MEMBRANE ANDROGEN RECEPTOR-INDUCED OXIDATIVE STRESS: MECHANISM INVOLVED IN NEURODEGENERATION

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ABSTRACT

Oxidative stress-associated neurodegenerative diseases, such as Parkinson's disease (PD), affect millions of people worldwide. Although aging is the greatest risk factor for PD, other significant factors may be implicated, such as sex hormones that can mediate sex differences. Men have a higher incidence and prevalence of PD than women. Therefore, testosterone, a primary male sex hormone and a known oxidative stressor, is implicated in PD pathophysiology. Since androgens can have negative effects on dopaminergic cells, it is imperative to understand the underlying mechanisms in order to determine what mediates the observed sex differences in PD prevalence.

NADPH Oxidase 1 and 2 are major oxidative stress generators in the brain, thus potential targets for testosterone-induced oxidative stress and cell death. This dissertation project therefore investigates the role of androgens and membrane androgen receptor activation on NOX1/2. We hypothesize that in dopaminergic cells, testosterone activates the membrane androgen receptor (AR45) that is complexed with NOX1/2 to increase oxidative stress. In an oxidative stress environment, androgen activation of this AR45-NOX complex leads to cell death. Results indicate that classical androgen receptor (AR) antagonists do not block testosterone's negative actions in an oxidative stress environment. The effects of AR45-NOX complex on cell viability can be blocked by either degrading AR45 protein or blocking NOX activation by apocynin. Further, these results show that testosterone's detrimental effect on cells is via a non-genomic mechanism, specifically via a novel membrane androgen receptor, AR45.

The findings of this study help identify key players in testosterone-induced neurodegeneration, which could serve as potential therapeutic targets for PD. Ultimately, this project provides novel mechanisms to explain thought provoking questions on male sex bias in PD.

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Specific Aims

Neurodegenerative diseases, of which Parkinson's and Alzheimer's disease are the most prevalent, affect millions of people worldwide. In fact, Parkinson's disease (PD) has been recorded as the second most common neurological disease. Generally, PD symptoms manifest when 80% of the cells within the substantia nigra are lost. Currently, the etiology of the disease remains elusive.

Although aging is the greatest risk factor for PD, other significant factors may be implicated, such as oxidative stress (OS). OS plays a key role in the pathogenesis of PD. Several studies have established that PD is sex biased, affecting more men than women. Testosterone, a primary male sex hormone and a known oxidative stressor, has been implicated in PD.

Evolution of science has brought about a drastic improvement in the motor symptoms of PD. Nonetheless, there are still growing controversies in the use of testosterone replacement therapies (TRT) in the management of non-motor symptoms of PD, such as decreased sexual libido and sexual dysfunction. It is unclear whether TRT in men with PD provides any substantial benefit. Previous studies in our lab indicate that testosterone via a non-genomic mechanism exacerbates OS damage in dopaminergic neurons. Specifically, testosterone acts through a membrane associated androgen receptor (mAR) variant – AR45, leading to the activation of OS that can result in cell death. However, the mechanism by which testosterone increases OS is unknown.

NADPH Oxidase (NOX) is a major OS generator in cells. In fact, both NOX 1 and NOX 2 (NOX 1/2) are associated with OS in dopaminergic cells, hence potential contributors to the pathogenesis of Parkinson's disease. It is possible that NOX 1/2 complexes with the membrane androgen receptor (AR45) to mediate testosterone-induced oxidative stress and cell loss.

Objective: Determine the underlying mechanisms by which testosterone increases OS in dopaminergic neurons.

Hypothesis: In dopaminergic cells, testosterone activates membrane androgen receptor (mAR), which is complexed with NOX1/2 to increase oxidative stress. In an OS environment, androgen activation of this AR45-NOX complex leads to cell death.

Long term goal: Identify pathways regulated by testosterone in dopaminergic neurons in order to provide effective pharmacological targets to enhance the treatment of PD.

Aim #1: Investigate androgen receptor (AR) involvement in testosterone- induced oxidative stress and cell loss in an oxidative stress environment

Aim #2: Determine the role of G protein subunit q and $InsP_3R$ in testosterone-induced neurodegeneration

Aim #3: Examine the role of NOX 1/2 in testosterone- induced oxidative stress and cell viability

Significance

More people are living longer, and an increased number of diseases are associated with aging. Aging is considered one of the biggest risk factors for PD, which has over 60,000 new cases diagnosed each year in the United States. One interesting fact about this disease is that there is neither a known cause nor cure for PD. Earlier studies revealed PD is sex biased, affecting more men than women. We established in our laboratory that testosterone can be neuroprotective or neurotoxic in dopaminergic cells, and these effects are dependent on the OS status of the cell. Since androgens, such as testosterone, can have negative effects on dopaminergic cells, it is imperative to understand the underlying mechanisms in order to determine what mediates the observed sex differences in PD prevalence. Once, we have identified the key players in this pathway, the exact therapeutic approach can be directed at the particular target, with limited unwanted effects (e.g. affecting reproductive physiology). Consequently, PD progression can be slowed down, thereby improving the quality of life of the patient.

Innovation

NOX is a major OS generator in most cells. With its recent identification in the brain, it has been linked to neurodegeneration, such as PD. Of note, in this research project we have established a relationship between androgens, the membrane androgen receptor, and NOX1/2 in OS generation and cell death. This project is innovative as it provides novel mechanisms to explain thought provoking questions on male sex bias in PD.

CHAPTER 1

SEX-RELATED DIFFERENCES IN OXIDATIVE STRESS AND NEURODEGENERATION

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Abstract

Oxidative stress has been implicated in a number of neurodegenerative diseases spanning various fields of research. Reactive oxygen species can be beneficial or harmful, depending on their concentration. High levels of reactive oxygen species can lead to oxidative stress, which is an imbalance between free radicals and antioxidants. Increased oxidative stress can result in cell loss. Interestingly, sex differences have been observed in oxidative stress generation, which may underlie sex differences observed in neurodegenerative disorders. An enhanced knowledge of the role of sex hormones on oxidative stress signaling and cell loss can yield valuable information, leading to sex-based mechanistic approaches to neurodegeneration.

Keywords: Alzheimer's disease, Parkinson's disease, testosterone, estrogen, menopause, aging

Oxidative Stress Generation

Oxygen is required by many living organisms for survival. Inefficient oxygen metabolism can be damaging and lead to oxidative stress [1]. Oxidative stress occurs when a biological system is overwhelmed by reactive oxygen species (ROS) due to its inability to counteract these free radicals [2]. Free radicals include peroxides, superoxides, hydroxyl radicals, and singlet oxygen [3, 4]. They are extremely unstable, reacting quickly with many biological products. Under normal homeostatic conditions, ROS plays a role in immunity, homeostasis, and signal transduction pathways [5, 6]. However, when antioxidants are inadequate to balance free radicals and ROS, oxidative stress occurs. These free radicals can trigger lipid peroxidation reactions [7], as well oxidative stress DNA mutations [8]. As the imbalance continues, oxidative stress can induce apoptosis, programmed cell death [9, 10].

Sex differences in oxidative stress have been observed in numerous basic and clinical studies, wherein males exhibit higher oxidative stress than females [11-17]. Interestingly, sex differences have been observed in the enzyme NADPH oxidase (NOX) [11, 13, 18-20], which is a major oxidative stress generator in cells [21]. NOX is a multi-subunit enzyme, consisting of membrane bound catalytic subunits and cytosolic regulatory subunits. The catalytic subunits are $gp91^{phox}$ and $p22^{phox}$, while the regulatory subunits are $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac. The regulatory complex translocates to the membrane upon $p47^{phox}$ phosphorylation, leading to subsequent activation of the enzyme [22]. The activated NOX complex catalyzes the transfer of electrons from NADPH to an oxygen molecule, resulting in the formation of reactive intermediates [23]. In different cell types, increased NOX has been observed in males compared to females. For example, using mesenteric arterioles microvessels, Dantas et.al found increased NOX subunits ($p47^{phox}$, $p22^{phox}$, $p67^{phox}$, and $gp91^{phox}$) in young adult male rats compared to females [11].

Similarly, young healthy male rats have increased oxidative stress and NOX expression in aortic and cerebral arterial cells compared to young healthy female rats [19, 20].

Oxidative stress can be influenced by homocysteine. Homocysteine, a non-protein α-amino acid, is an indicator of low folate and B-12 status [24], and has been used as a marker for oxidative stress [25, 26]. Studies have found homocysteine can increase NOX-mediated superoxide production, and this increase in superoxide production can be blocked by the NOX inhibitor, apocynin [18]. Similar to NOX, sex differences in homocysteine levels have been observed. Homocysteine levels are higher in men than women [14, 26-32]. Sex hormones can also influence homocysteine levels. In young healthy transsexuals homocysteine levels were influenced sex hormones. Specifically, male to female transsexuals receiving estrogen hormone therapy experienced decreased homocysteine, whereas female to male transsexuals receiving androgen hormone therapy had increased homocysteine levels [33]. These studies indicate sex hormones may underlie the observed sex differences in oxidative stress generation.

Although males generally have higher oxidative stress than females, oxidative stress is not always damaging. ROS can play a role in homeostasis, such as preconditioning. Preconditioning is a protective process, wherein exposure to a small insult allows the cells to better withstand a subsequent larger insult. This process has been observed in several types of cells (e.g. astrocytes, neurons, fibroblasts, muscle) [34-40]. In our basic science studies, physiological levels of testosterone can increase oxidative stress and be neuroprotective by preconditioning the cell against damage from subsequent exposures to oxidative stress [41, 42]. However, there appears to be a limit in testosterone's preconditioning capabilities. If oxidative stress is too high, then testosterone can be damaging.

Oxidative Stress and Aging

Oxidative stress has been linked with aging [43, 44]. Indeed, one of the major theories of aging is the Free Radical Theory of Aging. This theory proposes oxidative damage to cells, due to a buildup of free radicals in a biological system over a time period, results in aging and aging-associated diseases [45-47]. Sex differences in oxidative stress persist and worsen with aging in both animal and clinical samples, wherein males continue to have higher oxidative stress than females [48-50]. Aging, specifically menopause, plays a significant role in oxidative stress status in females. Menopause is a period characterized by a dramatic decline of estrogens, which normally occurs around 50-52 years of age in Caucasian women and 48-50 years of age in African-American and Hispanic women [51-57].

As expected, homocysteine increases with age [14, 26-32, 58], indicating elevated oxidative stress [25, 26]. Homocysteine levels further increase with menopause [59-63]. An elegant study by Hak et.al. used aged-matched post- and pre-menopausal women (46-55 years of age) and found homocysteine levels were significantly elevated after menopause [64]. Although other studies have not find this association [65, 66], it could be due a lack of adjusting for age. Similar findings have been observed in animal models, in which downstream targets of homocysteine, such as NOX [18], increased with aging, in general, and in female rats that have undergone ovariectomy, an experimental animal model for menopause [67, 68].

Our laboratory examined the role of aging and homocysteine levels using plasma samples from healthy Caucasian men (n = 700) and women (n = 1,061) over the age of 50 from the Texas Alzheimer's Research Care and Consortium (TARCC) funded by the state of Texas (Table 1). Our data showed homocysteine, used as a marker for oxidative stress, significantly increased with age

in both men and women. No differences in homocysteine levels were found between the men and women (Figure 1), which is consistent with prior studies indicating menopause increases homocysteine levels in healthy women to levels observed in healthy men [59-63]. Although hormone manipulations were not examined in the TARCC cohort, estrogen hormone replacement can decrease homocysteine levels in post-menopausal women [69-72]. Interestingly, estrogen hormone replacement therapy in post-menopausal women is associated with decreased testosterone, along with the expected increased estradiol [71, 73, 74]. Generally, menopause is associated with the loss of estrogen, but testosterone levels are maintained [75-78]. The effects of testosterone on menopause are understudied. Since estrogen hormone replacement therapy decreased testosterone levels in post-menopausal women and our prior studies showed that testosterone increased oxidative stress [41, 42], it is possible testosterone may mediate the elevation of homocysteine in post-menopausal women.

In 2001, approximately 40% of post-menopausal women were on some form of hormone replacement therapy [79]. Since there were not any conclusive trial data about the risks and benefits of hormone replacement therapy in post-menopausal women, the National Institutes Health (NIH) sponsored a large randomized, placebo controlled, double blind clinical trial called the Women's Health Initiative (WHI). Participants were post-menopausal women between the age of 50-79 years old at intake. This trial consisted of two arms: estrogen only replacement for post-menopausal women with a prior hysterectomy (n = 10,739) and estrogen + progesterone replacement for post-menopausal women with intact uteri (n = 16,608). Outcomes examined included stroke, venous thromboembolism, cancer, osteoporosis, and coronary heart disease [80]. Results from both arms found no protective effects of either estrogen only or estrogen + progesterone hormone replacement therapy, whereas adverse effects on cardiovascular disease

(e.g. pulmonary embolism, deep vein thrombosis, and ischemic stroke) were observed [81]. Due to these negative effects, the WHI study was terminated early [82]. Based on these results, use of hormone replacement therapy in post-menopausal women drastically declined [83].

Within the past few years, data from the WHI study have been reassessed. Researchers found 83% of the WHI participants were women several years from menopause. Furthermore, the average of age of participants was 63 years old, which is 12-13 years past menopause [84]. When the study outcomes were stratified by age of the participants, the results showed decreased cardiovascular risk and total mortality in younger women with hormone replacement therapy compared to the older women [85, 86]. No evidence of increased stroke was found in women 50-59 years of age in the estrogen only hormone replacement therapy group [87]. Thus, both a women's age and years from menopause are important factors for determining the impact of hormone replacement therapy. These factors are used to determine the "window of opportunity" for hormone replacement therapy in women, wherein the benefit/risk ratio is more protective in women less than 10 years from menopause [88].

Oxidative stress has been proposed as one mechanism underlying the "window of opportunity" for hormone replacement therapy. Several studies found estrogen can decrease oxidative stress [89-91]. However, estrogen's protective effects were found to be conditional [90, 92]. Based on these conditional effects of estrogen, Dr. Roberta Brinton coined the term "Healthy Cell Bias of Estrogen Action." Estrogen exposure to healthy cells results in estrogen being protective against subsequent insults, such as oxidative stress. However, this protective effect is not observed with estrogen exposure in unhealthy cells [90]. Indeed, we have found similar effects with testosterone in our *in vitro* studies [41], indicating the "Healthy Cell Bias" theory applies to all sex hormones in oxidative stress environments. Similarly, clinical studies examining the impact

of testosterone replacement therapy found conditional effects of testosterone, in which testosterone replacement therapy was associated with adverse effects in aged men (mean age is 74 years old) with chronic diseases [93, 94].

Oxidative Stress and Neurodegenerative Diseases

Oxidative stress has been implicated in several age-associated neurodegenerative diseases, including Alzheimer's disease [95], Parkinson's disease [96], and non-neurodegenerative diseases (e.g. cancers, sickle cell disease, cardiovascular diseases, and diabetes) [97-100]. Age is one of the greatest risk factors for both Alzheimer's and Parkinson's diseases. Furthermore, oxidative stress is a key feature in these progressive neurodegenerative disorders [96, 101]. Increased oxidative stress has been shown to be involved in cell loss in key brain regions (e.g. substantia nigra, cortex, and hippocampus) involved in the clinical manifestations of Alzheimer's and Parkinson's diseases [96, 102]. Interestingly, sex differences have been observed in both disorders.

Sex Differences in Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder, which affects millions of people universally. It has been recorded as the second most common neurological disease [103]. One characteristic feature of PD is neuronal death in the substantia nigra of the brain, specifically dopaminergic neurons. Mechanisms underlying this cell death include oxidative stress and inflammation [41, 104]. This results in the established symptoms of PD relating to the motor system, which include tremor, rigidity, bradykinesia and postural instability [105].

Generally, these symptoms manifest when 80% of the dopaminergic cells within the substantia nigra are lost [106]. The etiology of PD still remains elusive [107]. Aging is one of the principal risk factors for the development of idiopathic Parkinson's disease [108]. Along with aging, sex-related differences in PD have also been recognized [109]. Therefore, it is probable sex hormones play a vital role in this phenomenon, especially as men are 1.5-2 times more at risk of developing PD than women [110-112].

Several studies propose estrogen underlies this sex bias in PD. Women displayed a less severe PD phenotype than men at presentation. Indeed, studies found PD symptoms worsen for pre-menopausal women during menstruation, when estrogen levels are low [113]. Unsurprisingly, severity of PD increases in post-menopausal women compared to pre-menopausal women, due to the loss of estrogen during menopause. Clinical studies found estrogen hormone replacement can diminish the severity of early PD manifestations [114-116]. Therefore, estrogen was proposed to be neuroprotective for dopaminergic neurons in the substantia nigra, and can help mitigate PD progression [117-119].

Contrary to these reports, some studies were unable to find estrogen neuroprotection [120, 121], indicating another mechanism may be mediating this sex difference in PD. One possibility could be testosterone. Currently, the role of testosterone in neurodegeneration is understudied. Few studies have examined the impact of testosterone on PD, compared to studies on estrogen protection. Only one clinical study has been conducted on aged men with PD and treated with L-DOPA, and the results showed testosterone replacement therapy did not impact motor or non-motor PD features [122]. Further, this group observed no interactions between PD medications and testosterone levels [123]. Although this is an understudied area, basic science studies have yielded more information. Increased oxidative stress, via NOX, in the substantia nigral

dopaminergic neurons has been reported in male rats compared to female rats [124]. Studies from our lab found testosterone is an oxidative stressor in dopaminergic neurons, and its actions may be involved in this oxidative stress sex difference [41, 42]. In other studies using a 6-OHDA rat model, we observed testosterone can exacerbate oxidative stress damage, resulting in motor impairments [125]. It is possible testosterone may play a role in the increased PD incidence in post-menopausal women compared to pre-menopausal women [112], especially as post-menopausal women are more androgenic than estrogenic [126, 127]. Further research needs to be conducted on testosterone and PD.

Sex Differences in Alzheimer's disease

Oxidative stress plays a key role in the pathogenesis of Alzheimer's disease (AD) [128-130]. Indeed, increased NOX activity has been linked with AD progression and individuals converting from cognitively intact to dementia status [130-132]. Furthermore, associations between homocysteine and AD have been reported. Elevated homocysteine has been shown to contribute to dementia and AD progression [133-141]. Homocysteine can increase oxidative stress and cell loss in the hippocampus (one of the major brain regions affected in AD) [142]. AD risk is doubled in patients that have greater than 14 umol/L homocysteine, and thus homocysteine has been indicated as a potential AD risk factor [134, 140].

Sex differences have been reported in AD, wherein AD disproportionally affects women more than men in both prevalence and severity [121]. Based on this sex difference, several studies have examined the influence of estrogen on AD. In both *in vivo* and *in vitro* models estrogen

protected cells from AD-associated insults [143-149], such as β-amyloid and APP oxidative stress insults [150-152]. Furthermore, estrogen had a positive impact on cognition in surgically menopausal women [153, 154] and post-menopausal women [155-158]. Supporting the role of estrogen in neuroprotection, AD risk increases in post-menopausal women [152]. These studies indicate estrogen can act as a neuroprotectant in AD.

Interestingly, estrogen hormone replacement therapy on cognition in post-menopausal women is equivocal. Subsequent studies based on the WHI included the Women's Health Initiative Memory Study (WHIMS). The effects of hormone replacement therapy on cognition in post-menopausal women were assessed. Unlike the WHI study, the WHIMS study participants were at least 65 years old [92, 159]. Initial results indicated that hormone therapy had a negative impact on cognition. Specifically, estrogen + progesterone was linked with increased dementia and decreased verbal memory [92, 159-161]. Decline in verbal memory is one of the earliest predictors of AD [162, 163]. However, subsequent clinical studies that used peri-menopausal women, instead of post-menopausal women, found estrogen hormone therapy decreased dementia and AD risk [164, 165]. These studies indicate the beneficial effects of estrogen are conditional and may be biased toward protection of healthy cells [90].

Similar to estrogen, studies have indicated androgens can have protective and negative effects on AD. However, it appears testosterone effects are dependent on the cellular environment. One such variable that can result in androgens negatively impacting cells is oxidative stress [41, 166]. Using plasma samples from TARCC participants diagnosed with AD, we found oxidative stress increased with age in both men and women (Figure 2), consistent with other studies [167]. Interestingly, we observed men with AD have higher levels of homocysteine, used as a marker for

oxidative stress, than women with AD (Figure 2). In this cohort, hypertension and hyperlipidemia were more prevalent in men with AD than women with AD compared to cognitively intact men and women, respectively. Specifically, 61% (chi-squared $p < 0.05$) of men with AD had hyperlipidemia, unlike women with AD (50%; chi-squared $p = 0.967$). In addition, 58% (chi-squared $p < 0.05$) of men with AD had hypertension, whereas hypertension was present in 51% (chi-squared $p = 0.579$) of women with AD. Both hypertension and hyperlipidemia can increase oxidative stress [11, 168-171], which may increase the oxidative load enough to switch testosterone from a protective hormone to a damaging hormone. Indeed, our prior studies using the TARCC cohort showed endogenous testosterone levels were only associated with cognitive impairment under high oxidative stress (homocysteine levels >12 μmol/L) [172]. This effect of testosterone on cognition was lost when the cohort was not stratified based on oxidative stress, similar to findings in a recent study showing no effects of testosterone replacement therapy on age-associated memory impairment. This study by Resnick et.al. was a large, multi-site, clinical study, in which participants were men over 65 years of age (n = 788) and exposed to testosterone replacement therapy for one year. Regardless if the men were cognitively intact or impaired prior to hormone replacement therapy, no effects of testosterone were found [173]. No measures of oxidative stress were assayed in this study. Interestingly, majority of the participants in this study were on antihypertensives and phosphodiesterase inhibitors, which decrease oxidative stress [174-180]. Therefore, it is quite plausible testosterone replacement therapy affects cognition in men with elevated levels of oxidative stress.

Conclusion

Sex differences have been observed in oxidative stress and its related diseases. Estrogen and testosterone have been reported to contribute to sex differences in neurodegenerative diseases [181]. Elucidating sex hormone pathways in neurons may provide therapeutic targets to slow down the progression of neurodegenerative disorders by providing sex-based mechanistic approaches. Not only do studies indicate that sex is an important variable in oxidative stress and neurodegeneration, oxidative stress may be a key factor in determining how sex hormones impact neuronal function as either a neuroprotective or neurodamaging agent.

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Table 1

	Men			Women		
Variable	**N**	**Mean**	**St. Dev.**	**N**	**Mean**	**St. Dev.**
Age (years)	700	72.71	8.93	1061	72.21	9.74
	min. age		*max. age*	*min. age*		*max. age*
	51.00		94.00	50.00		102.00
	N	**%**		**N**	**%**	
hyperlipidemia	442	*63.14*		578	*54.48*	
hypertension	442	*63.14*		637	*60.04*	
obese	149	*21.29*		264	*24.88*	
Alzheimer's disease	405	*57.86*		516	*48.63*	
cognitively intact	295	*42.14*		545	*51.37*	
< high school diploma	82	*11.71*		168	*15.83*	
high school diploma	98	*14.00*		290	*27.33*	
≤ 4 yrs. college	315	*45.00*		432	*40.72*	
> college	205	*29.29*		171	*16.12*	

TABLE 1: Sample population characteristics. Plasma samples were obtained from Caucasian men and women enrolled in the Texas Alzheimer's Research Care and Consortium (TARCC). Cognitively intact controls performed within normal limits on all cognitive testing. AD patients met consensus-based diagnosis for probable AD based on NINCDS-ADRDA criteria [182]. Institutional Review Board approval was obtained at each TARCC site and written informed consent was obtained from participants and/or caregivers.

Figure Legends

Figure 1: Homocysteine levels (oxidative stress) increased with age in cognitively intact participants, regardless of sex. A significant regression equation was found ($F (2, 248) = 3.821$, $p < 0.05$), with an R^2 of 0.030. TARCC participants' homocysteine levels are equal to 7.455 + 0.086 (age) – 0.495 (sex), where age is measured in years and sex is coded as men and women. Cognitively intact participants' homocysteine levels increased 0.086 umol/L for every year and men had higher (0.495 umol/L) homocysteine (non-significant) than women. Only age was a significant predictor of homocysteine levels. Specific methods for sample collection are available in our prior publication [26]. Serum total homocysteine was assayed in the Atherosclerosis Clinical Research Laboratory at Baylor College of Medicine.

Figure 2: Homocysteine levels increased with age in both men and women with AD, with men have significantly higher homocysteine levels (oxidative stress) than women. A linear regression was calculated to homocysteine levels based on age and sex. A significant regression equation was found ($F (2, 95) = 11.220$, $p < 0.05$), with an R^2 of 0.191. TARCC participants' homocysteine levels are equal to -5.525 + 0.348 (age) – 3.788 (sex), where age is measured in years and sex is coded as men and women. AD participants' homocysteine levels increased 0.348 umol/L for every year and men had higher (3.788 umol/L) homocysteine than women. Both age and sex were significant predictors of homocysteine levels.

Figure 1

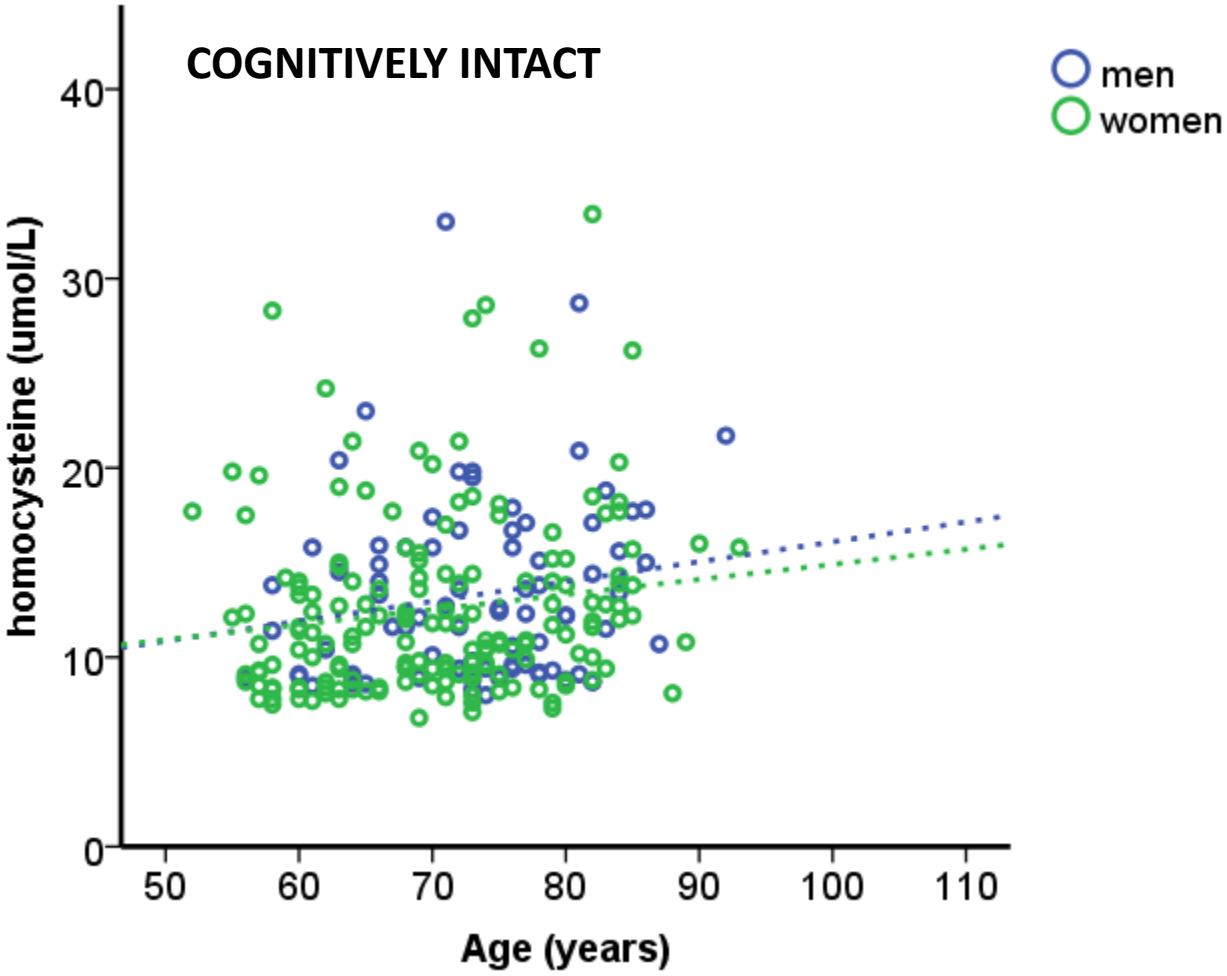
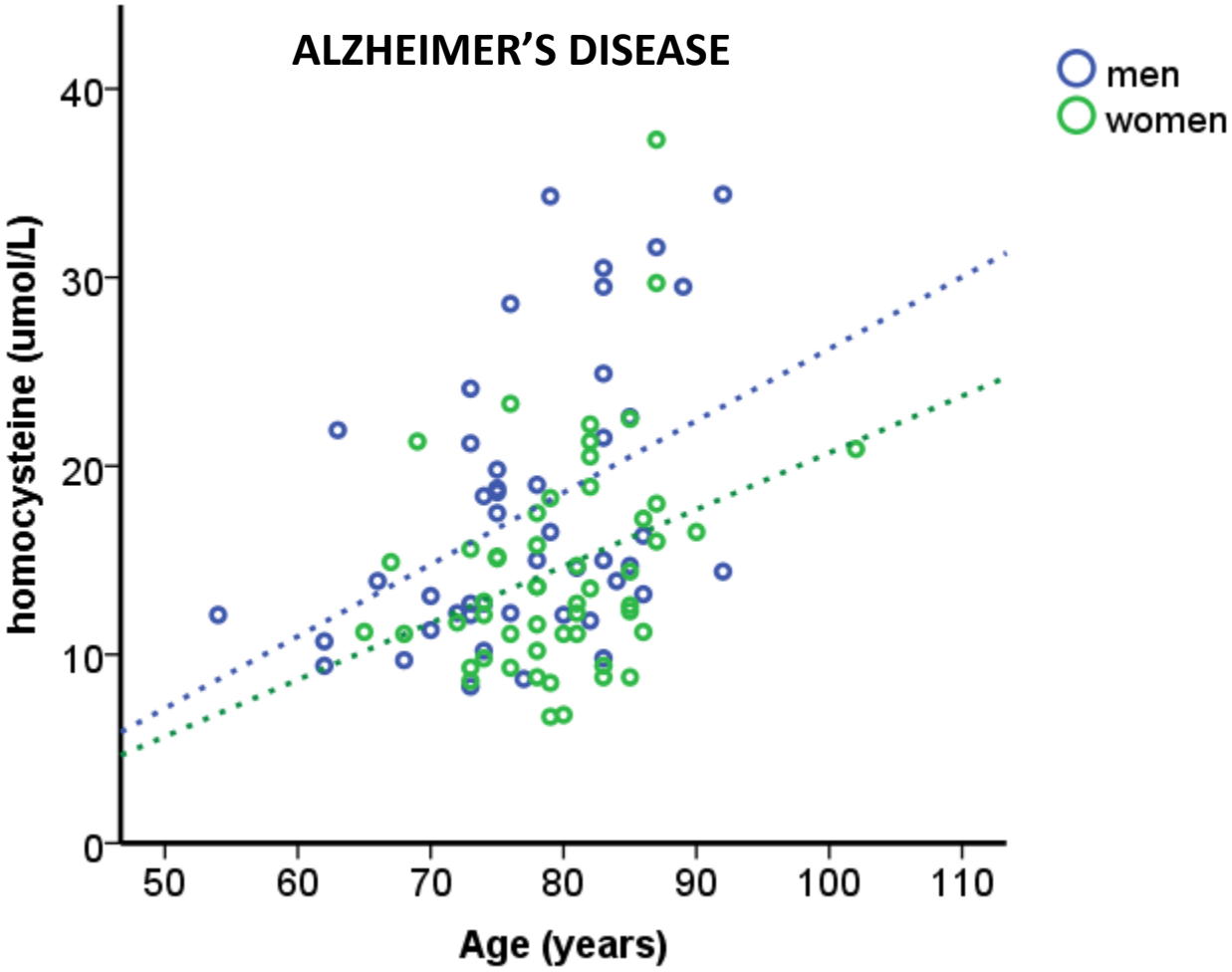

COGNITIVELY INTACT
men
women
homocysteine (umol/L)
0
10
20
30
40
50
60
70
80
90
100
110
Age (years)

Figure 2


ALZHEIMER'S DISEASE
men
women
homocysteine (umol/L)
0
10
20
30
40
50
60
70
80
90
100
110
Age (years)

CHAPTER 2

NEURODEGENERATIVE DISEASES

Neurodegenerative diseases continue to gain considerable attention. There have been remarkable discoveries within the field in recent times, which have further enhanced understanding of these diseases (1). Numerous neurodegenerative diseases have been mentioned, however Parkinson's disease (PD), Alzheimer's disease (AD), dementia, Amyotrophic Lateral Sclerosis, Huntington disease, and prion disease are the most studied (2-4). A common characteristic of these diseases is progressive neuronal death (5). Although, the etiology of neurodegeneration is unclear, aging and genetic predisposition remain important factors (6,7). Generally, the onset of neurodegenerative diseases occurs in the late stages of adulthood, raising health and economic concerns due to increased life expectancy (8-12). At present, PD treatment is symptomatic with the goal of improving motor symptoms but no cure is available. One possible way of identifying therapeutic strategies to treat neurodegenerative diseases is to first identify the mechanisms underlying the disease pathology. Not only do studies indicate that sex is an important variable in oxidative stress and neurodegeneration, oxidative stress may be a key factor in determining how sex hormones, such as androgens, impact neuronal function as either a neuroprotective or neurodamaging agent. Elucidating sex hormone pathways in neurons may provide therapeutic targets to slow down the progression of neurodegenerative disorders that show sex differences.

ANDROGENS

Androgens are produced in both males and females by the gonads (Leydig cells of the testes in males and ovaries in females) and the adrenal glands (13-16). As aging occurs, the Leydig cells of the testes decrease in function (17), and the amount of testosterone produced by the testes, specifically in aging men, decline (18). Low testosterone is associated with decreased sex drive, poor cognition, decline in learning and memory, bone mineral density, body fats and muscle strength (19,20).

Of all the major circulating androgens, testosterone and its more potent derivative dihydrotestosterone (DHT), bind to the androgen receptor (14,21-23). Testosterone serves as a prohormone for the synthesis of DHT, which is catalyzed by 5-alpha reductase (24-28). Testosterone can also be aromatized to the most potent estrogen; estradiol (29,30) by the aromatase enzyme, which stimulates estrogen receptors (31,32).

ANDROGEN RECEPTORS

Androgen receptors are highly expressed in many cells and tissues, hence mediate a wide range of biological effects (33-37). Androgens and its receptor complex are primarily responsible for reproduction and development of secondary sexual characteristics during puberty (23,38,39). However, multiple studies indicate that sex hormones, such as androgens, and their cognate receptors are involved in more than reproductive functions. Interestingly, the brain has been identified as a steroidogenic organ, with the ability to independently synthesize steroid hormones such as androgens and estrogens from cholesterol and other precursors (40). Androgens play

important roles in several brain regions, such as the entorhinal cortex, hippocampus, substantia nigra, amygdala and hypothalamus (41,42). In fact, androgens have a broad spectrum of activity in the brain; highly involved in cognition, mood, learning, and memory (43). In addition to androgens' role in reproduction, mood, memory, and movement, androgens play an essential role in adipose tissues wherein they influence fat redistribution (44). Androgens are also a major source of estradiol in men. Estradiol in men is also vital for modulating erectile function, libido, spermatogenesis and bone development (45)

Classical androgen receptors (AR) consists of three main domains: N-terminal variable domain for DNA transcription, a highly conserved DNA binding domain and the ligand binding domain (46,47). In canonical AR signaling, testosterone, a small lipophilic molecule, freely passes through the plasma membrane into the cytosol and binds to an androgen receptor, resulting in a conformational change in the receptor. This conformational change causes heat shock protein (HSP) to dissociate from the androgen receptor. The androgen/AR complex translocates from the cytoplasm to the cell nucleus, and bind to androgen response elements (ARE) after dimerization. This leads to the recruitment of co-activators, which enhance the interaction between DNA and the testosterone/AR complex. Thus, initiating gene transcription and protein synthesis, resulting in a biological response (48-50).

There is also compelling evidence of an alternative non-classical pathway, independent of DNA binding (51-53). This is known as the non-genomic pathway, which results in the fast actions of androgens, involving membrane androgen receptors (figure 1). Prior to the discovery of this

unconventional androgenic pathway, there were several reports on the fast actions of estrogens, resulting in the rapid release of intracellular calcium from the endoplasmic reticulum (54-58). Of note, membrane androgen receptors have not been studied extensively. Our lab has demonstrated the presence of a variant of the androgen receptor, AR45, which lacks the N-terminal domain and resides in the plasma membrane in N27 cells and multiple brain regions, such as the substantia nigra. Through co-immunoprecipitation studies (figure 2), we reported an interaction between membrane AR45 and $G\alpha_q$ (59), the G protein that can initiate intracellular calcium release from the endoplasmic reticulum. Therefore, this complex is likely to mediate some of the non-classical actions of testosterone including oxidative stress induced neurodegeneration.

ANDROGEN RECEPTOR ANTAGONISTS

AR antagonists, also known as antiandrogens, have primarily been used in the treatment of prostate cancer. Newer nonsteroidal antiandrogens (enzalutamide, bicalutamide) have gradually replaced the earlier AR antagonists such as flutamide (60-62). Both of these newer agents are more potent than the older antiandrogen, flutamide, in the treatment of metastatic prostate cancer (62,63). However, compared to bicalutamide, enzalutamide has a higher binding affinity for AR and is more efficacious. (64,65). Bicalutamide and enzalutamide act similarly by binding to AR and preventing it from adopting a transcriptionally active conformation. Additionally, enzalutamide prevents the translocation of enzalutamide-receptor complex into the nucleus, further inhibiting the binding of AR to DNA to initiate transcription (66). Bicaluamide has shown poor penetration across the blood-brain barrier, (67), however, high concentrations of enzalutamide were observed in the rat brain after oral administration (68). Because flutamide does not block membrane androgen receptors (69), there is the need to explore newer antiandrogens.

ANDROGEN RECEPTOR DEGRADER (ASC-J9)

Another effective way androgens can be down regulated is through protein degradation with receptor degraders (70). ASC-J9 (Dimethylcurcumin) is an androgen receptor degrader, which could potentially be used as first line therapy to manage or treat certain diseases largely influenced by androgen receptor signaling such as prostate cancer (71-77). ASC-J9 acts by disrupting the interaction between AR and AR co-regulators, subsequently promoting AR protein degradation (78). Several *in vitro* studies in prostate cancer cells have reported the effectiveness of ASC-J9 in degrading both full length and splice variant androgen receptors (78-82). ASC-J9, contrary to classical androgen receptor inhibitors, suppressed migration of macrophages and invasion of prostate cancer cells (83,84). Since ASC-J9 appears promising in castration-resistant prostate cancer (85), it is prudent to explore its therapeutic potential in Parkinson's disease.

ANDROGENS AND OXIDATIVE STRESS

Reactive oxygen species (ROS) are biologically and physiologically relevant, as it can significantly contribute to numerous disease pathologies, including neurodegenerative diseases (86). Low concentrations of ROS is important for intracellular signaling to occur (87). However, in excess, ROS may disturb homeostasis and precipitate oxidative stress (88). Similar to the healthy cell bias of estrogen, androgens prior to oxidative stress have been shown to be neuroprotective (69,89,90). However, androgens post neurodegenerative insult, such as oxidative stress, can be neurodamaging (90). It is possible that androgens may have an additive or synergistic effect in a cellular oxidative stress environment that can lead to cell death, as it is known that androgens exacerbate oxidative stress (69,90-92).

NADPH OXIDASE ENZYME

The NADPH oxidase (NOX) family is a major generator of ROS in most cells. NOX is a multi-subunit enzyme, with membrane and cytosolic components. Generally, the NOX family consists of seven isoforms; NOX1-NOX5, DUOX1/DUOX2 (93). NOX 1-5 consist of six transmembrane helices, whiles DUOX1/DUOX2 have an extra transmembrane domain (94). NOX is a multi-subunit enzyme, made up of membrane (gp91phox and p22phox) and cytosolic (p40phox, p47phox, p67phox) components (95,96). The different isoforms of NOX enzyme have been shown to be upregulated in various forms of cancers. NOX 1 is implicated in colon cancer, DUOX 1 and 2 in lung cancer, NOX 2 and 4 in ovarian cancer, and NOX 5 in melanoma (97). Further, pancreatic cancer cells, compared to normal cells have generated higher levels of NOX-induced ROS in certain *in vitro* studies (98,99). Although NOX 1 is predominantly expressed in neurons (100-102), NOX 2**,** NOX 3, and NOX 4 isoforms have been found to be associated with neurons, astrocytes, and microglia (97,103,104). There is also evidence to support increased mRNA and protein expression of NOX 2 and 4 in the ischemic rat brain (105).

NOX has been implicated in several disease pathologies including neurodegenerative disorders such as Alzheimer's disease, PD, and Multiple Sclerosis (106,107). In fact, NOX 2's involvement in age-linked oxidative stress has been reported, as aging-linked oxidative stress had no effect on NOX2 knock-out mice (108). Therefore, NOX can be a potential therapeutic target. NOX 1 knockdown decreased the expression and aggregation of α- synuclein protein, associated with PD (109). Meanwhile, there is growing evidence demonstrating neuroprotection after pharmacological inhibition of NOX (106).

NON-SPECIFIC NOX INHIBITORS

Apocynin and Diphenyleneiodonium chloride are the most commonly used NOX inhibitors (110). There have been increasing debates about their selectivity and specificity. Some authors have reported that these two agents show specificity for the various NOX isoforms such as NOX 1 and 2 (111). On the other hand, they have been described as non-specific NOX inhibitors (94,112,113).

Apocynin, apart from its anti-inflammatory and antioxidant properties (114-116), inhibits the assembly of the NOX enzyme (87). Specifically, apocynin prevents the translocation of cytosolic components to the membrane complex (117,118). Apocynin has shown to have protective effects in the brain upon administration in a rat model of traumatic brain injury. It effectively modulated neuronal autophagy and TLR4/NF-KB signaling pathway (119). In another study using traumatic brain injury rat model, apocynin was able to block traumatic brain injury -induced ROS production (120).

Diphenyleneiodonium chloride (DPI) does not only affect the NOX enzyme (121,122). DPI, similar to apocynin, exhibits free radical scavenging properties (123). DPI is also a potent inhibitor of cholinesterase enzyme, leading to prolonged smooth muscle contractions (124). In addition, it can also serve as an activator of transient receptor potential (TRP)A1 nociceptor, a channel for pain and hearing, in HEK-TRPA1 cells (125). An earlier study on DPI revealed its hypoglycemic actions (126). Interestingly, screening DPI for antimicrobial activity yielded positive results as DPI showed bactericidal activity against mycobacterium tuberculosis and staphylococcus aureus (127). Despite the fascinating findings on DPI, it has shown toxicity in N11 glial cells (128) and N27 dopaminergic neurons (82), due to its ability to induce oxidative stress (129).

Unlike DPI, apocynin has a very low toxicity profile (87). It is orally bioavailable and can cross the blood brain barrier (115,130-132). However, considering the lack of selectivity of apocynin, there has been a quest to discover selective inhibitors for the various NOX isoforms. Selective NOX inhibitors will eliminate issues with toxicity, potency, and specificity associated with the nonspecific small molecule NOX inhibitors (107,133-135).

SELECTIVE NOX INHIBITORS

ML-171, a modified phenothiazine has been identified as a selective NOX 1 inhibitor (136). Experimentally, ML171 has shown no toxicity profile against any cell type. NOX 1-dependent ROS generation is vital in regulating blood pressure, cell development, growth, proliferation and signaling (137-141). Interestingly, NOX 1 isoform in particular has been reported to be implicated in several disease pathologies including PD (142-146). In colon cancer cells, ML171, blocked the ROS-dependent formation of ECM- degrading invadopodia, implying the involvement of NOX 1 in cancer (136,146,147).

Another small molecule, GSK2795039, has been discovered as novel selective NOX 2 inhibitor. The selective NOX 2 inhibitory activity of GSK2795039 has been attributed to a sulphonamide group within its structure. This drug has been shown to be orally bioavailable and can cross the blood brain barrier, therefore may be a potential drug candidate for the treatment of NOX 2-mediated neurodegeneration (148,149).

NOX-MEDIATED PATHWAYS

In the vasculature, NOX activation can activate G protein-coupled receptor (GPCR) signaling, leading to either disease pathologies or important physiological functions such as gene expression, cell growth and proliferation (150).

In the CNS, G protein-coupled receptors (GPCRs) are ubiquitously expressed, and they mediate several neuronal processes (151-153). Indeed, drugs targeting GPCRs form the largest group of pharmacological agents on the market today (154,155). Recent advances attempting to provide insights in PD pathophysiology have highlighted the importance of GPCRs (156). Accumulation of calcium within brain neurons is known to contribute to the pathology of Parkinson's disease. Researchers have observed that an increase in calcium levels in neurons cause aggregation of α-synuclein proteins and potentially induce neuronal death (157). One non-debatable mechanism leading to activation of calcium permeable channel within a cell is via GPCR- phospholipase C signaling pathway. The reaction product IP_3 obtained from PIP2, functions as a second messenger and activates IP_3 receptor on the endoplasmic reticulum, which is permeable to intracellular calcium ions (158-164) (figure 3). Testosterone can rapidly induce release of intracellular calcium ions (69), resulting in long lasting oscillations (165). It is possible that testosterone is mediating its activity through NOX and IP_3 receptor.

NOX AND IP_3 RECEPTOR

Many signaling pathways leading to oxidative stress generation have been recognized. One important mechanism documented to induce oxidative stress and cell death involves the endoplasmic reticulum (ER) (166). Indeed, stress from ER contributes to neurodegenerative

diseases (167). Interestingly, Gang *et al* have reported a link between NOX and ER stress. NOX 2 deletion blocked oxidative stress and apoptosis in ER-stressed macrophages (168). In this same study, the authors emphasized the important role of NOX in IP_3 receptor activation (169). On the other hand, IP_3 can influence NOX. Calcium released by IP_3 receptor activation , is required by DAG to stimulate PKC, which in turn phospohorylate p47phox, a cytosolic subunit of NOX (170).

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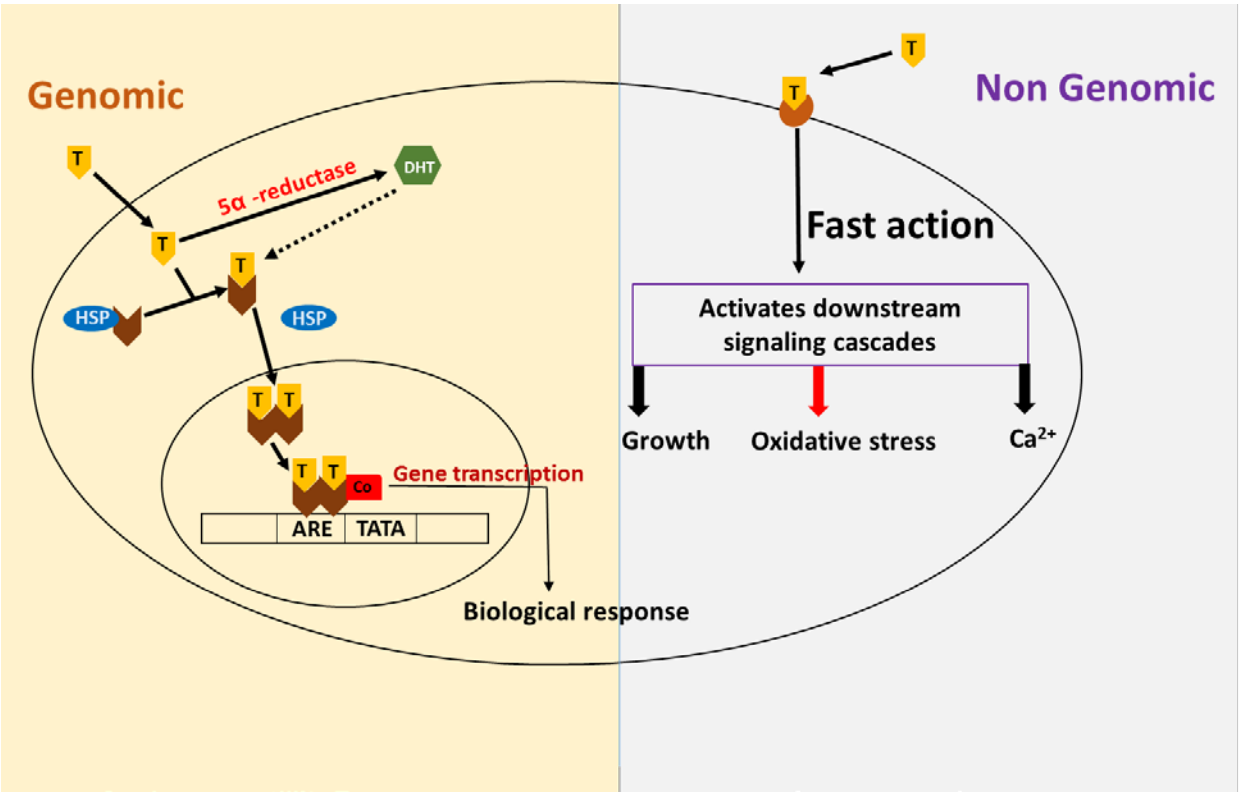

Figure 1. Androgen Pathways. In genomic androgen receptor (AR) signaling, free testosterone (T) cross the plasma membrane into the cytosol and bind to AR, resulting in a conformational change in the receptor, which causes heat shock protein (HSP) to dissociate from the AR. Testosterone can also be converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase. Androgen/AR complex translocates to the nucleus, and binds to androgen response elements (ARE) after dimerization. This leads to the recruitment of co-activators (or co-repressors), which enhance or repress, respectively, the interaction between DNA and testosterone/AR complex, initiating gene transcription and a biological response (left panel). In the non-genomic pathway, testosterone binds membrane AR and mediates its fast actions leading to downstream signaling cascades. Dark brown; classical AR, light brown; membrane AR (right panel).

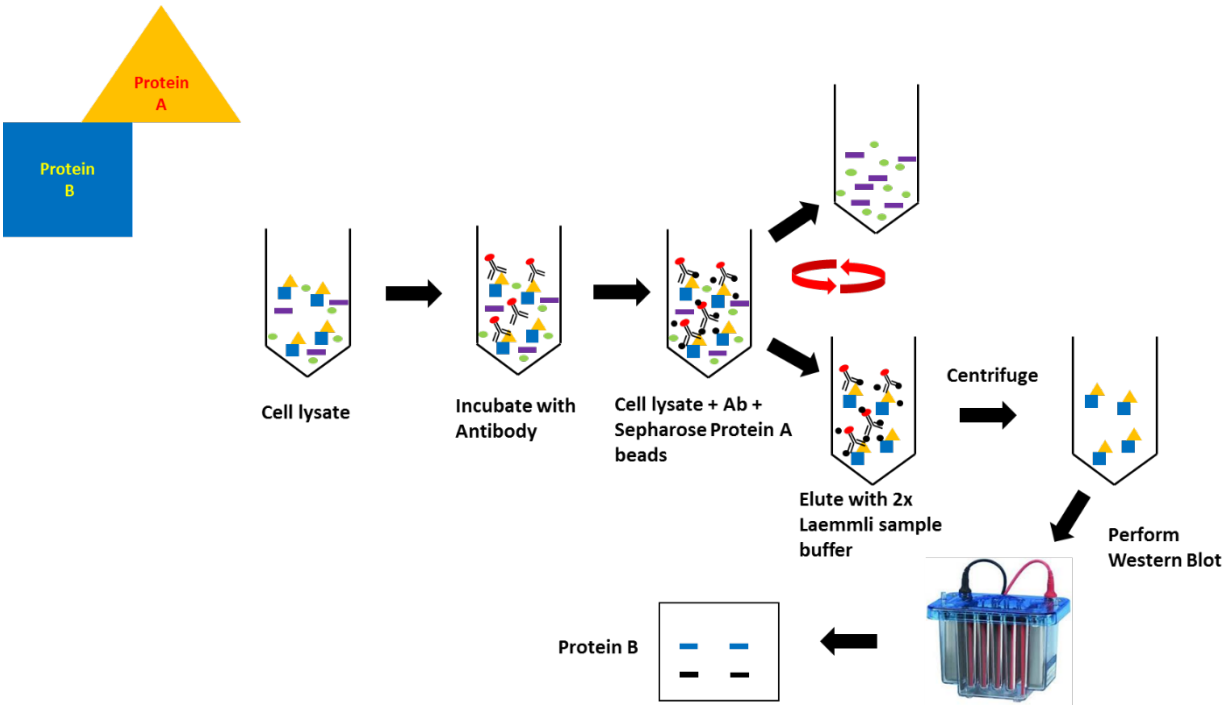

Figure 2. Co-immunoprecipitation. To determine if protein A and B interact, cell lysate is incubated with an antibody against protein A. The complex is then precipitated on sepharose protein A beads. Series of washing is done to remove proteins not precipitated on the beads. Laemmli sample buffer is added to elute the proteins from the beads. This is followed by centrifugation and the supernatant is collected for western blot analysis to detect protein B.

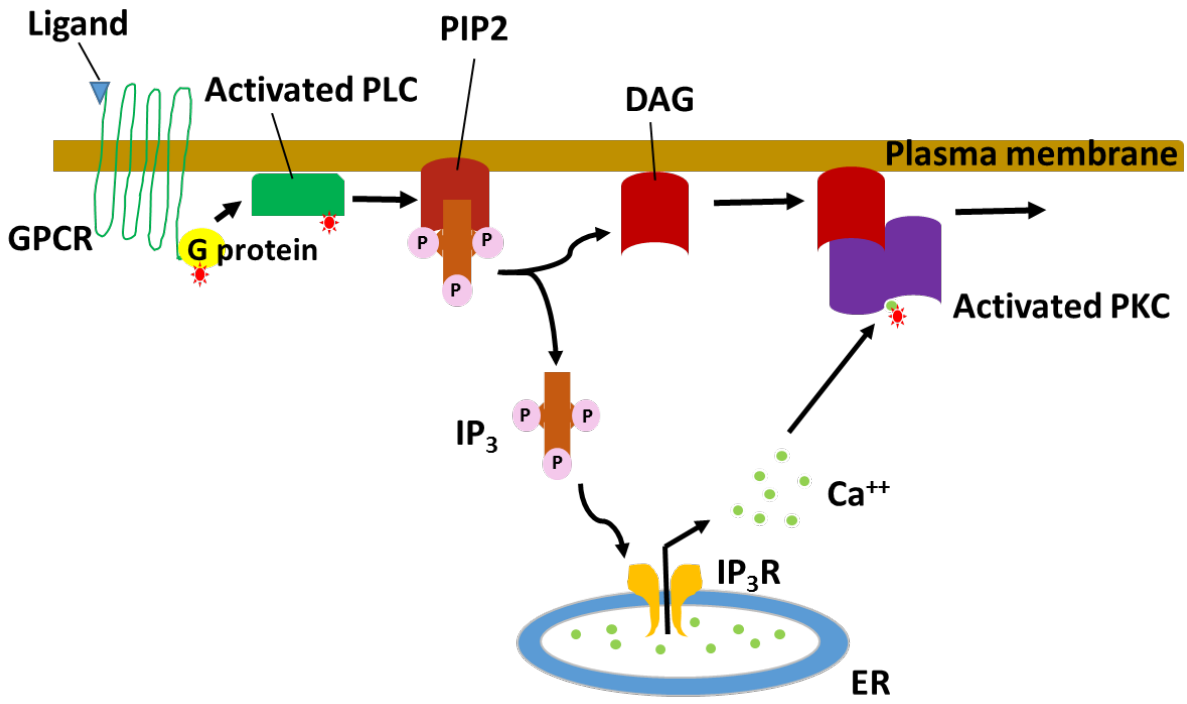

Figure 3. G Protein-Coupled Receptor (GPCR) Signaling Pathway through Phospholipase C. Ligand binds and activates the receptor coupled to G protein, leading to activation of phospholipase C (PLC). Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), resulting in the formation of diacylglycerol (DAG) and IP_3. IP_3 then activates IP_3 receptor (IP_3R) on the endoplasmic reticulum (ER), causing the release of Ca^{2+}, which in turn activates Protein kinase C (PKC).

CHAPTER 3

NADPH OXIDASE (NOX) MEDIATES MEMBRANE ANDROGEN RECEPTOR-INDUCED NEURODEGENERATION

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Abstract

Oxidative stress (OS) is a common characteristic of several neurodegenerative disorders, including Parkinson's disease (PD). PD is more prevalent in men than women, indicating the possible involvement of androgens. Androgens can have either neuroprotective or neurodamaging effects, depending on the presence of OS. Specifically, in an OS environment, androgens via a membrane-associated androgen receptor (mAR) exacerbate OS-induced damage. To investigate the role of androgens on OS signaling and neurodegeneration, the effects of testosterone and androgen receptor activation on the major OS signaling cascades, NADPH Oxidase 1/2 (NOX1, NOX2) and $G\alpha_q$/$InsP_3R$, were examined. To create an OS environment, an immortalized neuronal cell line was exposed to hydrogen peroxide (H_2O_2) prior to cell permeable/impermeable androgens. Different inhibitors were used to examine the role of G-proteins, mAR, $InsP_3R$, NOX1/2 on OS generation and cell viability. Both testosterone and DHT-BSA increased H_2O_2-induced OS and cell death, indicating the involvement of a mAR. Further, classical AR antagonists did not block testosterone's negative effects in an OS environment. Since there are no known antagonists specific for mAR, an AR protein degrader, ASC-J9, was used to block mAR action. ASC-J9 blocked testosterone's negative effects. To determine OS-related signaling mediated by mAR, this study examined NOX1, NOX2, $G\alpha_q$. NOX1, NOX2, and $G\alpha_q$ complex with mAR. Only NOX inhibition blocked testosterone-induced cell loss and OS. No effects of blocking either $G\alpha_q$ or G protein activation were observed on testosterone's negative effects. These results indicate that androgen-induced OS is via the mAR-NOX complex and not the mAR-$G\alpha_q$ complex.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease (1), and the prevalence of this disorder is expected to increase to approximately 2 million by year 2030 (2). The two major hallmark neuropathological characteristics of PD are the presence of Lewy Bodies and the loss of dopaminergic neurons in the substantia nigra pars compacta of the brain. The established motor symptoms (resting tremor, rigidity, bradykinesia) manifest when approximately 80% of the dopaminergic neurons within the substantia nigra are lost (3,4). Since no laboratory biomarker or imaging exist for PD diagnosis, diagnosis of PD is a clinical diagnosis that requires the presence of at least two established motor symptoms.

Although the etiology of PD remains elusive (5), oxidative stress has been linked to PD development and progression (6). Oxidative stress is an early event in PD pathogenesis and can precede dopaminergic neurodegeneration in the substantia nigra (7-9). The substantia nigra pars compacta is rich in dopaminergic neurons ~25,000 cells (10-12), and this composition may underlie its vulnerability to oxidative stress insults. Dopamine and its metabolites are highly reactive due to the auto-oxidation of dopamine that can generate free radicals, leading to cellular vulnerability of the substantia nigra (13,14). In addition to dopamine auto-oxidation, major cellular oxidative stress signaling cascades can contribute to dopaminergic neuronal vulnerability, such as NADPH oxidase (NOX) and calcium (Ca^{++}) neurotoxicity (15,16). NOX1 and NOX2 isoforms can increase oxidative stress generation and play a role in neurodegeneration (17-21), including dopaminergic neuronal loss in PD (22,23). Indeed, NOX can affect Ca^{++} release and vice versa (24-33). For example, oxidative stress can modulate ryanodine receptors and inositol trisphosphate receptors ($InsP_3R$) that are involved in intracellular Ca^{++} release from the endoplasmic reticulum

via the canonical GPCR Gαq signaling pathway (30-33). Further, NOX inhibition can block Ca^{++}signaling (28). Since these signaling pathways can induce a feed-forward loop, dysregulation could lead to substantia nigra vulnerability and ultimately PD. Therefore, determining mechanisms that can affect this feed-forward loop is paramount.

Sex differences in PD incidence and prevalence have also been recognized (34-38). Men are 1.5-2 times more likely to develop PD than women. (36,39,40). Furthermore, sex differences have been observed in oxidative stress signaling. Overall, men have a higher level of circulating oxidative stress and higher levels of NOX1 and NOX2 than women (41-45). In fact, sex hormones (estrogens, androgens) can influence oxidative stress generation. It is well-established that estrogens are protective against oxidative stress insults (46,47). However, the role of androgens, such as testosterone and dihydrotestosterone (DHT), in neurodegeneration is not extensively studied.

Current findings on the effects of androgens on cells are equivocal, wherein androgens were found to be protective or damaging to cells (48-52). Low testosterone is a risk factor for neurodegenerative disorders in men (53-55). However, other studies have found that testosterone can induce neuronal loss (56-60). Our lab observed that testosterone can have either neuroprotective or neurodamaging effects, depending on the presence of oxidative stress in the cellular environment (58,60). Specifically, we found testosterone is an oxidative stressor in both *in vivo* and *in vitro* studies (58-64). Depending on the level of oxidative stress in the cell, testosterone-induced oxidative stress could be neuroprotective via a pre-conditioning mechanism (58,65,66) or damaging by exacerbating existing oxidative stress damage (58-60,64,67-69). Using

cell impermeable androgens (e.g. testosterone bound to BSA), we found testosterone can increase cellular oxidative stress via a membrane associated androgen receptor (mAR), specifically an androgen receptor variant – AR45 that is missing its regulatory N-terminal domain (58,60,70). However, the specific oxidative stress signaling pathways initiated by mAR are not fully understood. To address this gap in knowledge, this study will investigate the mechanisms underlying mAR-induced neurodegeneration in the N27 dopaminergic cell line. Specifically, the role of NADPH Oxidase 1 and 2 (NOX1 and NOX2) and $G\alpha_q$/ $InsP_3R$ in mediating androgen-induced neurodegeneration in an oxidative stress environment was investigated.

Materials and Methods

Reagents

Androgen receptor (AR) antagonists, enzalutamide (#A3003) and bicalutamide (#B9061), were obtained from ApexBio Tech LLC and Sigma-Aldrich, respectively. AR degrader, (ASC J9; HY-15194), and NOX2 inhibitor (#GSK2795039) were purchased from Medchem express. Apocynin (#4663) and Diphenyleneiodonium chloride (DPI; #0504) were obtained from Tocris Bioscience. NOX 1 inhibitor (#ML171) was purchased from EMD Millipore. $InsP_3R$ inhibitor, (2-APB; #100065) and $G\alpha_q$ G Protein inhibitor (BIM-46187; #533299), were purchased from Calbiochem. GDPβS trilithium salt (#G7637) was obtained from Sigma-Aldrich/Millipore**.** Antibodies; MOX1 (sc-25545 for NOX1) (71), NOX2 (sc-130543) (72), Goat Anti Rabbit (sc-2004) (73), Goat Anti-Mouse (sc-2005) (74), androgen receptor C19 (sc-815) (75), and $G\alpha_q$ (sc-365906) (76) were from Santa Cruz Biotechnology. GAPDH antibody (GTX627408) (77) was purchased from GeneTex. DMSO was obtained from VWR, and phosphate buffer solution (PBS) from Quality Biological.

Pierce™ BCA protein assay kit was purchased from Thermo Scientific. Protein gels, Mini-PROTEAN TGX (#456-1093; #456-9036) were acquired from Bio Rad. Protein A sepharose (CL-4B) was obtained from GE Healthcare. RPMI 1640 was purchased from Hyclone. Penicillin-streptomycin (PS) and TrypLE select (10X) were acquired from Gibco. Fetal Bovine Serum (FBS) and L-glutamine were obtained from Corning, and Charcoal-stripped Fetal Bovine Serum (CS-FBS) from Atlanta Biologicals. Tert-butyl hydrogen peroxide (A13926) and Thiazolyl Blue Tetrazolium Bromide (MTT; #L11939) were purchased from Alfa Aesar. Fluorescent thiol detection kit (#FLTHIO100-2) was obtained from Cell Technology. Super signal West Femto chemiluminescent substrate was purchased from Thermo Scientific. Testosterone (#A6950-000) and dihydrotestosterone 3-CMO: BSA (DHT-BSA, #A2574-050) were obtained from Steraloids. Testosterone was made from a stock solution in 100% ethanol, whereas DHT-BSA was prepared in media. All inhibitors, except $G\alpha_q$ protein, were made from a stock solution in DMSO. Final concentrations of DMSO and ethanol were < 0.001% in all vehicle controls and treatment groups.

Cell Culture

The immortalized $1RB3AN_{27}$ (N27) dopaminergic neuronal cell line was harvested from fetal female rat mescencephalic tissue (78). This cell line expresses tyrosine hydroxylase (TH+), marker for dopaminergic neurons, and a membrane-associated splice variant of the androgen receptor, AR45, that is missing the regulatory N-terminus domain (58,70,79-82). N27 cells were cultured as previously published (60). Cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin (100 U/mL) and streptomycin (100 ug/ml) at 37℃ in 5% CO_2. Cells were seeded at a density of 6×10^4/well in 96-well cell culture plates (for cell viability assay), 6×10^4/100 mm dish (for oxidative stress assays) and 1×10^6 /100 mm dish (for co-immunoprecipitation and

western blot studies). To ensure the quality and integrity of the N27 cell line, we only used passages between 16 and 19 for all experiments. We also characterized these cells based on morphology, doubling time, and a well-characterized response to tert-butyl hydrogen peroxide (H_2O_2) and testosterone (58,60).

Experimental Design

Cells were plated into 96-well cell culture plates at a density of 6.0×10^4 cells/well in RPMI 1640 media supplemented with 10% FBS and 1% PS and incubated for 24 hours at 37°C, 5% CO_2. At approximately 80% confluency, media was changed to RPMI 1640 medium with charcoal-stripped FBS to remove confounding variables (e.g. hormones) from the serum. Maintaining cells in charcoal-stripped FBS does not negatively impact cell viability, as the media still contains salt, glucose, amino acids, and other nutrients (58,60,83). Inhibitors (and associated vehicle controls) used in this study were applied 2 hours prior to H_2O_2. Cells were exposed to 10 μM H_2O_2 for two hours. This concentration of H_2O_2 is sufficient to achieve 20% cell loss, which is necessary to achieve the oxidative stress threshold for androgens to negatively impact cells. After H_2O_2 exposure, androgens were applied to cells for 4 hours. Based on our previous studies (58,60), androgen concentrations used in this study were 100 nM testosterone and 500 nM DHT-BSA. A five-fold increase in the DHT-BSA concentration (ie. 500nM) was used to account for decreased DHT-receptor binding due to the structure of DHT-BSA (11 DHT molecules bound to 1 BSA molecule). Further, these androgen concentrations are appropriate to examine the androgen receptor splice variant, AR45, which exhibits a similar affinity as the full length androgen receptor to androgens (84).

Cell Viability

Cell viability was determined by MTT assay as previously described (60). Briefly, N27 cells were plated into 96-well cell culture plates at a density of 6.0 x 10^4 cells/ml in each well. Treatments were carried out as shown in Figure 1. Cell viability was examined by aspirating the media and incubating the cells in each well with 100 µL phenol red-free RPMI 1640 medium with charcoal-stripped FBS, followed by 20 µL of 5 mg/mL MTT at 37℃ and 5%CO_2 for a 3 hour period. Absorbance was read at 595 nm. Experiments were replicated at least three times on three separate plates; each n is a mean of eight (8) wells/treatment group on one plate. The colorimetric intensity is directly proportional to the number of live cells.

Oxidative stress

Fluorescent Thiol detection kit was used to measure the levels of reduced thiols, as previously published (60,79). Cells were plated in 100mm x 20mm cell culture dishes at a density of 6.0 x 10^4 cells per plate. Similar to our cell viability experiments, at 80% confluency, cells were treated with the inhibitor or vehicle for two hours prior to H_2O_2 exposure, but were only exposed to testosterone for 2 hours instead of 4 hours. This method allows oxidative stress to be measured prior to cell loss. Afterwards, cells were lysed with a mild lysis buffer (10X TrypLE select) on ice, collected into tubes, centrifuged for 1 minute at high speed, and then trypsin was removed. Fluoro thiol lysis buffer (200µl) was added to each tube, homogenized, and centrifuged (10,000 rpm) for 5 minutes. Supernatants were collected. Reduced thiols, an inverse measure of oxidative stress, were quantified in the cell lysates (1.0 x 10^5 cells/mL). At least three independent experiments were performed and fluorescence was measured at 488 nm excitation and 525 nm emission wavelengths.

Cell lysates and Homogenization

Following our experimental design (Figure 1), cells were placed on ice, washed with PBS, and lysed using NP40 lysis buffer with a cocktail of DTT (1µM), EDTA (1mM), and phosphatase and protease inhibitors (1:100). The lysates were homogenized and centrifuged at 12,753 rpm in 4℃ for 20 minutes. The supernatant was then removed and assayed for protein concentration. Protein concentrations were measured by using the Pierce BCA protein assay kit, according to the manufacturer's instructions.

Co-immunoprecipitation

For co-immunoprecipitation studies to determine protein-protein interactions of proteins with molecular weights around 40-50 kDA, the protocol was slightly modified to move the IgG bands from 45 kDa to 100 kDa. Cell lysate in NP40 lysis buffer was incubated with various primary antibodies overnight at 4ºC with agitation. The next day, the cell lysate + primary antibody mixture was placed on ice, and a bead slurry (Protein A sepharose beads with PBS) was added. The mixture was incubated at 4ºC for 4 hours, centrifuged at maximum speed for 2 minutes, and the supernatant aspirated. To separate the proteins attached to the primary antibody from the beads, 2x Laemmli sample loading buffer, containing β-mercaptoethanol, was added to the mixture and allowed to incubate at 37 ºC for 30 minutes in a water bath. After centrifugation at high speed for 2 minutes, protein-protein interactions were determined by Western blotting using respective antibodies specific to the proteins of interest.

Western Blot

For all experiments 20µg of protein was loaded on either Bio-Rad AnykD or 4-20% precast gels, except for co-immunoprecipitation that used 375µg of protein. Experiments were performed according to our previously published protocols (70). Proteins were separated by SDS-polyacrylamide gel electrophoresis at room temperature at 25mA. Next, proteins were transferred overnight at 50V onto polyvinylidene difluoride (PVDF) membrane in 4ºC. Membranes were quickly washed under agitation with TBS-Tween, and incubated in 5% nonfat milk in TBS-Tween at room temperature to block non-specific binding. Afterwards, the membranes were incubated with specific primary antibodies (1:500 dilution for MOX1, NOX2, ARC19, and 1:1000 dilution for $G\alpha_q$) (71,72,75,76) in TBS-Tween with 1% nonfat milk overnight at 4℃. The membranes were washed twice with TBS-Tween for 10-minutes on a shaker and incubated for 30 minutes in the corresponding secondary antibody (1:1000 dilution for goat anti-rabbit and goat anti-mouse) (73,74) in TBS-Tween with 1% nonfat milk at room temperature. After washing membranes twice for 10 minutes, the protein bands were detected using a super signal West Femto chemiluminescence assay. Protein band intensities were imaged using Syngene G: Box system together with FluorChem HD2 AIC software. Protein band densities were measured through densitometry with NIH Image J densitometer software and normalized to GAPDH (1:10000 dilution).

Statistical Analysis

All analyses were performed using IBM SPSS Statistics version 21 software. Results were expressed as mean $\pm$ SEM, and p value less than or equal to 0.05 ($p \leq 0.05$) indicates statistically significant differences. Comparisons were made by two or three-way ANOVA using inhibitors,

oxidative stressor and hormone as independent factors. Fisher's LSD post hoc analysis was used to assess differences between the various groups. Each experiment was replicated at least three times with different cell cultures.

Results

Membrane Androgen Receptor interacts with NOX1, NOX2, and $G\alpha_q$

Previously, we discovered that the mAR, AR45 splice variant, was localized to lipid rafts within the plasma membrane (70), indicating that the mAR is a component of a communication hub that could facilitate its interaction with membrane-associated proteins to initiate cellular signaling (e.g. oxidative stress signaling). To determine if the mAR (i.e. AR45) complexes with membrane-associated NADPH Oxidase (NOX1 and NOX2) and $G\alpha_q$/ $InsP_3R$ oxidative stress signaling cascades, we performed co-immunoprecipitation on N27 cell lysate. To immunoprecipate specific proteins of interest, primary antibodies for NOX1 (i.e. MOX1), NOX2, and AR45 (i.e. AR-C19) (71,72,75) were used. Next, we probed for protein-protein interactions by using primary antibodies for $G\alpha_q$, NOX1, NOX2, and AR45 (71,72,75,76), which showed AR45 complexed with NOX1 and NOX2. Consistent with our prior publication, AR45 complexed with Gαq (70). Although AR45 coupled with Gαq, no protein interactions between NOX1 or NOX2 with Gαq were observed (Figure 2A, B). Only the 45 kDa AR45 protein was observed, indicating that the co-immunoprecipitation studies were determining protein interactions with mAR.

Testosterone's detrimental effects are not mediated through the classical genomic pathway

We previously published that flutamide, a classical AR antagonist, did not block testosterone's negative effects (58). In this study, we strengthened our findings using two other classical AR

antagonists; enzalutamide and bicalutamide. Bicalutamide binds to the ligand-binding pocket of AR and inhibits AR by failing to induce the correct conformational change. Enzalutamide has a similar mechanism of action as bicalutamide, but with a higher affinity for the AR receptor (85,86). In addition to their common mechanisms of action, enzalutamide can prevent nuclear translocation of the AR and the binding of AR to DNA (85), hence more efficacious as an AR antagonist. Therefore, to confirm that testosterone's detrimental effects are not mediated through the classical cytosolic AR, enzalutamide and bicalutamide AR antagonists were used. As expected, testosterone did not affect cell viability (Figure 3A) and H_2O_2 significantly decreased cell viability ($F_{1, 16}$ = 799.2, $p < 0.05$). In the presence of oxidative stress, testosterone further decreased cell viability, as indicated by a significant interaction between oxidative stress and hormone ($F_{1, 16}$ = 165, $p < 0.05$). The AR antagonist, bicalutamide, had no effect on cell viability, regardless of the oxidative stress environment. Further, bicalutamide did not block testosterone's negative actions on cell viability in an oxidative stress environment. Similar results on cell viability using enzalutamide were observed (data not shown).

Since classical AR antagonists did not influence testosterone's effects, an AR degrader, ASC J9, was used to degrade both cytosolic and membrane-associated ARs. ASC J9 selectively promotes AR degradation by disrupting the interaction between AR and AR co-regulators, without impacting AR mRNA expression (87). To ensure that ASC J9 degraded mAR protein (45 kDa) expression, ASC J9 was used with and without testosterone, as previous reports stated testosterone can stabilize AR (70,88,89). Consistent with our prior findings (70), testosterone did not alter mAR protein expression (Figure 3B). In contrast, ASC J9 significantly reduced mAR expression ($F_{1, 8}$ = 10.6, $p < 0.05$), regardless of testosterone exposure (Figure 3B). To determine if degrading the

mAR impacts testosterone's negative effects in an oxidative stress environment, N27 cells were pretreated with ASC J9 for two hours prior to exposure to H_2O_2 and testosterone (Figure 3C). As expected, significant negative effects from oxidative stress exposure on cell viability were observed ($F_{1, 28} = 167.1$, $p < 0.05$). In addition, significant interactions between oxidative stressor and testosterone treatment ($F_{1, 28} = 8.2$, $p < 0.05$) and between oxidative stressor, hormone, and degrader ($F_{1, 26} = 4.5$, $p < 0.05$) were observed. Specifically, H_2O_2 induced approximately 20% cell loss and testosterone exacerbated H_2O_2 induced cell loss. The AR degrader blocked testosterone's negative effects on cell viability in an oxidative stress environment. However, the AR degrader did not impact H_2O_2 induced cell loss.

The role of G protein and $InsP_3R$ in testosterone-induced neurodegeneration

Since AR45 complexes with $G\alpha_q$ (Figure 2A) (70), it is of interest to determine if $G\alpha_q$ plays a role in testosterone's damaging effects in an oxidative stress environment. The canonical GPCR $G\alpha_q$ signaling pathway, which is involved in intracellular Ca^{++} release from the endoplasmic reticulum via ryanodine receptors and inositol trisphosphate receptors ($InsP_3R$), can increase oxidative stress (90). To examine this pathway, G protein activity was blocked with GDPβS trilithium, $G\alpha_q$ with BIM-46187, and $InsP_3R$ with 2-APB. In Figure 4A using GDPβS trilithium, a GDP analogue, to block G protein activity, significant effects of oxidative stressor ($F_{1, 29} = 206.8$, $p < 0.05$), hormone ($F_{1, 29} = 51$, $p < 0.05$) and an interaction between oxidative stressor and hormone ($F_{1, 29} = 40.6$, $p < 0.05$) was observed. As expected, we observed no effects of testosterone alone and significant effects of H_2O_2 on the cell viability ~ 20% cell loss. Testosterone, in the presence of an oxidative stressor, caused a further decrease in cell viability. However, GDPβS trilithium neither blocked H_2O_2's effects nor the negative effects of testosterone in an oxidative stress environment.

Next, $G\alpha_q$ was blocked with BIM-46187. We found significant effects of oxidative stressor ($F_{1, 23}$ = 303.1, $p < 0.05$), hormone ($F_{1, 23}$= 70.8, $p < 0.05$) and an interaction between oxidative stressor and hormone ($F_{1, 23}$ = 65.4, $p < 0.05$) (Figure 4B). Again, no effects of testosterone alone were observed. H_2O_2 significantly induced approximately 20% cell loss and testosterone worsened H_2O_2's damaging effects. Similar to the GDP analogue, $G\alpha_q$ inhibitor did not block testosterone's detrimental effects in an oxidative stress environment. These results indicate testosterone's damaging effects via the mAR are not through the $G\alpha_q$ protein pathway, although AR45 complexes with $G\alpha_q$.

Lastly, we looked at the effects of $InsP_3R$ inhibition on testosterone's negative effects on cell viability. We found significant effects of oxidative stressor ($F_{1, 48}$ = 314.10, $p < 0.05$), hormone ($F_{1, 48}$ = 58.8, $p < 0.05$), inhibitor ($F_{1, 48}$ = 35.1, $p < 0.05$), an interaction between oxidative stressor and hormone ($F_{1, 23}$ = 65.4, $p < 0.05$) and an interaction between oxidative stressor, hormone, and $InsP_3R$ inhibitor ($F_{1, 48}$ = 27, $p < 0.05$) (Figure 4C). Consistent with our prior studies, testosterone alone did not influence cell viability, whereas H_2O_2 did. Testosterone exacerbated H_2O_2's detrimental effects on the cells. $InsP_3R$ inhibition with 2-APB, alone, had no effect on cells, regardless of oxidative stress environment. In contrast to earlier observations with GDPβS trilithium and BIM-46187, 2-APB was able to protect the cells from testosterone's negative effects in an oxidative stress environment. This finding of $InsP_3R$ signaling, which mediates intracellular Ca++ release, extends our previous work showing mAR's negative effects on oxidative stress and cell viability in N27 cells was influenced by Ca++ influx into the mitochondria (58).

The effects of NOX inhibitor on oxidative stress generation and cell loss

To determine if NOX is involved in mAR-induced oxidative stress, we used the frequently used NOX inhibitors, apocynin and DPI, at various concentrations based on their IC_{50} (10 μM and 39 nM, respectively) (91,92). DPI was toxic to the cells, regardless of the concentration (10-80nM) used. However, apocynin did not impact cell viability in any of the doses (3-20μM) investigated (Figure 5A). Therefore, apocynin was used as a NOX inhibitor in the subsequent experiments.

Oxidative stress was measured by quantifying reduced thiols (an inverse measure of oxidative stress). Significant effects of oxidative stressor ($F_{1, 16} = 124.2$, $p < 0.05$), hormone ($F_{1, 16} = 28.7$, $p < 0.05$), inhibitor ($F_{1, 16} = 19.3$, $p < 0.05$), an interaction between oxidative stressor and hormone ($F_{1, 16} = 3.7$, $p < 0.05$) and an interaction between oxidative stressor, hormone, and inhibitor ($F_{1, 16} = 2.3$, $p < 0.05$) was observed (Figure 5B). Consistent with our prior publications (58-60,63), testosterone is an oxidative stressor. Both testosterone and H_2O_2 significantly decreased the level of reduced thiols, indicating an increase in oxidative stress. Further, testosterone exacerbated H_2O_2 induced oxidative stress by further decreasing reduced thiols. NOX inhibitor, apocynin, did not alter H_2O_2-induced cell loss, indicating H_2O_2 increases oxidative stress via a non-NOX mechanism. However, apocynin blocked testosterone induced decrease in reduce thiols, indicating that NOX mediates testosterone-induced oxidative stress generation.

A similar paradigm was observed for cell viability assay, except testosterone alone, had no effect on cell viability. Significant effects of oxidative stressor ($F_{1, 23} = 13.4$, $p < 0.05$), an interaction between oxidative stressor and hormone ($F_{1, 23} = 21.4$, $p < 0.05$) and an interaction between

oxidative stressor, hormone, and inhibitor ($F_{1, 23}$ = 10.3, $p < 0.05$) was observed (Figure 5C). Specifically, H_2O_2 decreased cell viability, inducing ~ 20% cell loss. Testosterone exacerbated H_2O_2 induced cell death, which was blocked by apocynin. Apocynin had no effect on cell viability, regardless of oxidative stress exposure. A similar pattern was observed using DHT-BSA. In Figure 5D, we found significant effects of oxidative stressor ($F_{1, 21}$= 513.9, $p < 0.05$), hormone ($F_{1, 21}$= 81.8, $p < 0.05$), inhibitor ($F_{1, 21}$= 57.8, $p < 0.05$), and an interaction between oxidative stressor and hormone ($F_{1, 21}$= 17, $p < 0.05$). H_2O_2 induced ~20% cell loss, and DHT-BSA further increased H_2O_2 induced cell loss. Apocynin did not alter H_2O_2's effects on cell viability. However, apocynin blocked DHT-BSA's detrimental effects in the presence of oxidative stress, implying the involvement of mAR and NOX on androgen's negative effects in an oxidative stress environment.

Since apocynin is a nonspecific NOX inhibitor (93,94), the role of NOX1 and NOX2 in testosterone mediated cell loss was examined. Cells were pretreated with selective NOX1 inhibitor, ML171 (95,96), followed by H_2O_2 and testosterone. Significant effects of oxidative stressor ($F_{1, 47}$= 321.5, $p < 0.05$), hormone ($F_{1, 47}$= 60.9, $p < 0.05$), inhibitor ($F_{1, 47}$= 8.9, $p < 0.05$), an interaction between oxidative stressor and hormone ($F_{1, 47}$= 64.1, $p < 0.05$) and between oxidative stressor, hormone, and inhibitor ($F_{1, 47}$= 9.3, $p < 0.05$) were observed. Similar to previous observations, H_2O_2 decreased cell viability and testosterone further exacerbated H_2O_2 induced cell loss. ML171, however, did not alter cell viability by itself or in the presence of testosterone. Further, ML171 did not protect the cells from H_2O_2. Contrary to observations with apocynin, ML171 partially protected against testosterone-induced cell loss in an oxidative stress environment (Figure 6A).

The role of NOX2 in testosterone-induced neurodegeneration was further examined using a selective NOX2 inhibitor, GSK2795039. Again, H_2O_2 decreased cell viability and testosterone further exacerbated H_2O_2 induced cell loss. However, GSK2795039, alone or in the presence of testosterone, did not alter cell viability. GSK2795039 did not protect the cells from H_2O_2, indicating GSK2795039 at this concentration does not exhibit general antioxidant properties. Similar to observations with ML171, GSK2795039 partially blocked testosterone-induced cell loss in the presence of H_2O_2. Significant effects of oxidative stressor ($F_{1, 28}$= 233.5, $p< 0.05$), hormone ($F_{1, 28}$= 34.6, $p< 0.05$), inhibitor ($F_{1, 28}$= 4.3, $p<0.05$), an interaction between oxidative stressor and hormone ($F_{1, 28}$= 24.5, $p< 0.05$) and between oxidative stressor, hormone, and inhibitor ($F_{1, 28}$= 4.6, $p < 0.05$) were observed (Figure 6B). Based on these findings using ML171 and GSK2795039, cells were exposed to both NOX1 and NOX2 inhibitors with the aim of achieving a full protection from testosterone's neurotoxic effects in an oxidative stress environment. Interestingly, antioxidant effects in the H_2O_2 group were observed, indicating non-specific antioxidant properties. This general antioxidant effect may be due to the phenolic structure of these inhibitors, which can have antioxidant effects at higher levels (97-99). Hence we were unable to examine the combined effects of these inhibitors on testosterone-induced cell loss in an oxidative stress environment (data not shown).

The effects of AR degradation on NOX1 and NOX2 protein expression

Since the mAR (i.e. AR45) complexed with NOX1 and NOX2, we wanted to ensure that mAR protein degradation did not alter NOX1 and NOX2 protein expression. Two-hour pretreatment of N27 cells with the AR degrader, ASC J9, did not alter NOX1 or NOX2 expression, irrespective of oxidative stress or testosterone exposure (Figure 7A, B). Although it appears mAR degradation

with ASC J9 affects NOX2 expression, there was no statistical significance across all treatment groups ($F_{1, 24}$= 3.95, p=0.059) (Figure 7B).

Discussion

More men than women are affected by PD, regardless of age (35,40,100,101). Sex hormones have been proposed to be involved in this disparity. Interestingly, the AR in a ligand-dependent manner can induce transcriptional activity of the TH gene (102) and increase TH activity (103). Thus, androgens can modulate dopaminergic neurons and dopamine synthesis, and ultimately oxidative stress generation via auto-oxidation of dopamine (13,14). This pathway may underlie androgen neuroprotection via oxidative stress preconditioning (58) and the increased incidence in PD for men. Notably, AR is expressed in the substantia nigra pars compacta, the region affected by PD, and not in the substantia nigra pars reticulata (104). ARs are known to mediate important physiological and biological actions, including mood and libido (105-107), sexual reproductive functions (108), learning and memory (109,110). Indeed, in men with PD, decreased testosterone has been associated with poor cognitive abilities (111), reduced sexual libido, and erectile dysfunction (112-114). Testosterone replacement therapy in men with PD significantly improved motor and non-motor symptoms (115,116). However, in light of reports that estrogen via the estrogen receptor alpha (ERα) can also upregulate TH activity (117) (118), androgen induced transcriptional regulation of TH does not fully explain the increased PD symptoms after disease onset observed in men compared to women (119-121). Therefore, in this study, other oxidative stress signaling cascades that can be modulated by androgens, such as NOX 1, NOX2, and $G\alpha_q$/ $InsP_3R$ were examined.

Low levels of androgens (1 nmol/L) via a non-genomic mechanism can exacerbate oxidative stress induced damage in dopaminergic neurons (58,60), supporting the role of a mAR in testosterone's negative effects in an oxidative stress environment. The function of mAR is poorly understood, but it is proposed to mediate fast, non-genomic actions of androgens (122-124), and is unaffected by classical AR antagonists (58). Furthermore, it is unclear if the mAR is a full-length AR, truncated AR, or an unknown AR localized to the plasma membrane. Recently, our lab discovered the presence of an AR splice variant, AR45, localized to lipid rafts within the substantia nigra pars compacta and the dopaminergic N27 cells (70). AR45 lacks the N-terminal regulatory domain, resulting in a molecular weight of 45 kDa unlike full length AR with a molecular weight of 110 kDa (84,108). Neither age nor testosterone affected AR45 expression (70). In this study, degradation of AR45 using ASC J9, unlike classical AR antagonists, did block mAR exacerbation of oxidative stress induced damage, supporting the role of AR45 as a mAR. This is of interest, as currently there are no known mAR antagonists. To the best of our knowledge, this is the first work to show inhibition of a mAR in CNS.

Based on previous publications showing AR45 interacts with $G\alpha_q$ protein within the lipid rafts (70) and testosterone increases intracellular Ca^{++} release in N27 cells (58), the role of $G\alpha_q/InsP_3R/Ca^{++}$ pathway on mAR-AR45 induced neurodegeneration was determined. Although AR45 complexes with $G\alpha_q$ protein, $G\alpha_q$ does not influence mAR induced neurodegeneration, as evidenced by no effects from either $G\alpha_q$ protein inhibitor or GDP analogue. Interestingly, inhibition of $InsP_3R$, which mediates intracellular Ca^{++} release, did block mAR-induced neurodegeneration. Clinical studies found therapeutic benefits of blocking Ca^{++}. Dihydropyridine Ca^{++} channel blockers have been shown to decrease PD risk, progression, and even mortality (125-

130). Further, there is an ongoing exploratory phase III clinical trial of isradipine (dihydropyridine Ca^{++} channel blocker), which may provide better insights to the association between Ca^{++} pathway and PD and its therapeutic potential (131).

In addition to the canonical GPCR $G\alpha_q$ pathway modulation of $InsP_3R$ for intracellular Ca^{++} release from the endoplasmic reticulum (30-33), NADPH oxidase (NOX) can affect $InsP_3R$ mediated Ca^{++} release (24-33,132). There are 7 members of the NOX family: NOX1, NOX2, NOX3, NOX4, NOX5, Duox 1, and Duox 2. It is well known that the NOX enzyme is associated with dopaminergic neurons (133,134) and plays an important role in normal cell physiology including differentiation, proliferation, and immune response (135-137). Indeed, several studies have reported that NOX is the predominant source of reactive oxygen species (ROS) in brain cells (16,22), aside from the mitochondria. Dysregulation of NOX can induce neuronal dysfunction. NOX1, NOX2, and NOX4 are expressed in neurons (138-141). In fact, NOX1 and NOX2 have been shown to be expressed in dopaminergic neurons from PD patients (20,142). Postmortem substantia nigra tissue from PD patients showed higher NOX2 expression than individuals without PD (133). In several PD experimental models (e.g. MPTP, rotenone, and 6-OHDA toxins), including the N27 dopaminergic cell line, NOX1 and NOX2 can mediate dopaminergic neuronal death (20,22,143-149).

The N27 cell line expresses NOX1, NOX2, NOX4, Duox 1, and Duox 2 homologues of NOX (20), and this study confirms the presence of NOX1 and NOX2 in N27 cells. This study extends these findings by showing that NOX1 and NOX2 complex with AR45. To determine the role of NOX on mAR neurodegeneration, two widely used nonspecific small molecule NOX inhibitors,

apocynin and diphenyleneiodonium (DPI) were used (92,144,150,151), as both have shown neuroprotective benefits in neurodegenerative diseases (152-157). Although prior studies using DPI observed protection from 6-OHDA-induced oxidative stress in N27 cells (20), it was not appropriate for our studies as DPI was toxic to N27 cells. DPI toxicity has been observed in other cells such as N11 glial cells and cultured cells isolated from rat heart, whereby DPI blocked a major antioxidant pathway, resulting in increased oxidative stress and apoptosis (158,159). In contrast, apocynin proved to be non-toxic in this cell line, making it an ideal NOX inhibitor. This is consistent with publications reporting the effectiveness, yet low toxicity of apocynin in both *in vitro* and *in vivo* models (160,161). Prior studies have shown apocynin decreased NOX activation, inflammation, and apoptosis in experimental models of PD (134,162). Indeed, in this study apocynin blocked testosterone's neurodamaging effects in an oxidative stress environment, suggesting that both NOX1 and NOX2 are involved. Apocynin had no influence on H_2O_2 induced cell loss. This effect was not unexpected, as tert-butyl hydrogen peroxide exerts its effects on oxidative stress via two pathways: cytochrome P450 for peroxyl and alkoxyl radical production and glutathione peroxidase for lipid peroxidation (163). It is also important to note that sub-lethal concentrations of tert-butyl hydrogen peroxide that does not affect NOX protein expression were used in this study.

Apocynin's mechanism of action is controversial. It can act on NOX1 and NOX2 (164), as well as other flavoprotein enzymes (93,165,166). Since increased expression of NOX1, and not NOX2, has been shown in response to 6-OHDA and rotenone PD toxins in N27 cells (20,149), this study examined both NOX1 and NOX2. NOX1 was examined by using a NOX1 specific inhibitor, ML171, which does not affect NOX2 activity (95,167). ML171, an unsubstituted phenothiazine

has shown very high potency in blocking NOX1-dependent ROS generation (95). Contrary to observations with apocynin, this selective NOX1 inhibitor only partially inhibited testosterone's negative actions in an oxidative stress environment in N27 cells. These results indicate another NOX subunit is involved, such as NOX2 that also complexes with AR45. Indeed, selective NOX2 inhibition by GSK2795039 partially blocked testosterone induced cell loss in an oxidative stress environment. Although NOX2 is generally associated with astrocytes and microglia (16,133,168,169), NOX2 is expressed in dopaminergic neurons (20,142) and can influence oxidative stress (146). Unlike apocynin that is orally bioavailable (170-172), i*n vivo* use of NOX2 inhibitors is limited due to the poor oral bioavailability and the need for i.v. administration (167).

Since there has been a lack of success of mitigating mitochondrial-associated oxidative stress in clinical trials (173), NOX inhibitors may be potential therapeutics for PD. The NOX1, NOX4, NOX5 inhibitor, GKT137831, is orally bioavailable and well-tolerated in humans. Further, this is the only NOX inhibitor that has progressed into clinical trials with more than 170 patients (174,175). Apocynin has also been proposed as a possible therapeutic. Using the MPTP PD model, apocynin was shown to protect dopaminergic neurons and improve motor function in nonhuman primates (176). Although apocynin has not been tested in patients with neurodegeneration, it decreased oxidative stress production in asthmatic patients (177). These preclinical and clinical studies appear promising. Since NOX1 and NOX2 complexes with AR45, NOX inhibition may be a potential therapeutic to slow PD progression in men or in postmenopausal women that exhibit an androgenic hormone profile compared to premenopausal women (178,179).

In conclusion, this study provides evidence that 1) AR45 located in lipid rafts complexes with NOX1, NOX2, and $G\alpha_q$, 2) $G\alpha_q$ does not complex with NOX1 and NOX2, 3) androgens' negative effects are mediated via AR45 and NOX, 4) $G\alpha_q$ does not mediate testosterone's negative effects, and 5) $InsP_3R$ is involved in testosterone-induced neurodegeneration (Figure 8). The findings of this study help identify key players (e.g. mAR, NOX, and $InsP_3R$ mediated Ca^{++}) in testosterone-induced neurodegeneration, which could serve as potential therapeutic targets for PD.

Declaration of Interest

None

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Author contributions

Mavis Tenkorang has full access to all of the data in the study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

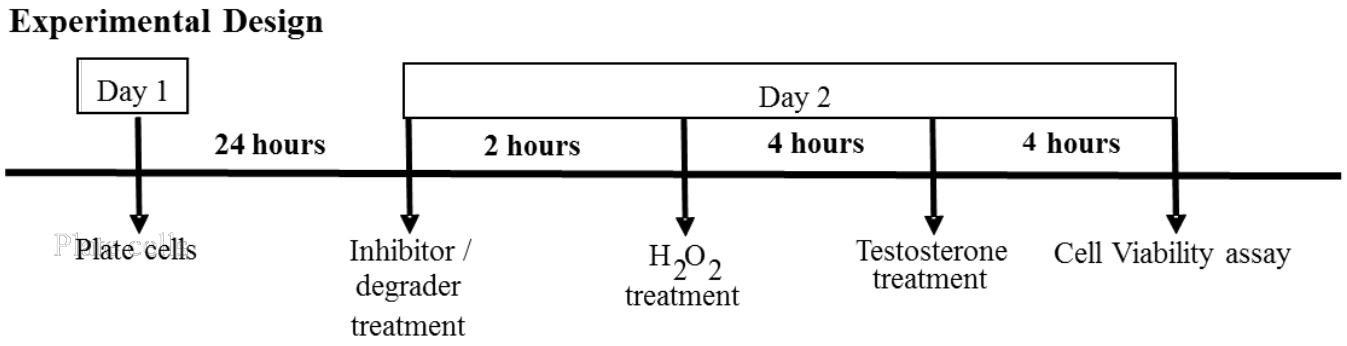


Figure 1: **Experimental design**. N27 cells were plated in 96-well cell culture plates and incubated at 37ºC in 5% CO_2 for 24 hours. 2-hour pretreatment was conducted for all inhibitors and AR degrader. At 80% confluency, cells were exposed to the oxidative stressor, H_2O_2, for 4 hours followed by testosterone treatment.

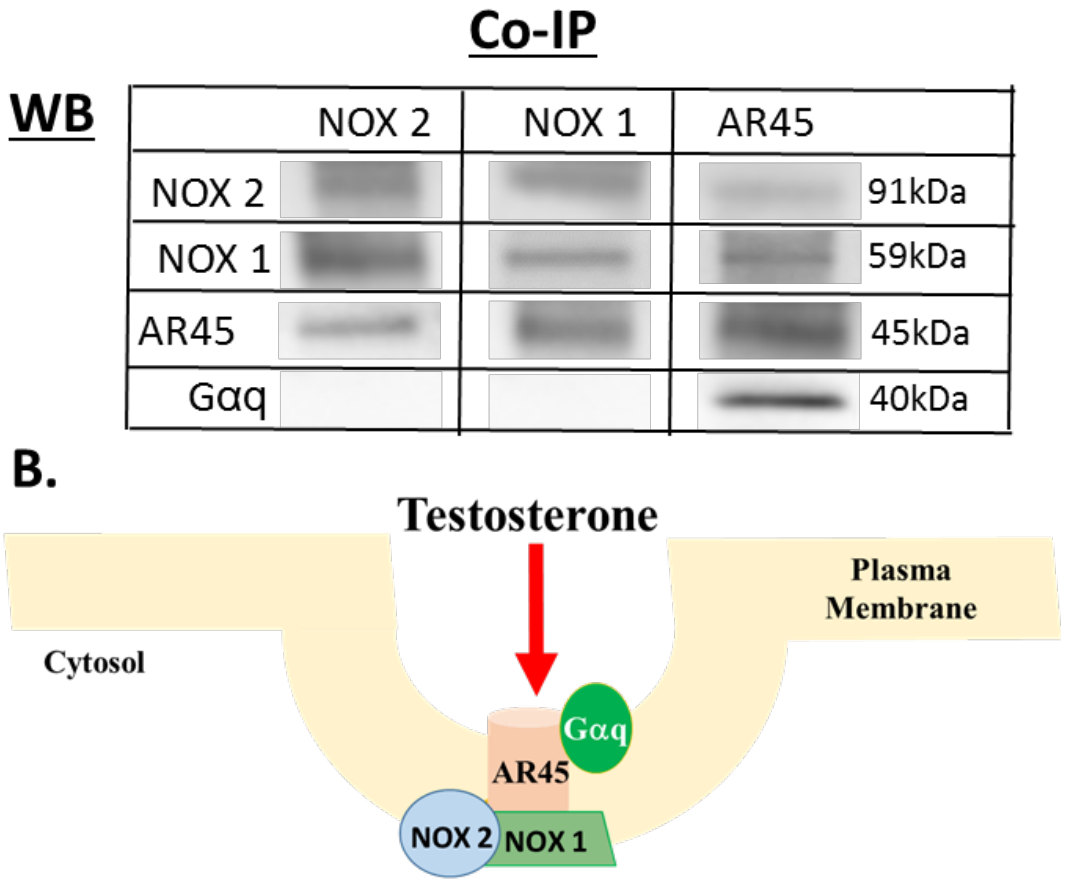
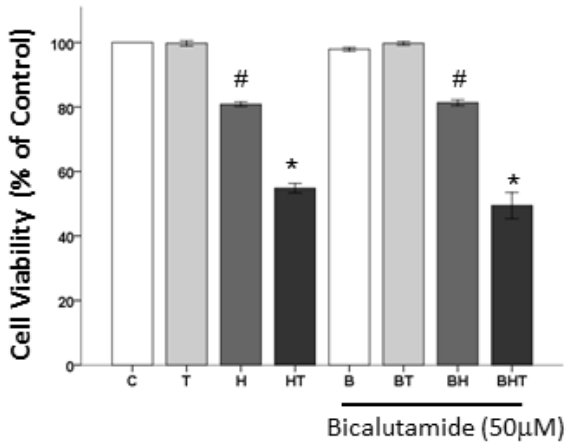
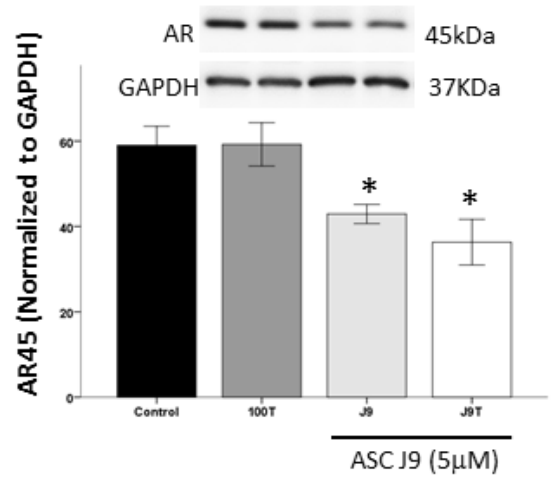


Figure 2: **Co-immunoprecipitation of AR45, NOX1, NOX2 to determine protein complex.** NOX1, NOX2, and AR45 proteins were precipitated using specific antibodies, and then probed with respective antibodies for NOX1, NOX2, and AR45 to determine protein-protein interactions. NOX1, NOX2, and AR45 interact to form a protein complex. $G\alpha_q$ couples with AR45 but does not interact with either NOX1 or NOX2 (A). Testosterone can bind to the mAR, AR45, and activate multiple signaling pathways via its protein interactions with NOX1, NOX2, and $G\alpha_q$ in a lipid raft (B). Co-IP, co-immunoprecipitation; WB, Western Blot.

A.


Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
*
C
T
H
HT
B
BT
BH
BHT
Bicalutamide (50μM)

B.


AR
45kDa
GAPDH
37KDa
AR45 (Normalized to GAPDH)
60
40
20
0
*
*
Control
100T
J9
J9T
ASC J9 (5μM)

C.

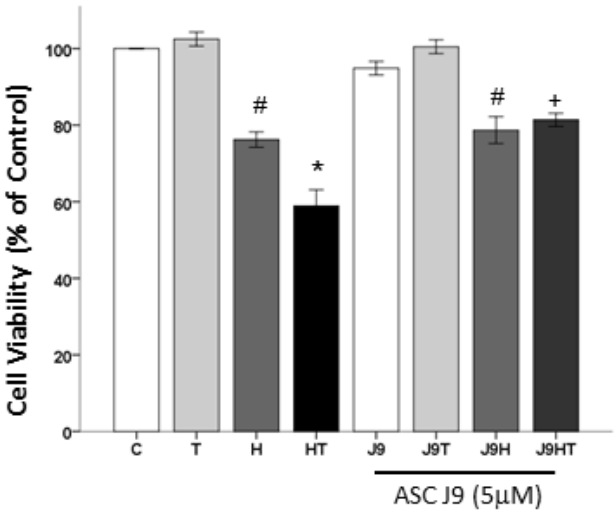

Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
+
C
T
H
HT
J9
J9T
J9H
J9HT
ASC J9 (5μM)

Figure 3: **Testosterone's detrimental effects are not mediated through the classical genomic pathway.** Testosterone, alone, does not affect cell viability. H_2O_2 induced ~ 20% cell loss, which was exacerbated by testosterone. The AR antagonist, bicalutamide, did not block testosterone's negative effects in an oxidative stress environment (A). Testosterone did not alter AR45 expression. The AR degrader, ASC J9, significantly decreased the expression of AR45, irrespective of the presence of testosterone (B). ASC J9 blocked testosterone-induced cell loss in an oxidative stress environment, but did not influence H_2O_2 induced cell loss (C). C, vehicle control; T, 100nM testosterone; H, H_2O_2; HT, post-treatment T; B, Bicalutamide; J9, ASC J9. Results were determined by ANOVA followed by Fisher LSD post hoc test. Results are reported as mean $\pm$ SEM. $p \leq 0.05$; *versus all groups, # versus control, + versus HT groups.

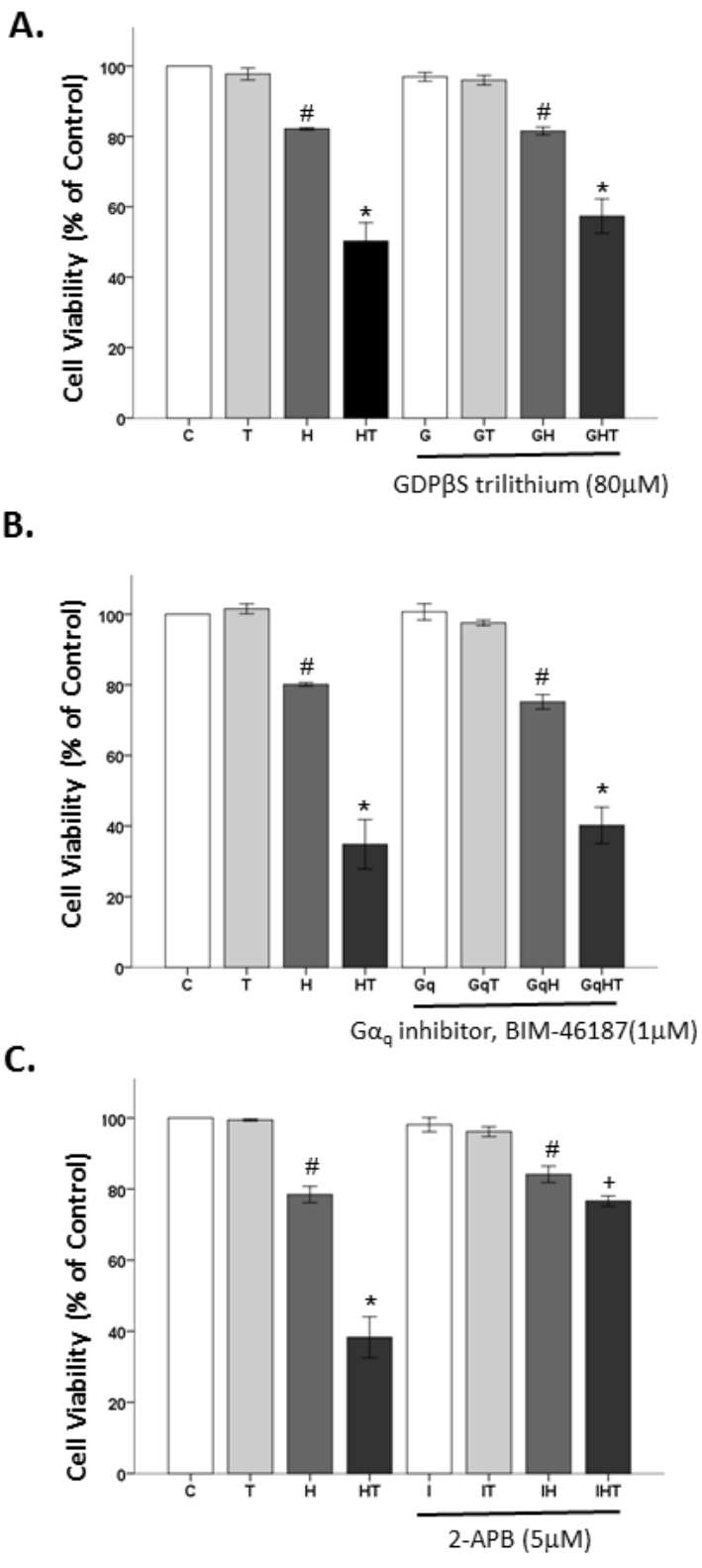

A.
Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
*
C
T
H
HT
G
GT
GH
GHT
GDPβS trilithium (80μM)
B.
Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
*
C
T
H
HT
Gq
GqT
GqH
GqHT
Gαq inhibitor, BIM-46187(1μM)
C.
Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
+
C
T
H
HT
I
IT
IH
IHT
2-APB (5μM)

Figure 4: **The role of G protein and $InsP_3R$ receptor in testosterone-induced neurodegeneration.** GDPβS trilithium, GDP analogue and BIM-46187, $G\alpha_q$ inhibitor, did not protect the cells from testosterone's detrimental effects in an oxidative stress environment (A, B). $InsP_3R$ inhibitor, 2-APB was able to block testosterone's damaging effects in an oxidative stress environment (C). C, vehicle control; T, 100nM testosterone; 500nm DHT-BSA; H, H_2O_2; HT, post-treatment T; G, GDPβS trilithium; Gq, BIM-46187; I, 2-APB. Results were determined by ANOVA followed by Fisher LSD post hoc test. Results are reported as mean $\pm$ SEM. $p \leq 0.05$; * versus all groups, # versus control, + versus HT groups.

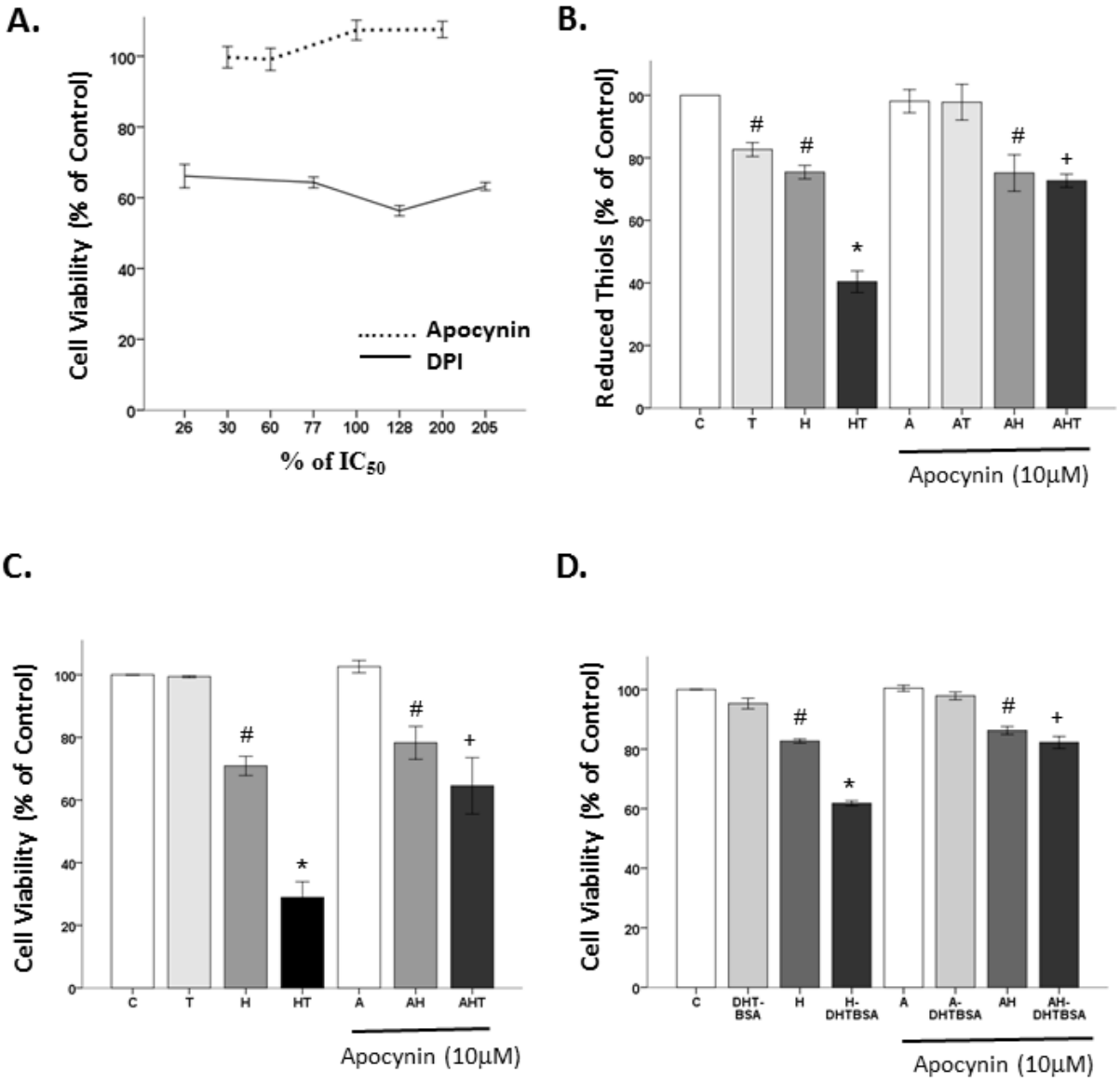

A.
Cell Viability (% of Control)
100
80
60
40
20
0
26
30
60
77
100
128
200
205
% of IC50
Apocynin
DPI
B.
Reduced Thiols (% of Control)
C
T
H
HT
A
AT
AH
AHT
Apocynin (10µM)
C.
Cell Viability (% of Control)
Apocynin (10µM)
D.
Cell Viability (% of Control)
DHT-BSA
H-DHTBSA
A-DHTBSA
AH-DHTBSA
Apocynin (10µM)

Figure 5: **The effects of non-specific NOX inhibitor on oxidative stress generation and cell loss.** DPI was toxic to N27 cells, regardless of the concentration used. Apocynin did not show toxicity (A). Testosterone, alone, increased oxidative stress generation, as evidenced by decreased reduced thiols. Apocynin blocked testosterone's effects on oxidative stress generation (B). Testosterone, alone, had no effect on cell viability. Apocynin did not protect cells from H_2O_2's effects. Apocynin blocked testosterone induced cell loss in an oxidative stress environment (C). Similarly, apocynin blocked DHT-BSA exacerbation of H_2O_2 induced cell loss (D). C, vehicle control; T, 100nM testosterone; 500nm DHT-BSA, H, H_2O_2; HT, post-treatment testosterone; A, apocynin. Results were determined by ANOVA followed by Fisher LSD post hoc test. Results are reported as mean $\pm$ SEM. $p \leq 0.05$; * versus all groups, # versus control, + versus HT groups.

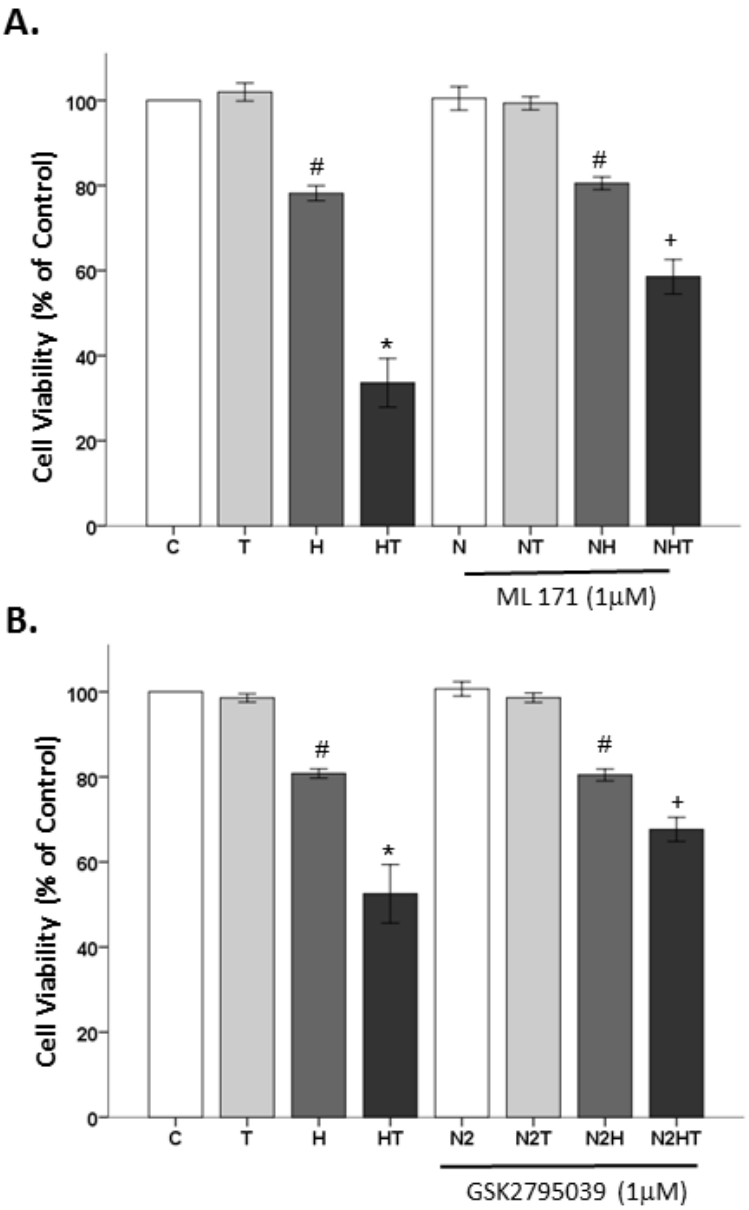
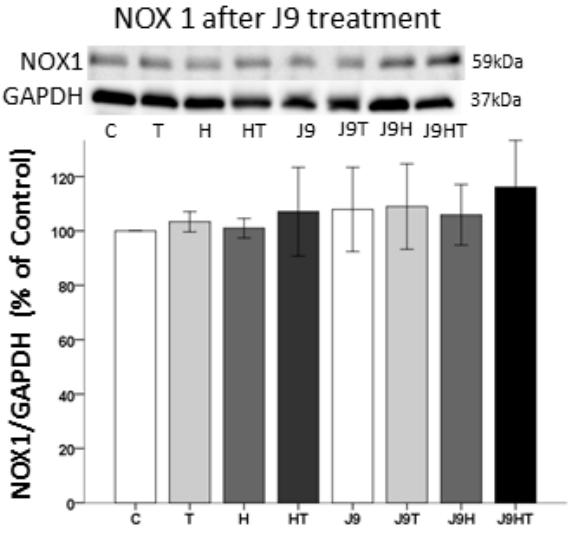

A.
Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
+
C
T
H
HT
N
NT
NH
NHT
ML 171 (1μM)
B.
Cell Viability (% of Control)
100
80
60
40
20
0
#
*
#
+
C
T
H
HT
N2
N2T
N2H
N2HT
GSK2795039 (1μM)

Figure 6: **The effects of selective NOX inhibitors on testosterone-induced cell loss.** Selective NOX1 inhibitor, ML171, had no effect on cell viability. H_2O_2-induced cell loss was not blocked by ML171. ML171 partially blocked testosterone induced cell loss in the presence of oxidative stress (A). Selective NOX2 inhibitor, GSK2795039, had no effect on cell viability. H_2O_2-induced cell loss was not blocked by GSK2795039. GSK2795039 partially blocked testosterone induced cell loss in the presence of oxidative stress (B). C, vehicle control; T, 100nM testosterone; 500nm DHT-BSA, H, H_2O_2; HT, post-treatment T; N, ML 171; N2, GSK2795039. Results were determined by ANOVA followed by Fisher LSD post hoc test. Results are reported as mean $\pm$ SEM. $p \leq 0.05$; * versus all groups, # versus control, + versus HT groups.

A.


NOX 1 after J9 treatment
NOX1
GAPDH
59kDa
37kDa
C T H HT J9 J9T J9H J9HT
NOX1/GAPDH (% of Control)
0 20 40 60 80 100 120
C T H HT J9 J9T J9H J9HT

B.

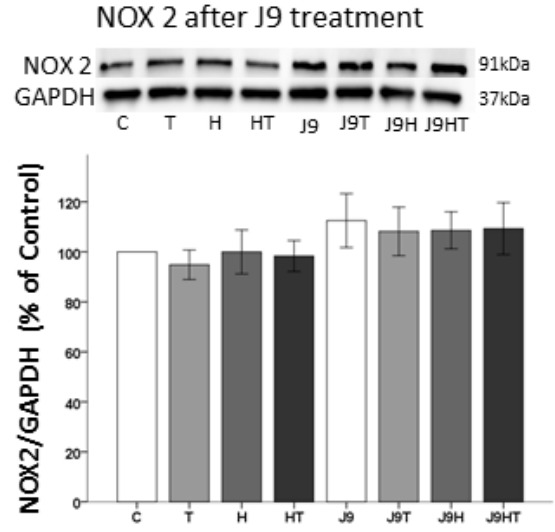

NOX 2 after J9 treatment
NOX 2
GAPDH
91kDa
37kDa
C T H HT J9 J9T J9H J9HT
NOX2/GAPDH (% of Control)
0 20 40 60 80 100 120
C T H HT J9 J9T J9H J9HT

Figure 7: **The effects of AR degradation on NOX1 and NOX2 protein expression.** Degrading the AR45, using ASC J9, did not affect NOX1 or NOX2 expression. Data were expressed as a normalized ratio of protein band density of NOX1 (A) and NOX2 (B) against GAPDH, and presented as mean ± standard deviation. Results were determined by ANOVA followed by Fisher LSD post hoc test. C, vehicle control; T, 100nM testosterone; H, H_2O_2; HT, post-treatment T; J9, ASC J9. Results are reported as mean $\pm$ SEM. $p \leq 0.05$.

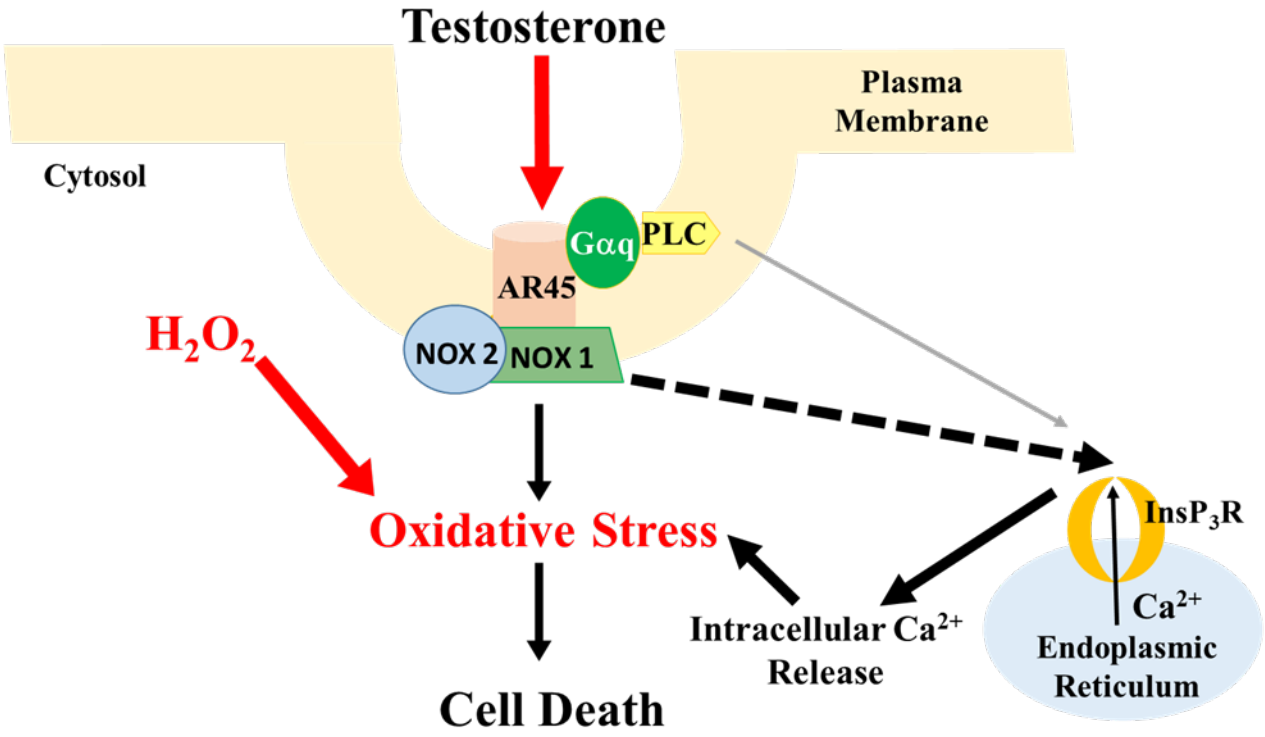


Figure 8: **Working Model.** Membrane androgen receptor (i.e. AR45) resides in a lipid raft within the plasma membrane and complexes with NOX1, NOX2, and $G\alpha_q$. Testosterone can bind and activate AR45, which in turn stimulates NOX1 and NOX2, resulting in oxidative stress generation. Alternatively, the AR45-NOX complex can upregulate $InsP_3R$ activity to increase intracellular calcium release, resulting in increased oxidative stress. Dysregulation of this pathway can lead to neurotoxicity, such as during PD. The AR45-$G\alpha_q$ pathway is not involved in testosterone induced cell loss in oxidative stress environments.

CHAPTER 4

SUMMARY DISCUSSION

PD has been commonly described as the progressive loss of dopaminergic neurons in the substantia nigra pars compacta of the brain. Early PD diagnosis is a huge challenge because the observable motor symptoms appear when 80-90% of dopaminergic neurons are lost, resulting in significant disease progression (1). Oxidative stress is one of the major characteristics of PD. Multiple variables can influence oxidative stress generation, such as α-synuclein. Aggregates of α-synuclein proteins, known as Lewy bodies, are key histopathological feature of PD (2). There have been several reports on mutations in the α-synuclein gene, hence its implication in PD (3). Strikingly, such mutations in α-synuclein gene trigger free radical accumulation in yeast cells by causing mitochondrial dysfunction. Potentially, α-synuclein aggregates make holes in the outer wall of the mitochondria, causing a leakage of ROS into the cytoplasm (4). There is still ongoing research on α-synuclein protein as potential target in PD pathology (5). Another contributor to oxidative stress generation is androgens, such as testosterone and dihydrotestosterone. Androgen-induced oxidative stress may be an important contributor underlying the observed male-sex bias in PD. Therefore, it is essential to study androgen pathways.

Until recently, molecular mechanisms of androgens centered on classical androgen receptors, gene transcription, and protein synthesis. Discovery of the fast non-genomic actions of androgens has paved way for more membrane androgen receptor (mAR) -focused research to be conducted in the endocrine field (6). Although the genomic pathway of androgens is very well understood,

researchers are still gaining insights into the novel non-classical action of androgens, especially in the central nervous system. Nonetheless, extensive research has been conducted on mAR in the field of prostate cancer. Indeed, current studies in prostate cancer have demonstrated the involvement of the non-genomic pathway of androgens in disease progression (7). Several variants of the androgen receptor (AR) have been identified in human prostate tissue: 1) a novel mAR, ZIP9, which also functions as is a zinc transporter protein and can induce apoptotic signaling pathways (8-10), 2) AR-V7 that lacks the C-terminal ligand-binding domain because of alternative splicing of the AR mRNA, 3) AR-V9 (commonly coexpressed with AR-V7), which was obtained by contiguous splicing of AR exons 1/2/3, exerts its activity independent of ligand binding and can promote resistance to drugs targeting AR (11-13), and 4) AR45, an naturally occurring AR splice variant, first identified in human placenta tissue via 5′ Rapid amplification of cDNA ends (RACE). This AR45 consists of a DNA binding domain, a hinge region, and a ligand-binding domain, but is missing the entire region encoded by exon 1 of the AR gene. In this same study, tissues from the skeletal muscle, heart, prostate, lungs and breast were found to strongly express AR45 mRNA. However, there was a weaker AR45 mRNA signal in the testis (14). One purported role of AR45 in prostate cancer cells (e.g. LNCaP cells) is its ability to negatively regulate AR signaling by inhibiting the activity of full length AR after nuclear translocation. Upregulation of AR45 in these cells inhibited proliferation (13).

Unlike estrogen receptor splice variants that have been reported in the central nervous system (15-18) androgen receptor variants were not observed until 2017 when our lab identified the presence of putative AR45 in plasma membrane lipid rafts (membrane signaling hubs) within the rat brain regions of the substantia nigra, entorhinal cortex, and hippocampus (19). Interestingly, AR45 was

not co-expressed with full length AR in the substantia nigra, unlike its expression profile in the prostate (19,20). Therefore, AR45's function in the brain may be different from its function in the prostate. Indeed, using a N27 cell line that only expresses AR45; we were able to determine that AR45 can induce oxidative stress generation. Specifically, AR45 increases oxidative stress generation by complexing with NOX 1 and NOX 2, major oxidative stress generators in cells. Androgens via this AR45/NOX complex may induce a feed-forward cycle to further increase oxidative stress generation that could lead to the loss of dopaminergic neurons in the substantia nigra, and thus increased risk for PD in men or even post-menopausal women and pre-menopausal women with polycystic ovarian syndrome, who exhibit an androgenic phenotype (i.e. low estrogens and normal androgens).

CLINICAL IMPLICATIONS

Current medications used in the treatment of PD include levodopa in combination with benserazide or carbidopa, monoamine oxidase (MAO) B inhibitors (eg. selegiline), dopamine agonists (eg. bromocriptine, pergolide, apomorphine and the newer agent cabergoline), amantadine, anticholinergics (eg. benztropine, procyclidine) and Catechol O-Methyl Transferase, COMT Inhibitors (eg. Tolcapone, entacapone) (21,22). Although, these medications especially levodopa have proven to be very potent in PD treatment (23-26), adverse effects from prolonged use cannot be over emphasized. Products from levodopa metabolism may speed up neuronal degeneration, thereby worsening the condition (27). Amantadine has demonstrated effectiveness in reducing levodopa-induced dyskinesia (uncontrolled involuntary movement), as well as delay the onset of dementia in PD patients (28-30). Nonetheless, it significantly causes untoward effects such as hallucinations

and insomnia (26,31). COMT inhibitors are also known to cause dyskinesia (21). There are reports that tolcapone, in particular, induces hepatotoxicity in both *in vitro* and *in vivo* models (32,33).

Although men are more prone to PD than women are, there is little or no sex differences in PD progression (34,35). Sex differences in PD treatment is equivocal. One study recommended a decrease in the dosage of levodopa medication prescribed for women, as they tend to slowly metabolize the drug. Further, women experienced higher rate of levodopa-induced dyskinesia than their male counterparts (36). Another study revealed no sex differences in the use of early PD medications (37). Interestingly, usage of non-aspirin nonsteroidal anti-inflammatory drugs have been associated with decreased PD risk, whereas aspirin usage has been associated with increased PD risk in men but not women (38). However, other studies have found no effects of anti-inflammatory drugs on PD risk (39,40). Regardless, more information about sex-specific management of PD could be potentially vital.

Currently, several research studies, including ours, are geared towards identifying alternative pharmacological approaches with improved side effects profiles of current PD therapy. Lately, attention has been focused more on strategies to protect dopaminergic neurons in order to halt PD disease progression. Selegiline (MAO-B inhibitor) and amantadine (antiviral agent with mild antiparkinsonian activity) have been postulated to be neuroprotective by potentially decreasing ROS generated from dopamine metabolism (27,41-44). In particular, selegiline has been reported to be the most successful PD therapy, due to its neurotrophic factor-like effects on dopaminergic neurons, thereby slowing the progression of the disease (45).

At present, PD treatment is focused on symptom management instead of treating the disease itself (46). Following reports that oxidative stress is critical in PD pathology, antioxidants have been used in both *in vitro* and *in vivo* research studies and have shown promising results (47). Coenzyme Q10, a naturally occurring potent antioxidants that is primarily in the mitochondria, was reported to decrease dopaminergic neuronal loss in mice treated with the dopaminergic neurotoxin MPTP (48). However, antioxidant use in clinical research has been unsuccessful or inconclusive (48,49). For example, Tocopherol, a lipid soluble antioxidant, in a controlled clinical trial showed no beneficial effects in ameliorating PD symptoms. It could be that tocopherol is less effective in scavenging free radicals (50). Perhaps, pharmacological inhibition, rather than free radical scavenging, may be a more effective approach. Since NOX and mAR have been identified as key players in mediating testosterone's negative effects on oxidative stress generation, they could be potential therapeutic targets in PD treatment. The NOX inhibitor, apocynin, is readily available on the market for the management of asthma, due to its anti-inflammatory properties (51-53). Apocynin is orally bioavailable (54,55) and can cross the blood brain barrier (56). Hence, it can be retooled for PD therapy.

This dissertation presented findings on mAR mediated oxidative stress generation in N27 cell line. Interfering with this pathway could translate into a neuroprotective strategy for PD. Currently, there are not specific mAR antagonists. However, if a pharmacotherapy targeting the mAR was designed, patient compliance would increase as there would be limited untoward effects such as erectile dysfunction that is usually associated with classical AR antagonists (57,58). Furthermore, a mAR specific antagonist will allow the use of testosterone replacement therapy to improve PD mood and motor symptoms by acting on ARs in the muscles to induce anabolic effects.

FUTURE STUDIES

We have been able to identify neural pathways underlying androgen-induced neurodegeneration, using N27 cells. The N27 dopaminergic cell line is tyrosine hydroxylase positive (TH^+). This enzyme is key in the rate-limiting step in dopamine synthesis. Therefore, its presence in this cell line is indicative of dopaminergic neurons (59,60). In addition, N27 cells express AR45, which was found to be localized in the plasma membrane, coupled with NOX1/2 and Gαq. These characteristics of N27 cell line make it a good model for the experiments in this dissertation project. However, one universal limitation of using *in vitro* cell culture model is the exclusion of the local environment of the cells, such as the cells' interactions with other cell types. This could potentially affect the hypotheses tested. Our future studies will therefore use animal models to confirm our findings. We plan to use chronic intermittent hypoxia (CIH) as our oxidative stressor. The CIH protocol is composed of repeated, alternate periods of normoxia and hypoxia, which mimics sleep disturbances commonly observed in PD (61-64). Prior studies by our lab used this particular CIH model, with apnea-hypopnea index (AHI) of 10, to induce mild hypoxia in Long Evans male rats (65,66). Consistent with other publications, there was significant rise in oxidative stress in the substantia nigra, entorhinal cortex, and dorsal hippocampus, indicative of early stage neurodegenerative diseases (66). Along with increased oxidative stress, increased circulating proinflammatory markers were observed in rats subjected to CIH (65,67).

Androgens' contribution to CIH-induced oxidative stress response and inflammation have also been observed in our lab. Notably, CIH significantly increased oxidative stress in key brain regions

associated with neurodegenerative diseases in gonadectomized rat receiving androgen supplementation (66). This demonstrates the synergistic impact of androgens and CIH on oxidative stress pathways. Given that NOX plays a critical role in mediating androgens' negative effects in N27cells (68), it would be interesting to examine the influence of androgens and CIH on NOX activity and levels of inflammatory cytokines in both the plasma and specific brain regions (figure 1). It is possible that AR45-NOX complex activates a COX2 inflammatory cascade based on our prior study showing COX2 mediates testosterone induced oxidative stress and apoptosis (69). This would indicate that the AR45-NOX complex is upstream of COX2 signaling. Thus COX2 may be another mechanism underlying the feed-forward regulation of AR45-NOX on oxidative stress generation, especially as it is known that inflammation can exacerbate oxidative stress generation (70,71).

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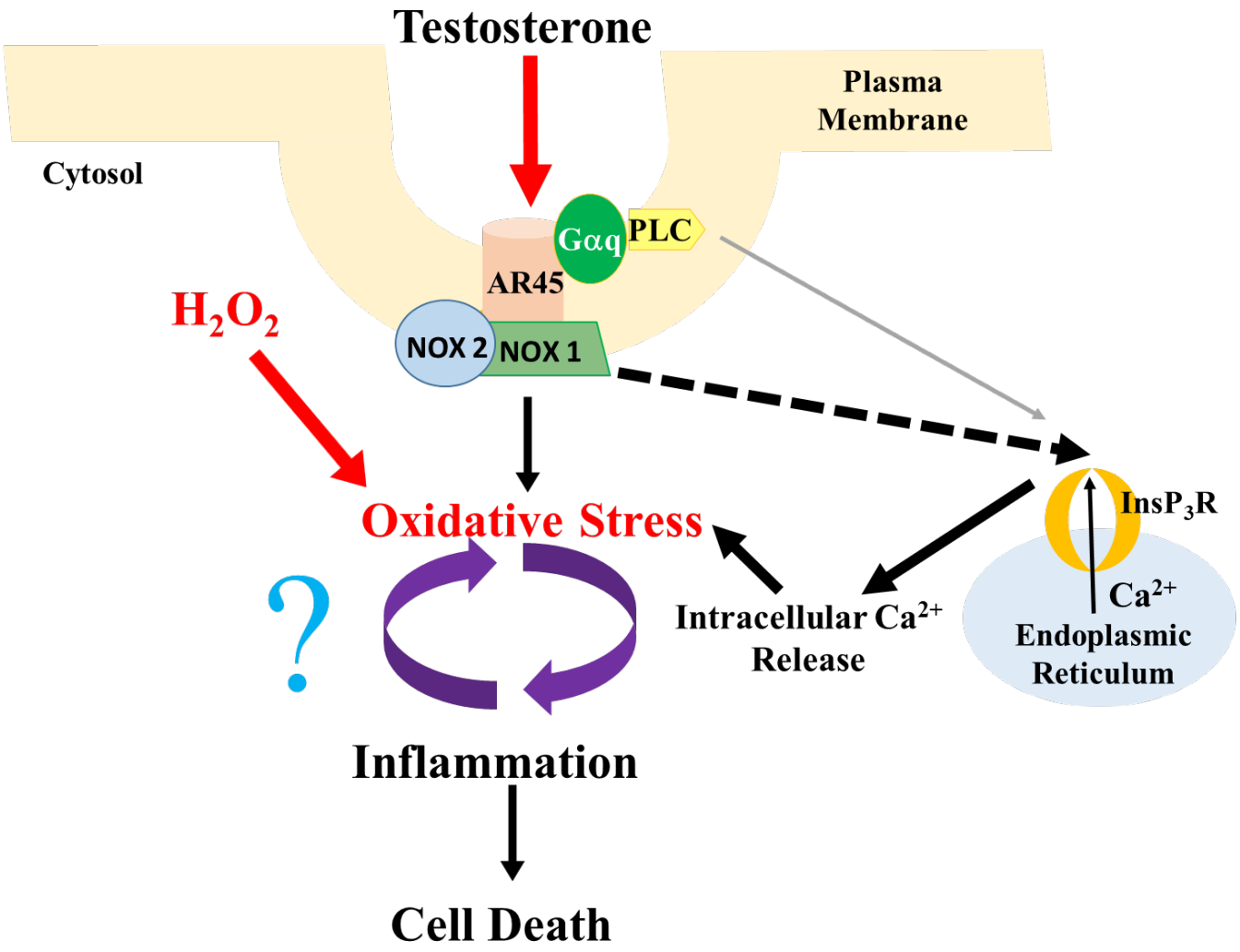

Figure 1. Feed forward regulation of membrane androgen receptor/NOX complex on oxidative stress generation. Membrane androgen receptor/NOX complex mediates the interdependence between oxidative stress and inflammation, leading to cell death. Modified from Tenkorang et al (68).