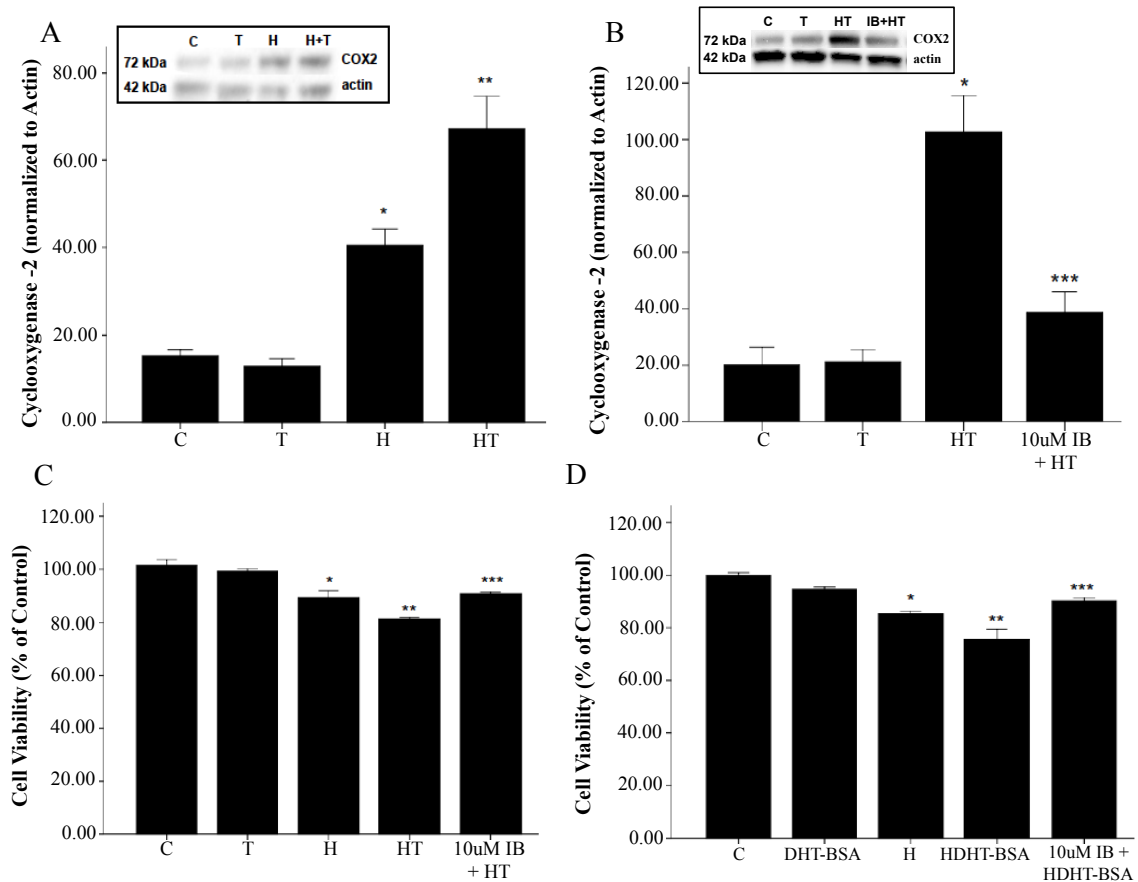


Figure 22. **Testosterone increases COX2 expression in N27 dopamine neurons.** A) Testosterone significantly increased COX2 protein expression in cells pretreated with tert-butyl hydrogen peroxide. B) IB significantly decreased COX2 protein expression in cells post-treated with testosterone. C) IB significantly increased cell viability of N27 dopamine neurons. D) IB significantly increased cell viability in oxidative stressed cells treated with DHT-BSA. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone; DHT-BSA: post-treatment dihydrotestosterone-bovine serum albumin; IB: 10uM Ibuprofen. ANOVA followed by

Tukey post hoc test. Significance symbols: * compared to C, ** compared to H, *** compared to HT/HDHT-BSA. n=3 per treatment group, at least three independent replications were performed.

IBUPROFEN INCREASES SURVIVAL IN DOPAMINE NEURONS

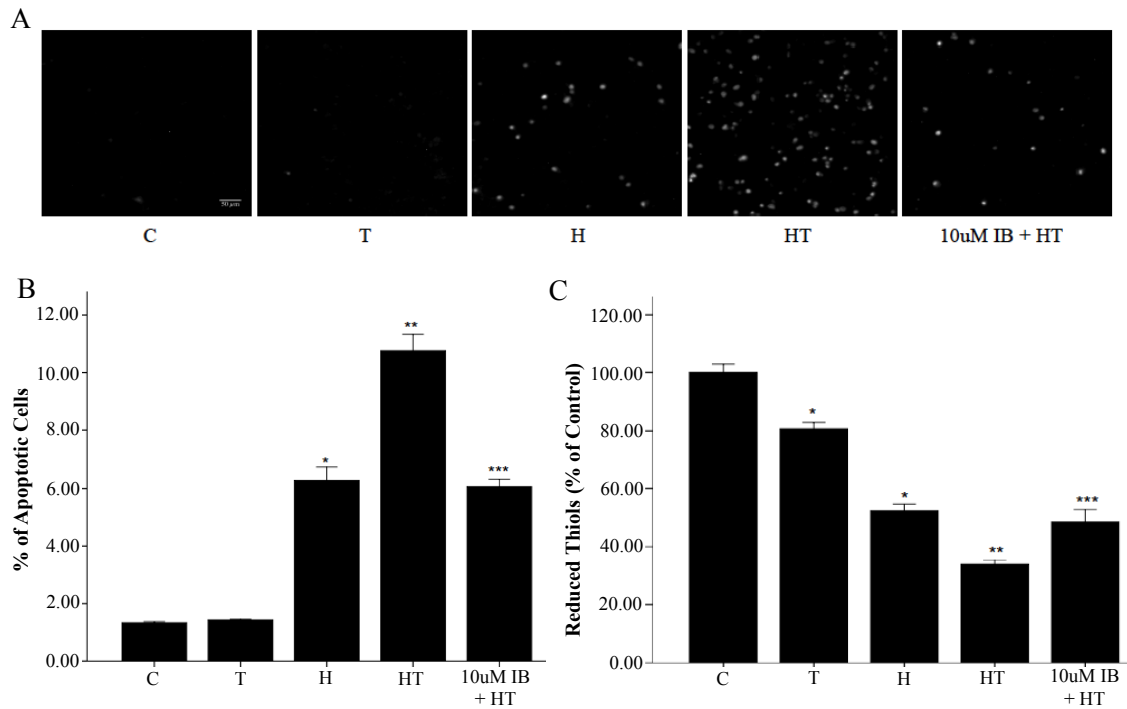


Figure 23. **Ibuprofen increases survival in dopamine neurons.** A) Cells treated with Ibuprofen significantly increased reduced thiols in oxidative stressed dopamine neurons. B) IB decreased apoptosis in oxidative stressed dopamine neurons. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone; IB: 10uM Ibuprofen. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, ** compared to H, *** compared to HT. Scale bar = 50 micrometers (uM). n=3 per treatment group, at least three independent replications were performed.

TESTOSTERONE INCREASES ALPHA SYNUCLEIN EXPRESSION IN N27

DOPAMINE NEURONS

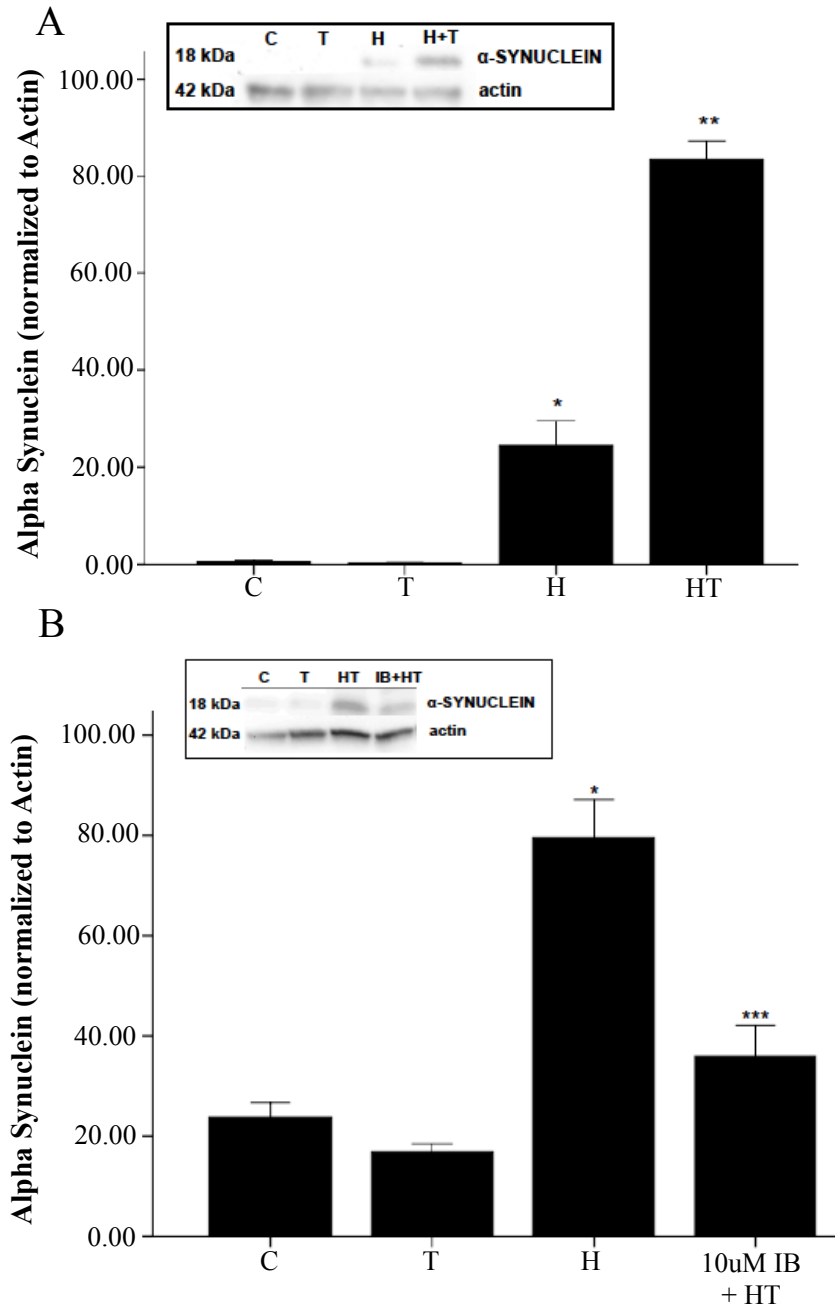


Figure 24. **Testosterone increases alpha synuclein expression in N27 dopamine neurons.** A) Testosterone significantly increased the protein expression of alpha synuclein in cells pretreated with tert-butyl hydrogen peroxide. B) IB significantly

decreased COX2 protein expression in cells post-treated with testosterone. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone; IB: 10uM Ibuprofen. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, ** compared to H, *** compared to HT. n=3 per treatment group, at least three independent replications were performed.

IBUPROFEN DECREASES OXIDATIVE STRESS INDUCED BY
TESTOSTERONE

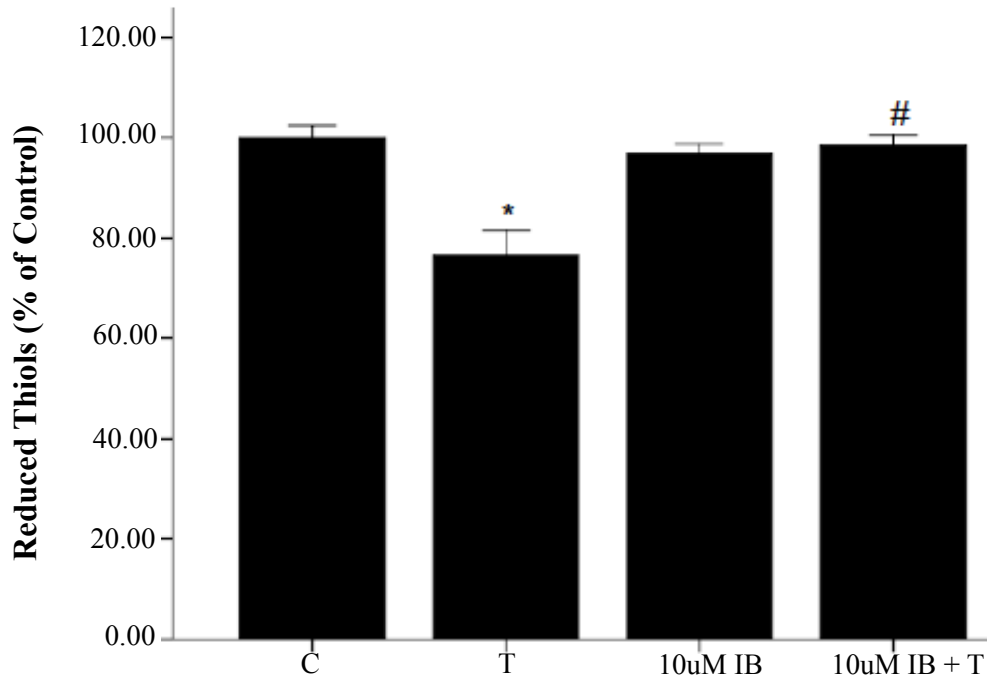


Figure 25. **Ibuprofen decreases oxidative stress induced by Testosterone.** Ibuprofen significantly increased reduced thiols in the presence of testosterone. C: vehicle control; T: 100nM testosterone; IB: 10uM Ibuprofen. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, # compared to T. n=3 per treatment group, at least three independent replications were performed.

**OXIDATIVE STRESS AND TESTOSTERONE SIGNALING ON
NEUROINFLAMMATORY MARKERS IN DOPAMINE NEURONS.**

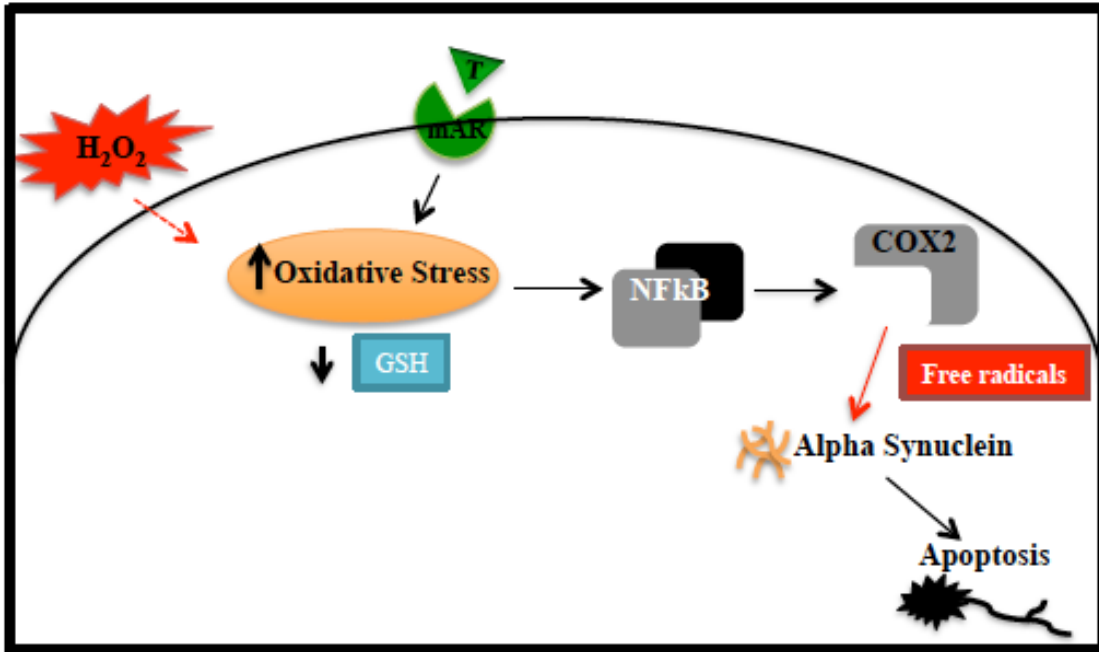


Figure 26. **Oxidative Stress and Testosterone signaling on neuroinflammatory markers in dopamine neurons.** In the absence of an oxidative stress environment, T through the mAR increases oxidative stress but has no effect on cell death. In contrast, T in an oxidative stress environment decreases GSH and increases oxidative stress. This increase in oxidative stress induces the oxidative responsive transcription factor NFkB to promote COX2 signaling. COX2 can signal as a peroxidase releasing free radicals into the cytoplasm contributing to oxidative stress and alpha synuclein formation, and thus apoptosis through oxidative DNA damage in dopamine neurons. GSH: glutathione; mAR: membrane associated androgen receptor; T: testosterone; H₂O₂: hydrogen peroxide.

SUPPLEMENTAL

This supplemental section is composed of unpublished data that was used as a foundation for signaling cascades and inflammatory inhibitor data, in addition to lab literature. For signaling cascades, preliminary data shows that NFkB p50 nuclear localization was increased in response to oxidative stress (Figure 27). Thus, this study examined the activity of NFkB in oxidative stress conditions. Also, dopamine neurodegeneration is positive for apoptotic cells, thus caspase 3 expression was examined in response to oxidative stress conditions. Using Western blot analysis, the expression of pro-caspase 3 was decreased in response to oxidative stress (Figure 28, $F_{3,7}=85.306$, $p<.001$). Lastly, this study examined the oxidative pre-treatment of ibuprofen, which found that ibuprofen blocks the effects of testosterone ($F_{6, 21}= 51.536$, $p <.001$, Figure 29).

All drugs were dissolved in the vehicle DMSO (.0005%), which has no effect on cell viability ($F_{7, 70}=144.245$, Figure 30). Unfortunately, the inflammatory inhibitor celecoxib showed very poor solubility under our conditions, and could not be used. Therefore, other inflammatory inhibitors were used to determine the correct concentrations for the N27 cell line. Studies were conducted using concentration response graphs. Cell viability was used to determine the survival rate of inhibitors, as these inflammatory inhibitors can be toxic. As a result, 10uM IB ($F_{10, 56}= 66.302$, Figure 31A and B) and 250nM CAPE ($F_{7,43}=522.577$, $p<.001$, Figure 32A and $F_{3,31}= 14.477$, $p<.001$, Figure 32B) were found to be non-toxic.

OXIDATIVE STRESS INDUCES NUCLEAR NFkB

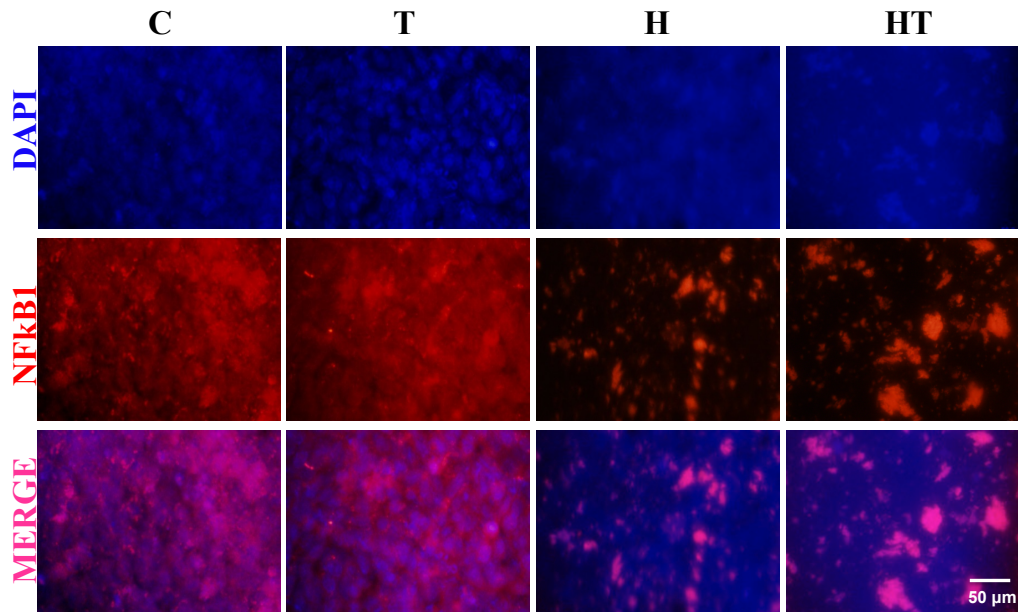


Figure 27. **Oxidative stress induces nuclear NFkB.** This preliminary data shows that oxidative stress induces the nuclear localization of NFkB in N27 dopaminergic neurons. Testosterone exacerbates the expression of nuclear NFkB in oxidative stressed dopamine neurons. Primary antibody is NFkB p105/50, and secondary antibody is Alexa Fluor 594 with an excitation/emission at 591/614, pictures obtained from a confocal microscope. DAPI: nuclear control; NFkB1: NFkB p105/50; C: control; T: testosterone; H: Hydrogen peroxide; HT: post-treatment testosterone. Scale bar = 50 micrometers (uM). n=1 per treatment group.

CASPASE 3 IN OXIDATIVE STRESSED DOPAMINERGIC NEURONS

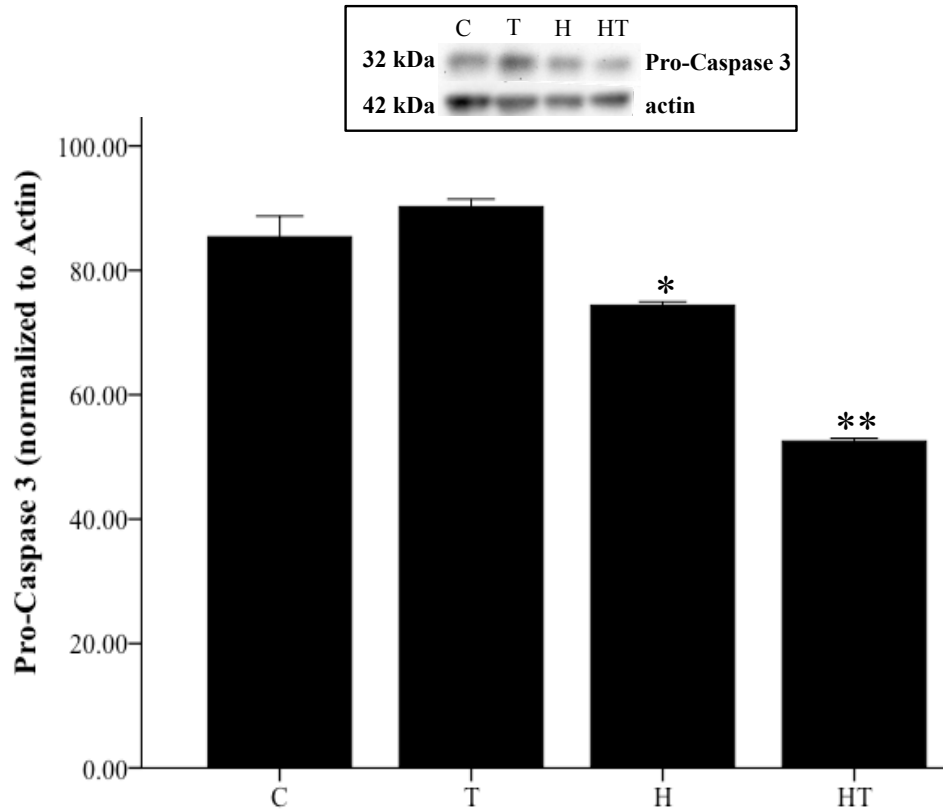


Figure 28. **Caspase 3 in oxidative stressed dopaminergic neurons.** This data shows that hydrogen peroxide decreases pro-caspase 3 expression in N27 dopaminergic neurons. Testosterone decreases the expression of caspase 3 in oxidative stressed dopamine neurons. C: control; T: testosterone; H: Hydrogen peroxide; HT: post-treatment testosterone. * compared to control, ** compared to hydrogen peroxide. n=3 per treatment group.

IBUPROFEN POST-TREATMENT INCREASES NEURONAL SURVIVAL

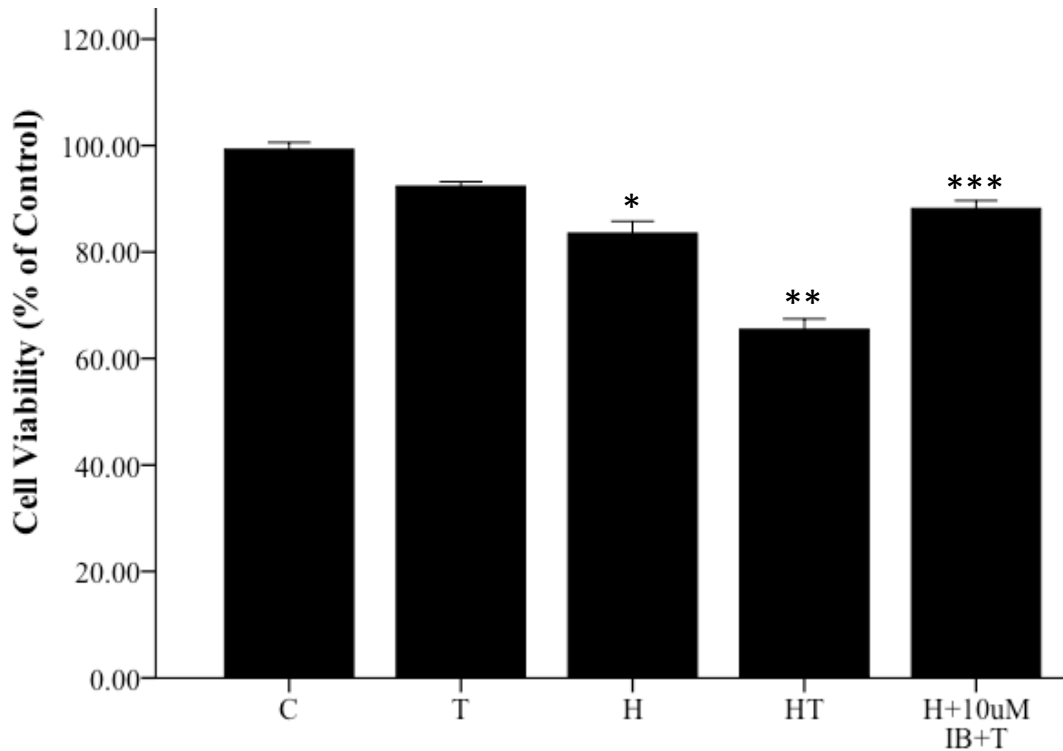


Figure 29. **Post-treatment ibuprofen increases neuronal survival.** Ibuprofen post treatment increases dopamine neuronal cell viability. C: vehicle control; T: 100nM testosterone; H: hydrogen peroxide; HT: post-treatment testosterone; IB: 10uM Ibuprofen. ANOVA followed by Tukey post hoc test. Significance symbols: * compared to C, ** compared to H, *** compared to HT. n=3 per treatment group, at least three independent replications were performed.

OXIDATIVE STRESS DECREASES NEURONAL CELL VIABILITY

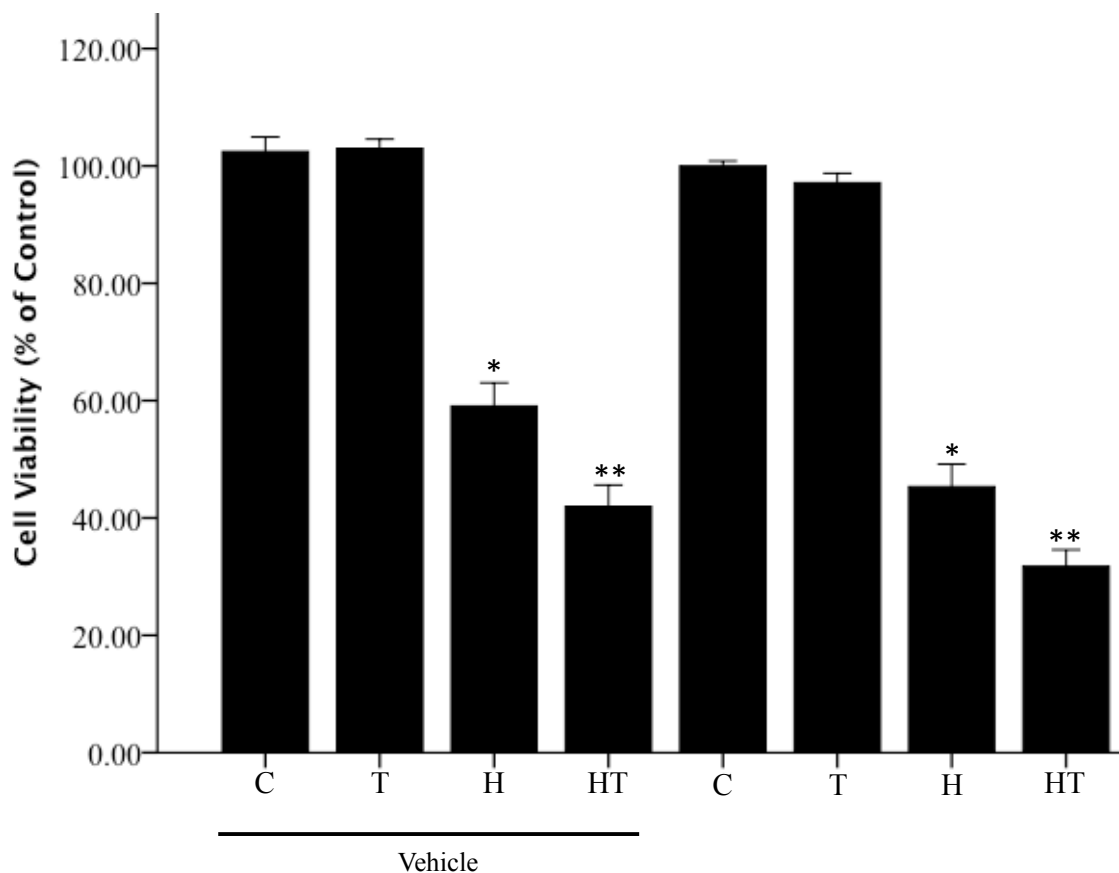


Figure 30. **Oxidative stress decreases neuronal cell viability.** This data shows that DMSO does not compromise dopamine cell viability in oxidative stress. Testosterone exacerbates the decrease in oxidative stressed dopamine neurons. Vehicle: DMSO, dimethyl sulfoxide (.0005%); C: control; T: testosterone; H: Hydrogen peroxide; HT: post-treatment testosterone. * compared to C, ** compared to H. n=3 per treatment group, at least three independent replications were performed.

IBUPROFEN CONCENTRATIONS ON N27 CELL VIABILITY

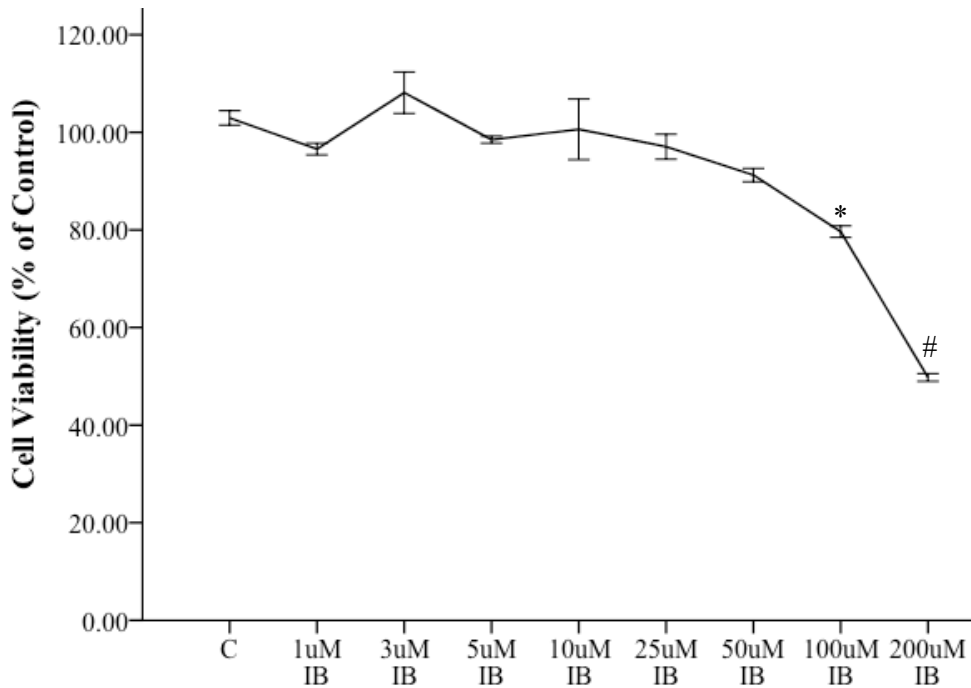


Figure 31. **Ibuprofen concentrations on N27 cell viability.** Ibuprofen, a non-selective inhibitor of COX1/COX2, inhibits COX2 activity. There are alterations in cell viability between 1uM-10uM of ibuprofen, but no significant effect, at 25 micromolars cell viability decreases with increasing concentrations of ibuprofen. C: vehicle control; IB: ibuprofen. * compared to control, # compared to 100uM IB. n=3 per treatment group, at least three independent replications were performed.

CAPE CONCENTRATIONS ON N27 CELL VIABILITY

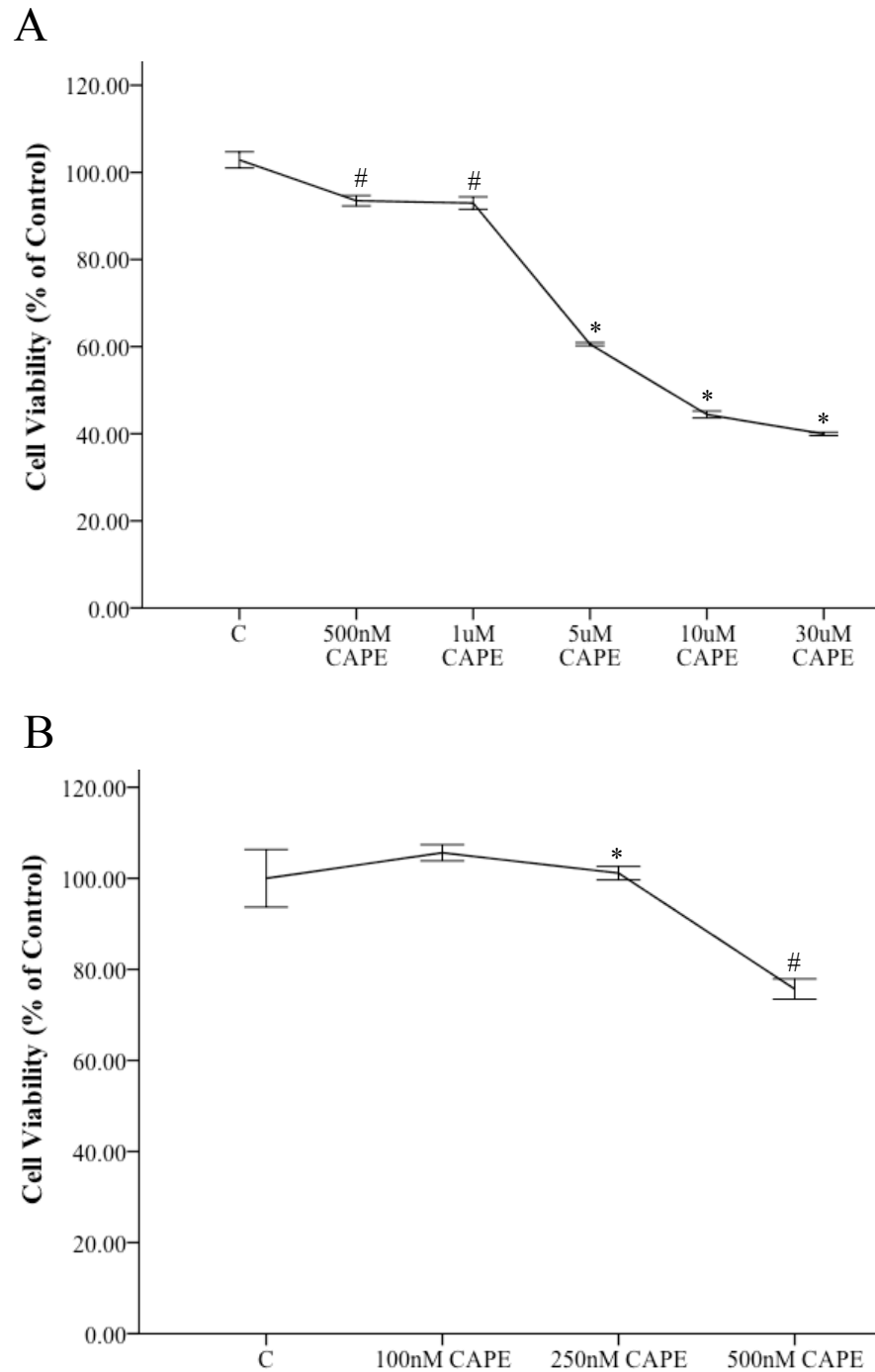


Figure 32. **CAPE concentrations on cell N27 cell viability.** CAPE is a potent inhibitor of NFkB activity. A) CAPE concentrations significantly decreased cell viability exceeding 500nM of CAPE. B) 250nM of CAPE had no significant effect on cell

viability. C: vehicle control; CAPE: caffeic acid phenyl ester. # compared to C, * compared to 500nM. n=3 per treatment group, at least three independent replications were performed.

CHAPTER IV

DISCUSSION

OVERALL FINDINGS

In these studies, testosterone had either protective or damaging effects on cell viability and inflammation. Testosterone, alone, can be neuroprotective. However, under oxidative stress conditions, testosterone can increase inflammation and induce oxidative stress resulting in increased cell death. Moreover, testosterone can elicit its effects on cell viability and inflammation through a membrane androgen receptor as indicated by 1) insensitivity to the androgen receptor antagonist, flutamide and 2) the ability of membrane impermeable testosterone conjugated to BSA to mimic the effects of testosterone on cell viability and inflammation.

MEMBRANE ANDROGEN RECEPTOR

Since the membrane androgen receptor has not been cloned, there is speculation that the membrane androgen receptor is an intracellular androgen receptor embedded in the membrane [332, 333]. If the membrane androgen receptor were a classical androgen receptor, then this receptor would be sensitive to the classical androgen receptor

antagonists. Other androgen receptor antagonists should be used in addition to flutamide to confirm that the membrane androgen receptor is insensitive to classical androgen receptor antagonists. Gatson et al., 2007 show that one of the properties of the membrane androgen receptor is its insensitivity to flutamide, which is consistent with our results that showed the classical androgen receptor antagonist, flutamide, did not affect the signaling of testosterone [164]. Therefore, these results indicate the membrane androgen receptor could be a novel membrane androgen receptor. Consistent with a membrane mediated mechanism, our results show that testosterone can increase calcium levels. Thus, it is possible that the membrane androgen receptor may be a G-protein coupled receptor coupled to Gq that stimulates endoplasmic reticulum calcium release. Thus, the membrane androgen receptor may be a valuable therapeutic target.

TESTOSTERONE CONTROVERSY: PROTECTION OR DAMAGE

A. PROTECTION

The effects of testosterone on neurons have been controversial. Some studies have found testosterone to be protective, while others find testosterone to be damaging. Fargo et al., 2007 and 2009 showed that testosterone can attenuate motor neuron atrophy, and restores dendritic morphology and motor neurons [334, 335]. In addition, Chi-Fai Lau et al., 2014 showed that testosterone can protect hippocampal neurons against amyloid beta damage [336]. Moreover, Pike et al., 2014 showed that testosterone could decrease inflammation in the cortex of mice [337].

B. DAMAGING

Other studies have shown damaging effects. Yang et al., 2002 shows that testosterone increases neuronal cell death through glutamate toxicity [303]. In comparison, Estrada et al., 2006 shows that testosterone increases calcium concentrations and apoptosis in human neuroblastoma cells [167, 338]. Further, Cunningham et al., 2009 shows that testosterone induces apoptosis through a caspase-3 dependent mechanism [48]. Moreover, the damaging effects of testosterone are not limited to neurons. Gatson et al., 2006 shows that testosterone's active metabolite, dihydrotestosterone conjugated to BSA decreases the phosphorylation of the protective AKT/ERK pathway in glioma cells [216]. In addition, Gatson et al., 2007 shows that a reduction of AKT phosphorylation by dihydrotestosterone conjugated BSA in oxidative stress increases cell death in primary cortical astrocytes [164]. These studies show that testosterone has different mechanisms of action that may be dependent on the environment. In addition, this dissertation addresses the controversy behind testosterone because there is a male sex bias observed in Parkinson's disease neurodegeneration.

PARKINSON'S DISEASE (PD)

PD is predominantly a late-onset sporadic disease affecting millions of people 60 and older in the United States [339, 340]. In fact, the risk of PD increases with aging in both men and women [22]. Men have a two-fold increased prevalence of PD over women, identifying a gender bias that suggests testosterone may be involved in PD [22]. PD is also characterized by oxidative stress and inflammation that can induce neuronal cell death. PD is associated with low testosterone in aging men [341]. Thus, testosterone

has been prescribed as a therapy for non-motor symptoms in PD [342]. However, studies of testosterone are controversial because testosterone has been shown to play both a protective and damaging role in neurons. The question remains, what facilitates these different effects of testosterone? Could oxidative stress determine the function of testosterone as a neuroprotectant or neurotoxin?

N27 CELLULAR MODEL FOR PD

N27 cells are derived from the rat mesencephalon [48, 152, 213]. This cell line has been used as a dopamine neuronal model because it is positive for tyrosine hydroxylase, dopamine and dopamine transporter [48, 152, 212, 343], Table 3. As a neuronal model, this cell line expresses low levels of synaptophysin, a major synaptic vesicle protein expressed in immature neurons, and high levels of the neuron specific enolase marker [213, 344]. In addition, N27 cells have resembling properties and characteristics of midbrain dopaminergic neurons compared to any other cell line [152, 213, 215, 345-347]. Moreover, this cell line has been identified as a useful dopamine neuronal model to study chronic conditions of oxidative stress associated with PD neurodegeneration [211]. Approximately 109 papers reference the use of N27 cells according to the PubMed database on November 4, 2015, identifying that this cell line is widely used as a cellular model for PD [348]. The N27 cell line expresses the androgen receptor, and estrogen receptor alpha and beta [48, 152]. Thus, this cell line was used in these studies to identify whether the effects of oxidative stress on dopamine cells contributes to the different effects of testosterone, Figure 33.

RESEARCH FINDINGS OF TESTOSTERONE'S EFFECTS

A. PROTECTION

Testosterone has been shown to play a protective role against subsequent later insults of oxidative stress. Studies have identified that testosterone's protective effects can be mediated through estrogen receptor alpha and beta dependent mechanism [349, 350]. In contrast, our studies show that testosterone, through an androgen receptor, can induce non-toxic levels of calcium in the cellular environment resulting in neuroprotection via a preconditioning mechanism. This increase in calcium is buffered by the mitochondria increasing its energy production and increasing reactive oxygen species in the cellular environment. As a result, the environment has the antioxidant capacity to tolerate later insults. For example, testosterone pretreatment to oxidative stress (hydrogen peroxide) protects cells from the detrimental effects of subsequent oxidative stress. Thus, indicating that testosterone can be protective for men that have low testosterone levels.

B. DAMAGING

Testosterone has been described to play a damaging role under oxidative stress conditions. For example, under oxidative stress conditions, testosterone can further increase oxidative stress induced apoptosis, as testosterone is an oxidative stressor. For men with high oxidative stress levels, testosterone therapy would not be beneficial because testosterone can exacerbate the oxidative stress load and thus increase oxidative stress associated disease progression.

Potential drug targets could be developed to antagonize the membrane androgen receptor to block the negative effects of testosterone. Another potential therapy is the use of antioxidants in conjunction with testosterone replacement therapy. Antioxidants such as vitamin C and E are associated with decreased PD progression, and creatine is in phase 3 clinical trials for PD patients [44, 339, 351-353].

High oxidative stress levels can trigger multiple pathways of cell death, such as inflammation. Pro-inflammatory markers such as NFkB and COX2 can initiate the inflammatory pathway. This study identified that the oxidative stressor, hydrogen peroxide, induced cellular signaling of NFkB, COX2 and apoptotic cell death in N27 dopaminergic cells. Testosterone post-treatment under oxidative stress conditions further increased signaling of NFkB, COX2 and apoptotic cell death in N27 dopaminergic cells.

Current treatments for PD use dopamine replacement therapy to alleviate motor dysfunctions in PD. However, these therapies do not address the cellular insults responsible for dopamine loss. Dopamine neurons continue to degenerate in the midbrain without amelioration. Therefore, signaling cascades responsible for dopamine neurodegeneration could be a target for therapeutic interventions in decreasing dopamine loss and motor deficiencies in PD.

Research studies have used the selective COX2 inhibitor, celecoxib, to examine its effects in PD models. Celecoxib is a safe COX2 inhibitor because it has no observed cardiovascular risks [354, 355]. Celecoxib used in PD models increases neuronal survival and protects animals from neurobehavioral deficits [356, 357]. Unfortunately, the use of celecoxib is difficult for *in vitro* experiments. Celecoxib precipitates out of solution, which makes it unreliable for *in vitro* experimental studies.

A clinical drug of use is ibuprofen that nonselectively inhibits COX2. Clinical studies of ibuprofen were associated with a decreased risk of PD [358-360]. In addition, ibuprofen was inversely associated with PD [347, 358, 359]. These clinical studies show that ibuprofen can reduce the risk of PD. However, clinical studies have yet to identify whether PD patients benefit from the use of ibuprofen after disease onset. In cellular models, ibuprofen was associated with dopamine cell survival [129]. Ibuprofen was used in this study to examine its protective effects in oxidative stress and testosterone induced dopamine neurodegeneration. Consequently, this study shows that ibuprofen protects dopamine neurons from the effects of testosterone in an oxidative stress environment. Thus, we identify that COX2 is a potential drug target for lowering the risk and decreasing the progression of PD in men.

Another inflammatory target upstream from COX2 is NFκB. CAPE is a potent inhibitor of NFκB and has been used in PD models. CAPE was shown to increase dopamine survival and alleviate behavioral deficits associated with dopamine neurodegeneration [122]. Consequently, the results in this dissertation showed that CAPE increased dopamine cell viability by blocking testosterone's negative effects in an oxidative stress environment. Thus, we identify that testosterone in oxidative stress environment activates COX2 via NFκB to result in dopamine neurodegeneration. However, NFκB is a poor drug target as it can modulate other downstream signaling cascades that could provide cellular protection.

In these studies, the use of inflammatory inhibitors did not completely attenuate the effects of oxidative stress signaling, although it did block testosterone's effects in inflammation and oxidative stress. Thus, signifying that different signaling cascades

contribute to dopamine neurotoxicity. This study identified that testosterone can be protective, but in an oxidative stress environment it can initiate an inflammatory response. Moreover, it showed that testosterone's mechanism of action on inflammation is different than hydrogen peroxide's mechanism of action.

TESTOSTERONE LEVELS IN MEN

The findings in these studies indicate that testosterone is an important factor for neuronal health. Therefore, knowledge of physiological testosterone levels in aging men is important, as testosterone may be a risk factor for PD. In these studies, testosterone was used in concentrations within 1-100 nM, which represents the equivalence of physiological testosterone in men. For example, testosterone levels in young men range from 350 ng/dL (12 nmol/L) to 750 ng/dL (26 nmol/L), which can increase to approximately 41.64 nmol/L in response to erotic stimulation and exercise [168, 263-265, 361-363]. Studies show that in healthy aging men, testosterone levels do not differ significantly from that of younger males [185].

The Endocrine Society defines low testosterone as less than 200- 300 ng/dL in young men, which is approximately 1 nM of testosterone in our studies [364]. Approximately 30% of the aging male population is affected by low testosterone, a condition characterized by nonspecific symptoms and generally observed in unhealthy men [364]. Even though testosterone levels can decline, it is important to note that testosterone levels are still higher in males than females.

The fact that testosterone levels are still within the normal range in healthy men signifies that testosterone is vital to the aging male physiology in cases of libido, health

and overall wellbeing. Thus, testosterone hormone therapy is shown to be a therapeutic for young males to improve sexual function and stamina, and prevent metabolic syndrome and chronic illnesses [188, 197, 342, 365, 366]. However, there is not much information on testosterone levels and testosterone therapy in aging men. Due to this lack of information, the NIH Institute of Medicine recommended more research on testosterone therapy in older men [367].

TESTOSTERONE SUPPLEMENTS

The objective of testosterone replacement therapy is to safely restore testosterone to normal physiological levels and attenuate the symptoms of low testosterone [364]. Young men suffering from untreated low testosterone are described to be at risk for mortality, coronary artery disease and stroke [365, 368, 369]. In younger men, testosterone replacement therapy improves sexual function, muscle mass, strength and energy [366, 370-375]. Moreover, testosterone therapy is not an indication for borderline levels of testosterone in men [376]. Thus, the Endocrine Society has provided guidelines for healthcare providers to avoid testosterone replacement for men requiring fertility, history of untreated sleep apnea, prostate cancer, breast cancer, and elevated prostate specific antigen [188].

In addition, testosterone replacement therapy is prescribed for males with low testosterone suffering from erectile dysfunction to support a healthy lifestyle [369, 377]. Interestingly, low testosterone in aging males with is associated with erectile dysfunction, metabolic syndrome and chronic illnesses [191]. It remains unclear whether low testosterone is a cause or consequence of illness predisposing males to these conditions.

In fact, there are studies that identified low testosterone as a consequence of illness. Remarkably, inflammatory factors can inhibit testosterone production supporting the hypothesis that low testosterone could be a consequence of oxidative stress induced signaling [378].

The commercialized health risks associated with untreated low testosterone in men drives the increase in testosterone replacement therapy in the United States [369]. In fact, the sales of testosterone supplements are on the rise, reaching 1.6 billion in 2011 [190, 369, 379]. Clinical studies show that health risks of supplements include cardiovascular disease, sleep disturbances and mortality [192, 369, 378]. Testosterone remains controversial in the aging population because there is no large-scale clinical trial that addresses health risks of low testosterone and long-term use of testosterone replacement therapy in aging men [190, 369, 379]. Despite the limitation of testosterone studies, the American Academy of Anti-Aging Medicine Society advocates for the use of hormone supplements to improve skin health, lean body mass and libido, and improve aging [191, 380, 381]. In addition to clinicians prescribing testosterone, there is a wide use of off-label hormones to prevent and reverse the effects of aging [191]. This controversy surrounding low testosterone and hormones should bring awareness to any use of steroids. Guidelines for the sale and use of hormones should be strictly regulated as far as pre-existing conditions in the aging population and adverse effects of cardiovascular issues, prostate cancer, sleep disturbances and mortality [369].

CONTRAINDICATIONS OF TESTOSTERONE REPLACEMENT THERAPY

A. CARDIOVASCULAR

Aging males can have pre-existing conditions such as metabolic syndrome and cardiovascular disease (CVD) that fits into this umbrella of chronic illnesses [382]. Illnesses such as metabolic syndrome encompass a cluster of factors including high blood pressure and insulin resistance [382]. Even though these metabolic factors affect the periphery, they increase the risk of CVD and neurodegeneration due to blood circulation feeding into the central nervous system. CVD is the most common cause of mortality that affects more than 1 in 3 men [383]. CVD characterized by oxidative stress includes conditions of congestive heart failure and stroke [384]. CVD produces reactive oxygen species creating high levels of oxidative stress that can induce inflammation [384]. Thus, endogenous testosterone and testosterone treatment could contribute to this oxidative stress signaling of reactive oxygen species. Low endogenous testosterone could potentiate CVD due to the loss of testosterone's preconditioning effect. Further, hormone therapy has been proposed as a treatment for CVD in those with low testosterone, but it could exacerbate oxidative stress induced signaling. Moreover, clinical studies identify testosterone replacement therapy as a contraindication in CVD [385]. Vasoconstriction and inflammation are potential mechanisms by which testosterone can mediate the effects of oxidative stress in the periphery [382]. In other words, testosterone effects on oxidative stress are not limited to the central nervous system.

B. PROSTATE CANCER

Prostate Cancer is the second most common cause of mortality in men [386]. Oxidative stress and testosterone play an important role in prostate carcinogenesis and progression [382, 386]. Prostate cancer can induce a hypercoaguable state of abnormal blood clots [382]. As a result, this condition and oxidative stress can be exacerbated by the treatment of testosterone recommended to treat men with low testosterone. Thus, testosterone replacement therapy should be cautioned in men with prostate cancer or a family history of prostate cancer.

C. SLEEP APNEA

Sleep apnea (SA) is characterized by irregular episodes of complete breathing cessation for ten seconds or more [387, 388]. Approximately 4% of the aging male population is diagnosed with SA [387, 388]. In addition, SA is associated with low testosterone [389]. SA is common in patients with dementia because memories are consolidated during sleep [390]. Thus, SA can lead to the decline of memory associated with dementia. SA patients have high levels of lipid peroxidation a result of excessive reactive oxygen species and insufficient antioxidants [391]. Most importantly, low testosterone and testosterone therapy can exacerbate SA [391, 392]. Therefore, testosterone therapy is a contraindication in SA.

STUDY CONTRIBUTION TO CONTRAINDICATIONS

Men with low testosterone have a higher incidence of stroke, a condition characterized by oxidative stress [393]. In fact, testosterone replacement therapy

increases the incidence of stroke and neurodegeneration in men [193, 394, 395]. The mechanism of stroke induces death of brain cells giving rise to neurodegeneration such as PD, identifying that stroke may be a risk factor for PD [393]. PD is the most common motor dysfunction prevalent in men. Our studies show that under oxidative stress conditions, testosterone can induce a concentration dependent damaging effect on neuronal cell viability. Our work is consistent with other studies showing a dose-dependent effect of testosterone replacement therapy on increasing the number of adverse events in older men [192]. In summary, these findings suggest that for men over 50 years of age, testosterone replacement therapy may influence neurodegeneration.

TESTOSTERONE IN WOMEN

There is an increased incidence of PD in postmenopausal women around the age of 50 [396]. Interestingly, testosterone therapy has been prescribed in postmenopausal women for issues with libido, motivation and overall wellbeing [171, 172]. However, the effects of testosterone replacement therapy on brain function in postmenopausal women are unknown. Based on clinical studies, testosterone has been associated with breast cancer, cardiovascular issues and sleep apnea [397]. Therefore, testosterone therapy may not be appropriate for postmenopausal women, especially because there is a lack of long-term studies of testosterone in women [398]. Moreover, the studies in this dissertation were conducted in a female dopaminergic N27 cell line, suggesting that our data on testosterone and oxidative stress could be applicable to women. With the increase in testosterone replacement therapy by women, it is possible that the incidence of PD in women may increase and bridge the gender gap in neurodegeneration.

SUMMARY

Based on published literature and these studies, testosterone can have state-dependent protective and damaging effects. Testosterone's damaging effects may underlie the increased incidence of PD in men versus women. In comparison, Figure 34 shows that under normal conditions, physiological testosterone is associated with low oxidative stress, low inflammation and increased antioxidants in healthy neurons. With chronic illness, testosterone levels can decline, resulting in higher oxidative stress and inflammation due to the loss of antioxidant capacity. Moreover, these studies indicate that besides antioxidants and inflammatory inhibitors, the membrane androgen receptor could be a novel therapeutic target for decreasing the progression of PD in men. Also, this putative membrane androgen receptor could be relevant to postmenopausal women that use testosterone replacement therapy to prevent increased PD risk.

The increase in testosterone replacement may further contribute to the increased risk of neurodegeneration in aging men. A major concern for testosterone replacement therapy is a potential surge in the incidence of PD relative to the increased prescriptions in the baby boomers aging population. Currently, baby boomers are turning 65 years of age at a rate of approximately 10,000 a day, and by 2060 we will have twice as many people 65 and older in 2015 [399]. Thus, to avoid the possible increase in neurodegeneration such as PD, the current contraindications for testosterone therapy should include examining the levels of oxidative stress.

FUTURE DIRECTIONS

Based on this study, testosterone can increase the release of intracellular calcium, oxidative stress, inflammation and apoptosis in dopaminergic neurons. Since, preliminary data in the Cunningham lab indicates that testosterone signals via a non-genomic pathway associated with G-proteins, future studies will identify the G-proteins associated with the membrane androgen receptor in dopaminergic neurons. Consequently, identifying the G-proteins associated with the membrane androgen receptor could provide a clear understanding of testosterone's signaling in neurotoxicity and neuroprotection. These experiments will be tested in an *in vivo* rat model to determine if similar mechanisms of actions occur in a complete system of biological structures, such as microglia and astrocytes. In addition, testosterone should not be used in environments of high oxidative stress.

In clinical populations oxidative stress can be quantified by measuring homocysteine. In our recent publication, we showed that oxidative stress, as indicated by homocysteine, increased with age and neurodegeneration in men over 50 years of age [370]. Further, testosterone increased cognitive impairment in men with high homocysteine levels ($>12 \mu\text{mol/L}$), consistent with our studies showing that testosterone in a high oxidative stress environment is damaging [370]. Our *in vitro* and clinical findings are applicable to the testosterone replacement therapy debate, and suggest that homocysteine levels should be assayed prior to testosterone therapy to ensure that only men with low oxidative stress receive testosterone. By defining the subset of men with increased oxidative stress, the adverse effects of testosterone therapy may be diminished.

PRIMARY VS. IMMORTALIZED MIDBRAIN CELLS

Primary Midbrain Cells	
TH ⁺	+
DA	+
DAT	+
DAR	+
GABA	+
Neuron	+
N27 Midbrain Cells	
TH ⁺	+
DA	+
DAT	+
Neuron	+

Table 3. **Primary vs. Immortalized Midbrain cells.** These cell lines are both female in origin, and dopamine characteristics and neuronal properties [48, 152, 214, 215, 345]. Adapted from Ding et al., 2004; Su et al., 2012; Cunningham et al., 2009; Rao et al., 1991; Meador-Woodruff et al., 1991. TH: Tyrosine Hydroxylase, DA: Dopamine, DAT: Dopamine Transporter, DAR: Dopamine Receptor, GABA: Gamma-Aminobutyric acid.

EXPERIMENTAL DESIGN

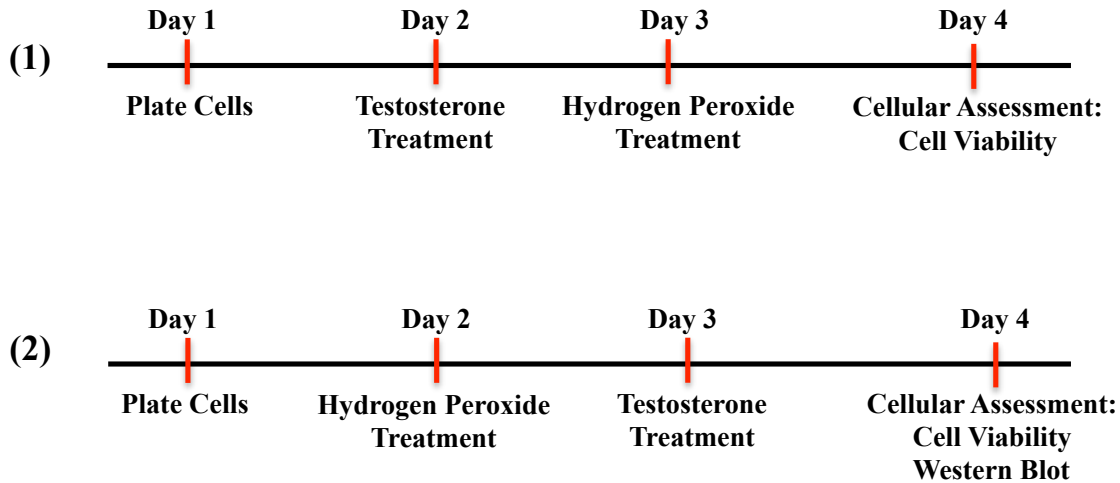


Figure 33. **Experimental Design.** Cells were plated for 24 hours before experiments. (1) Day 2 media was replaced with charcoal stripped media to avoid confounding factors before testosterone treatment. On Day 3 hydrogen peroxide was added to the experiment for 24 hours before study collection and assessment. (2) Cellular media was replaced with charcoal stripped media to avoid confounding factors before hydrogen peroxide treatment. On day 3, cells were treated with testosterone for 24 hrs. Day 4 experiments were collected and analyzed using cellular assessments.

TESTOSTERONE AND OXIDATIVE STRESS

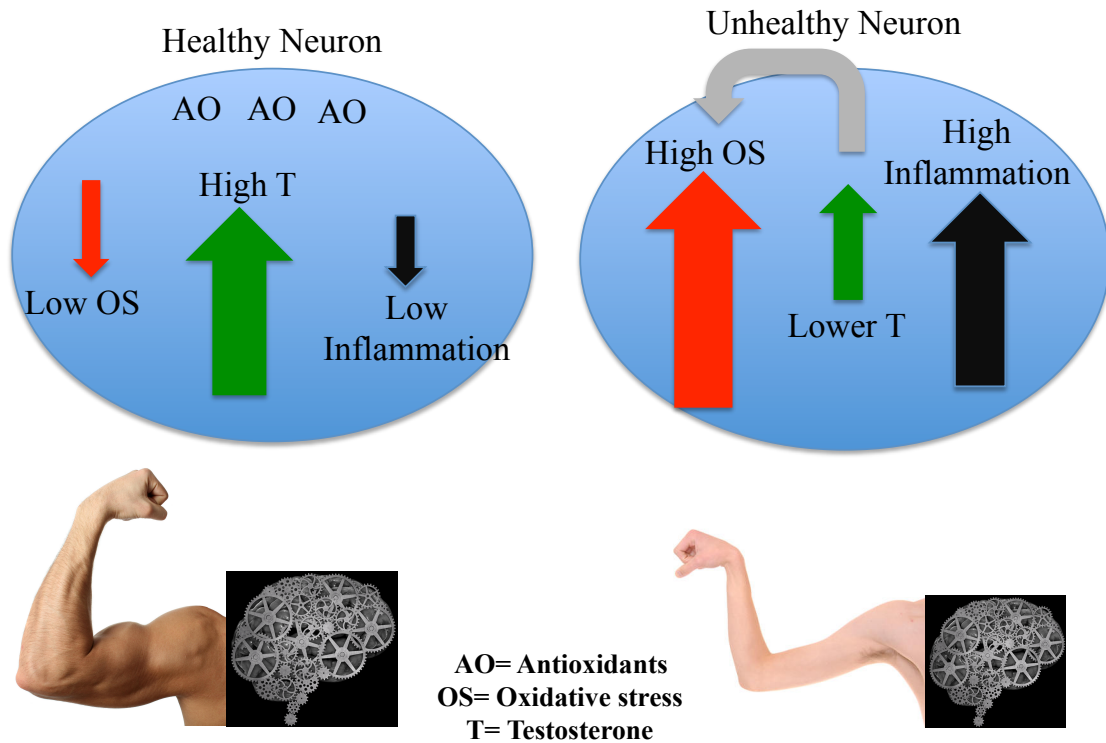


Figure 34. **Testosterone and Oxidative Stress.** Under normal conditions, physiological testosterone is associated with low oxidative stress, low inflammation and increased antioxidants in healthy neurons. With chronic illness, testosterone levels can decline, resulting in high oxidative stress and inflammation due to the loss of antioxidant capacity.

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