NOVEL ANDROGEN RECEPTOR SPLICE VARIANT IN THE SUBSTANTIA NIGRA

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Jo Garza Contreras, B.S.

Fort Worth, Texas

June 2017

ACKNOWLEDGEMENTS

I would like to thank Dr. Rebecca Cunningham for allowing me to join her lab. She has been extremely patient and understanding throughout my graduate school journey. I value her guidance and wisdom, and I will take that with me on my future journey. I would also like to thank my lab members who have been supportive and helpful throughout this project. This project would not have been possible without their help, and I am truly grateful for all they have done for me. Furthermore, I appreciate Dr. Tom Cunningham for being so kind and providing his time and equipment to our lab.

I would also like to thank Dr. Derek Schreihofer for his advice and guidance on this project. I also thank Dr. Schreihofer for allowing me to learn new skills and teach them to other lab members. I have found that teaching others has given me great joy and I look forward to passing my knowledge down to future scientists. I also thank my committee members Dr. Salvatore and Dr. Basha for their time and commitment to me.

Lastly, I would like to thank my mother Cruz for being my rock throughout this rollercoaster ride. She has been incredibly supportive and understanding, especially when I was too busy to be with family. My family kept me going forward during the tough times and my friends kept my head above water when things were tough. I appreciate all those who were there for me and supported me on my journey to graduate school, through graduate school and beyond.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

CHAPTER II

LIST OF ABBREVIATIONS

ABP	Androgen Binding Protein
AF2	Activation Function 2 Region
AR	Androgen Receptor
ARE	Androgen Response Element
DBD	DNA Binding Domain
DHT	5α-Dihydrotestosterone
FL-AR	Full-length Androgen Receptor
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GPCR	G-protein Coupled Receptor
HAT	Histone Acetyltransferase
HPG	Hypothalamic Pituitary Gonadal
HSP	Heat Shock Proteins
LBD	Ligand Binding Domain
LH	Luteinizing Hormone
LNCaP	Human Prostate Carcinoma Cells
mAR	Membrane Androgen Receptor
NR3C	Nuclear Receptor subfamily 3 group C
NTD	N-Terminal Domain
PD	Parkinson's Disease
ROS	Reactive Oxygen Species
SHBG	Sex Hormone Binding Globulin
SN	Substantia Nigra
SRY	Sex Determining Region
StAR	Steroidogenic Acute Regulatory Protein
TAU	Transcription Activation Unit
T-BSA	Testosterone Bound to Bovine Serum Albumin
TH	Tyrosine Hydroxylase

CHAPTER I

INTRODUCTION AND BACKGROUND

1.1 STEROID HORMONES

Steroid hormones are a class of hormones that act as signaling molecules to function in maintaining homeostasis in the body by regulating physiological systems. Steroid hormones are classified into two groups: corticosteroids and sex hormones. Corticosteroids are synthesized in the adrenal cortex, whereas sex hormones are synthesized in the gonads. These hormones are further classified by the type of receptor they bind. Corticosteroids bind to either the mineralocorticoid or glucocorticoid receptors. Androgen, estrogen and progesterone bind to sex hormone receptors [1]. These receptors belong to a superfamily of nuclear receptors, which act as transcription factors. There are 48 receptors categorized into six subfamilies and grouped by similarities of their conserved DNA and ligand binding regions. Steroid hormone receptors are classified as nuclear receptor, subfamily 3, group C (NR3C) [2].

Steroid hormones are derived from cholesterol, a lipophilic molecule that is insoluble in water. Cholesterol can be synthesized locally from acetate when it is then shuttled into the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein. A multistep process is initiated by desmolase, a P450 enzyme that cleaves the side chain of cholesterol to transform it into pregnenolone. Desmolase and the StAR protein are both rate limiting step enzymes [3, 4]. Pregnenolone is the precursor molecule of all steroid molecules and conversion into a specific hormone depends on the enzyme catalyzing the molecule. Upon synthesis, the

steroid hormones are not stored, rather they immediately diffuse out of the cell and act locally or enter the bloodstream. Steroid hormones traveling via the bloodstream require carrier molecules due to their hydrophobic nature [3].

1.2 ANDROGEN PHYSIOLOGY

Androgens, such as testosterone and DHT, are the primary sex hormones that regulate an extensive range of physiological processes in males, such as sexual differentiation, spermatogenesis, and the maintenance of secondary sex characteristics. Testosterone and its metabolite 5α-dihydrotestosterone (DHT) play separate roles in the formation of the male sex organs. Testosterone initiates the development of the vas deferens, seminal vesicles, ejaculatory duct, and epididymis. Development of the penis, scrotum, urethra and prostate gland are controlled by DHT [5]. During embryogenesis, testes formation is initiated by the expression of the SRY gene on the Y chromosome [4, 6]. The testis is primarily composed of seminiferous tubules comprised of Sertoli cells. The interstitial space of the testis contains Leydig cells, which synthesize testosterone. Phenotypic sexual differentiation occurs when Sertoli cells release anti-Müllerian hormone to prevent the development of female genitalia [4, 5, 7].

Puberty in males begins when hypothalamic neurons release gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system in a pulsatile fashion to the anterior pituitary. GnRH stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream [8]. LH and FSH stimulate the increase of cholesterol and its transport into the mitochondria where it will be converted to pregnenolone. LH binding to the Leydig cells signals testosterone production [3, 4]. Sertoli cells promote the synthesis of androgen-binding protein (ABP) when stimulated by FSH. Testosterone bound to ABP acts locally on Sertoli

cells to initiate spermatogenesis [8, 9].

Testicular androgens account for about 90-95% of all androgens, and the remaining androgens are produced by the adrenal glands in the zona reticularis [8]. Testosterone and its bioactive metabolites DHT and estrogen can be synthesized locally or secreted into the periphery to act on androgen-sensitive cells. The irreversible enzymatic conversion of testosterone to DHT is catalyzed by 5α-reductase. Also, testosterone can be converted by aromatase, which facilitates the metabolism of testosterone into estradiol [8].

Circulating testosterone can either be bound or free. Due to its lipophilic nature, testosterone must be bound to a carrier protein such as albumin, sex hormone binding globulin (SHBG) or androgen binding protein (ABP). The binding of testosterone to a carrier protein renders it inactive because it is prevented from reaching its target cell and binding to its receptor. Free testosterone can enter the target cell freely because it is not bound to a carrier protein. Free testosterone is considered biologically active, and it represents only 2% of the total testosterone [3]. Total testosterone includes both free and bound hormone. In human males, the total testosterone level is between 270-1070 ng/dL [10].

Testosterone levels are maintained by metabolic inactivation and negative feedback. Testosterone is primarily inactivated in the liver by phase I and phase II metabolism. These inactivated metabolites are then excreted via the urinary or biliary system [3]. When testosterone levels are sufficient, production is regulated via a negative feedback loop. Testosterone inhibits the production of GnRH by binding to androgen receptors in the hypothalamic neurons. GnRH pulsing into the anterior pituitary is decreased which causes a decrease in LH. Inhibin, produced by the Sertoli cells, functions solely in down-regulating FSH production [3, 11]. Regulating testosterone levels is vital for the maintenance of the secondary sex characteristics [11].

1.3 CLASSICAL ANDROGEN RECEPTOR

Androgens mediate their effects via the androgen receptor (AR or NR3C4) [2].Testosterone and DHT are capable of binding to the same receptor. However, testosterone dissociates from the receptor at a faster rate compared to DHT. The binding affinity of DHT to a receptor is significantly stronger, and therefore, DHT is more potent as an agonist than testosterone [12-14]. The DHT-receptor complex stability allows the complex to persist longer in the cell [15].

In the late 1980's, several research groups cloned the human AR [16-19]. Cloning of the human AR was made possible due to the conserved homology and groundbreaking cloning of the human glucocorticoid receptor. The AR gene is located on the X chromosome at Xq11.2-q12 and spans approximately 90kb of DNA [16, 20]. The AR is the only nuclear receptor located on the X chromosome. The AR gene contains eight exons which code for a protein with three major functional domains and a hinge region (Figure 1). Exon 1 is located at the 5', N-terminal end. The N-terminal domain (NTD) is the least conserved region and contains multiple trinucleotide repeats. Tau-1 and Tau-5 (transcription activation unit) are regions in the NTD that are important for transcription activation [21]. Depending on the cell, corepressors and or coactivators bind to the NTD to influence transcription [22, 23]. The DNA binding domain (DBD) located in exons 2 and 3 contains two zinc fingers. The zinc fingers and the hinge region are involved in high-affinity DNA binding at the androgen response element (ARE) in target promoters [23]. The ARE is a 15 base pair palindromic sequence that is selective for the androgen receptor [24]. The DBD is the most conserved region and shares approximately 80 percent homology with the progesterone receptor [17, 19]. The hinge region located in exon 4 is poorly conserved yet contains a nuclear localization sequence similar to other nuclear receptors [23]. Lastly, exons five through eight code

for the ligand binding domain (LBD) followed by the C- terminal 3' end [16, 20, 25-27].A cleft in the ligand binding domain (LBD) is known as the Activation Function 2 (AF2) region [23]. Ligand binding to the receptor activates the protein to initiate transcription [28]. An androgen receptor that does not have a functional LBD is rendered constitutively active and can lead to a diseased state [29].

The human AR protein is 919 amino acid residues. However, this number varies due to polymorphic polyglutamine and polyglycine residues in the N-terminal domain (NTD) [30-32]. Biologically active androgens quickly traverse the plasma membrane due to its lipophilic nature [3]. Upon entry into the cell, testosterone binding to the receptor occurs either immediately or after conversion to DHT [24]. In the cytoplasm, unbound AR is bound to chaperone molecules, such as heat shock proteins (HSP). Upon androgen binding, the AR undergoes a conformational change, and the heat shock proteins are released [24, 33]. This AR complex is then shuttled into the nucleus and dimerizes with another AR complex [24]. Binding of AR homodimer complex to the androgen response element promotes the recruitment of co-activators which facilitate transcription [24]. SRC1 and TIF 2 are coactivators that bind to the AF2 region to promote transcription [34].Histone acetyltransferase (HAT) initiates the unraveling of the nucleosome. The exposed DNA allows RNA polymerase II enhanced transcription initiation [21, 35]. When the AR no longer binds its ligand, it can either be shuttled back into the cytoplasm for reuse or degraded by proteasomes [36].

The AR structure, stability, and activity are achieved by post-translational modification. AR modification occurs via phosphorylation, acetylation, methylation, SUMOylation, and ubiquitination. Each AR domain is capable of being phosphorylated. Most phosphorylated sites function in transcription activity. However, phosphorylation can function to stabilize and localize the AR. Acetylation and methylation sites on the DBD, hinge, and LBD domains function in

transcription activity. SUMOylation of the N-terminal domain functions in transcriptional activity and stabilization of the AR. Ubiquitination of Lys845 and Lys 847 on the LBD promote the degradation of the AR by proteasomes. These post-translational modifications are important in the maintaining the life cycle of the AR [37].

Androgen receptors are nearly ubiquitously expressed throughout mammalian tissues [38]. However, in the brain, there is region specific-expression. For example, hypothalamic neurons express substantial amounts of AR-positive cells, due to androgen regulation [39]. Other midbrain areas, such as the substantia nigra and ventral tegmentum, express few AR-positive cells when using an N-terminal directed antibody [40]. Studies in our lab show when a C-terminal antibody is used to detect AR expression in dopaminergic cells we see high AR expression [41]. It is likely these differences in expression may be due to a splice variant.

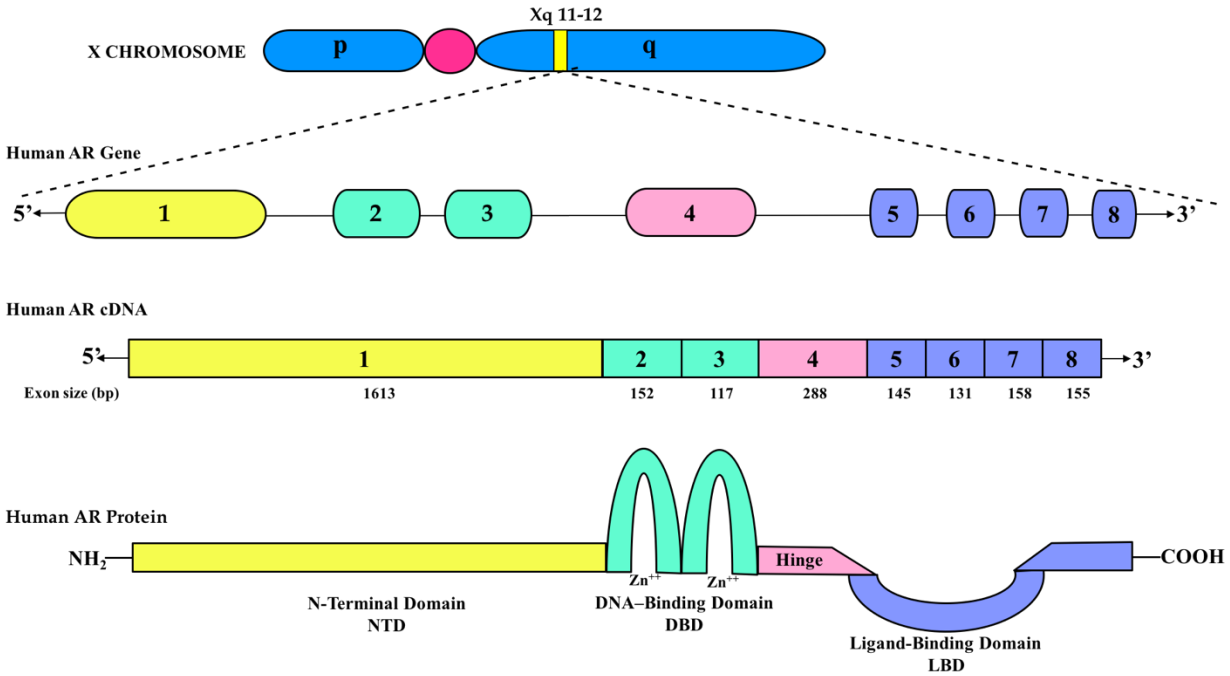

FIGURE 1. Androgen Receptor Gene, cDNA, And Protein.

The human androgen receptor gene is mapped to the X chromosome (Xq 11-12). The AR contains 4 domains encoded by 8 exons. The N-terminal domain (NTD) is derived from exon 1. The DNA-binding region (DBD) including 2 zinc fingers is encoded from exons 2 and 3. Exons 4-8 encode the hinge region, and ligand-binding domain (LBD) at the C-terminal end. The classical AR is a 110 kDa protein composed of 919 amino acids. Adapted from Galani et al.[42] and Quigley et al. [33]

1.4 ANDROGEN RECEPTOR VARIANTS

In 2005, a novel, naturally occurring, transcript variant was identified in human placenta tissue [43]. Approximately 22.1kb downstream the canonical exon 1 is a previously unreported exon, exon 1b. Alternative splicing of exon 1b yielded a 45 kDa protein. Due to its molecular weight, the novel variant was dubbed AR45 [44]. Exon 1b produces a unique seven amino acid N-terminus. The DNA binding domain, hinge region, steroid/ligand binding domain, and C-terminal region of AR45 are identical to the classical AR [43, 45]. AR45 mRNA has been identified in several human tissues such as placenta, prostate, breast, heart and lung tissues [46]. AR45 mRNA has also been observed in the macaque heart, skeletal muscle and lung [47]. The highest expression of AR45 mRNA was in the heart, with no expression in the brain [43, 47]. AR45 protein was not observed in those tissues but was observed in the LNCaP human prostate cancer cell line [43]. Ectopic expression of the AR45 protein was capable of binding to androgens, translocating to the nucleus and interacting with the FL-AR. Transcriptional activity of FL-AR was inhibited by AR45. Also, overexpression of AR45 in LNCaP cells inhibited cell proliferation. These data suggest AR45 is a negative regulator FL-AR signaling [29, 43].

AR variants AR-V3, AR-V4, AR-V7 and AR V-12, have been observed in breast and prostate cancer cells [48]. Alternative splicing, exon skipping, or cryptic exon inclusion are mechanisms which these variants are generated [49] These variants contain a complete N-terminus and DBD but lack the LBD domain. An N-terminal antibody has been used to detect these proteins due to the lack of the LBD [48, 50]. The loss of the LBD renders these variants constitutively active and therefore are ligand-independent proteins [48, 50]. Due to its ligand independence, these variants do not behave like the FL-AR.

1.5 ANDROGEN RECEPTOR NONGENOMIC SIGNALING

Many authors have hypothesized the existence of a putative membrane androgen receptor (mAR). Membrane mediated effects have been observed in multiple studies, yet the mAR has not been purified or cloned. One approach to examining nongenomic signaling is by using cells that lack the classical AR, such as Mouse IC-21 macrophages. However, in the presence of testosterone, a rapid increase in calcium from intracellular calcium stores were observed in these cells [51]. Another approach is the use of membrane-impermeable testosterone conjugated to BSA (T-BSA). The large BSA molecule restricts the steroid hormone from entering the cell [52]. Therefore, any effects seen in the presence of testosterone must be due to a receptor in the membrane. An additional approach is to use an antiandrogen such as flutamide which is known to block the classical intracellular androgen receptor [53]. Studies using T-BSA and flutamide have shown fast cellular effects, such as an increase in intracellular calcium [51, 54-59]. A nongenomic effect in the presence of androgen is the rapid modulation of intracellular calcium levels [51, 54, 56, 59-69].

Calcium functions as a second messenger and regulates gene expression and cell proliferation [70]. Previous studies in our lab show increased calcium levels in N27 dopaminergic cells when testosterone alone was added. In addition, this study was performed using calcium free buffer. Therefore, the calcium is attributed to intracellular calcium stores, possibly mobilized from the endoplasmic reticulum [52]. Interestingly, a rapid influx of intracellular calcium is implicated as mediating an increase in oxidative stress which can lead to neurodegeneration [71]. These fast effects on calcium signaling are indicative of signaling mediated by G-proteins [54, 64, 66, 67, 72-75]. GPCR's and G-proteins are commonly found in signaling domains such as lipid rafts [46]. The GPCR α subunits Gαq and Gαo regulate intracellular calcium [54, 64, 66, 67, 72, 73, 75, 76].

Androgen's fast nongenomic effects on calcium signaling suggest that this could be mediated through a mAR or by some other mechanism such as a GPCR.

Several theories proposing a possible candidate for the mAR are AR shuttling, AR associated with G-proteins, and an AR variant. The classical AR has been shown to shuttle to the plasma membrane via LBD mediated palmitoylation, the addition of fatty acids to cysteine residues [77]. Palmitoylation facilitates the association of caveolin-1 with AR, which is crucial for membrane localization and signaling [77]. It is possible once AR is shuttled to the membrane it may interact with its ligand.

Lipid rafts are specialized membrane microdomains that regulate the trafficking of membrane proteins and direct the assembly of signaling proteins such as G proteins involved in calcium signaling [46, 78-81]. Studies have shown an increase in intracellular calcium in the presence of androgens [51, 54, 56, 59-61, 63, 65, 76]. Therefore, it is probable the mAR is present in lipid rafts and interacts with G proteins to promote calcium signaling. In the substantia nigra (SN), differences in AR expression when using site-specific antibodies suggests AR45 variant in this brain region [40, 41]. In addition, our lab has observed T-BSA induced calcium mobilization in dopaminergic neurons [52]. It is likely AR45 may be a membrane bound receptor associated with a G protein or possibly the FL-AR shuttled to the membrane.

1.6 ANDROGENS AND DISEASE

Sex differences have been observed in neurodegenerative diseases such as Parkinson's disease (PD). PD is a progressive neurodegenerative disease classified as a loss of dopaminergic neurons in the *substantia nigra pars compacta* (SN). Substantia nigra translates to "black substance" due to melanin, a dark pigment produced during dopamine synthesis [82]. The SN is a

primary regulatory component of the basal ganglia, which is a collection of nuclei in the brain that allows movement. Normal movement is contingent on the neurotransmitter, dopamine. Neurodegeneration of SN leads to a significant loss of dopamine, which causes a loss of fine motor functions. Patients with PD can exhibit such symptoms as a resting tremor, bradykinesia, muscle rigidity and a shuffled gait [82]. PD is the second most common neurological disorder, affecting 1.2% of people over the age of 65 [83].

Aged men are two times more likely to be diagnosed with PD than women [84, 85]. Clinical studies have shown men with elevated testosterone and previously diagnosed with a neurodegenerative disorder have an increase in cognitive impairment [86]. In *vivo* studies show testosterone exacerbates neurodegeneration, motor asymmetry and oxidative stress generation in the presence of a neurotoxin [86]. Within seconds, impermeable testosterone can mediate an influx of intracellular calcium in dopaminergic neurons [52]. Furthermore, the influx of intracellular calcium activates oxidative stress generating enzymes culminating in increased oxidative stress [71, 87, 88]. Calcium signaling is tightly regulated in the cell because calcium dysregulation can lead to neurodegeneration. Interestingly, calcium dysregulation in the SN is one of the key features in Parkinson's disease.

In 2010, it was estimated that approximately 630,000 people in the United States were diagnosed with PD. As the baby boomer generation ages, the number of neurodegenerative diseases will also increase. The medical expenses incurred by patients with PD is approximate $14 billion. The indirect cost of PD, such as loss of income is estimated at $6.3 billion [83]. Elucidating the pathology of the sex differences in PD may lead to potential therapeutic target to slow down the progression of PD.

1.7 SIGNIFICANCE AND SPECIFIC AIMS

Numerous studies support the existence of a membrane androgen receptor, which is involved in fast nongenomic effects via calcium signaling. The ***goal*** of this study was to identify the membrane androgen receptor and its signaling mechanisms in dopaminergic neurons. We ***hypothesize*** that the mAR is the androgen receptor variant AR45, which is present in the membrane of dopaminergic neurons and associated with G proteins.

The following ***specific aims*** were used to test our hypothesis:

SPECIFIC AIM 1: Distinguish the location and presence of androgen receptors in dopaminergic cells.

SPECIFIC AIM 2: Determine the association between membrane androgen receptors and G proteins Gαq, Gαs, Gαo and Gαi.

CHAPTER II

PRESENCE OF ANDROGEN RECEPTOR SPLICE VARIANT IN THE SUBSTANTIA NIGRA

Jo Garza Contreras, Phong Duong, Brina Snyder, Joel Little, Rebecca L. Cunningham

The University of North Texas

Health Science Center

3500 Camp Bowie Blvd.

Fort Worth, TX

*To be submitted to eNeuro – Disorders of the Nervous System

2.1 ABSTRACT

Fast, non-genomic androgen actions have been described in various cell types, including neurons. However, the receptor mediating this cell membrane initiated rapid signaling remains unknown. This study found an androgen receptor splice variant in dopaminergic neurons from an immortalized female fetal N27 cell line and substantia nigral tissue from gonadally intact and gonadectomized (young and aged) male rats. This splice variant protein has a molecular weight of 45 kDa and lacks an N-terminal domain, indicating it is the AR45 splice variant. In dopaminergic neurons, AR45 is localized to plasma membrane lipid rafts, a microdomain involved in cellular signaling. Further, AR45 protein interacts with membrane associated G-proteins, Gαq, and Gαo. Neither age nor hormone levels altered AR45 expression. Interestingly, full-length classical androgen receptors were not observed in the substantia nigra, unlike AR45 expression that was observed throughout this brain region. These results provide the first evidence of AR45 protein expression in the brain, specifically plasma membrane lipid rafts. AR45 presence in lipid rafts indicates that it may function as a membrane androgen receptor to mediate fast, non-genomic androgen actions

2.2 SIGNIFICANCE STATEMENT

Evidence has been building for the existence of a membrane androgen receptor, but this receptor remains elusive. We predicted that the membrane androgen receptor is a splice variant that is present in lipid raft microdomains within the neuronal plasma membrane. Indeed, an androgen receptor that is lacking an N-terminal domain and complexed to Gαq and Gαo G-proteins was highly expressed in lipid rafts in dopaminergic neurons. This splice variant provides a potential target for mediating androgen's non-genomic actions.

.

2.3 INTRODUCTION

Previous studies indicate that androgens and androgen receptors (AR) may be involved in sex differences observed in neurodegenerative diseases, such as Parkinson's disease (PD) [41, 52, 86, 89-93]. It is unknown what mechanism(s) underlies this sex difference. Recent studies support the involvement of a putative membrane androgen receptor (mAR) [41, 52, 55, 57, 89].

The classical AR is expressed in a wide variety of human tissues, including the substantia nigra (SN), the brain region associated with PD [40, 94-96]. Classical AR is involved in genomic transcription [35, 97-99]. This AR is composed of eight exons is 110 kDa molecular weight [23]. The classical AR has four distinct regions: regulatory NH_2 terminal domain (NTD) [23, 30-32, 100], DNA-binding domain (DBD), hinge domain, and the ligand binding domain (LBD) that is in the COOH terminal domain (CTD) [23].

Evidence for a non-genomic mAR has been accumulating since the 1990's. Investigators have found that androgens, even cell-impermeable androgens, can have fast cellular effects, such as rapid changes in intracellular calcium, that are unaffected by AR antagonists [51, 52, 54, 60, 62, 65]. It is possible the mAR is unaffected by the AR antagonist due to its location in the membrane. Unlike the classical AR, the mAR has not been cloned or purified. Different theories have proposed that the mAR could be the classical AR anchored to the plasma membrane [77], an unknown AR [58], or even a splice variant of the classical AR [101].

Numerous AR splice variants have been found outside the nervous system. These variants originate from alternative splicing at different promoters and thus increase AR complexity and biological functions [43, 102-106]. Most of these AR splice variants have a truncated LBD in the CTD, which can result in a constitutively active AR [105, 107, 108]. In contrast to the loss of LBD,

some of these AR splice variants exhibit partial to full loss of the NH_2 regulatory domain. For example, both AR splice variants (AR8 and AR45) have truncated NTD [20, 43, 109, 110].

The AR splice variant, AR45, is the least understood variant and has only been characterized in humans. This splice variant lacks the entire NTD. This deletion decreases the protein molecular weight of AR from 110 kDa to 45 kDa [43]. Functionally, in the periphery AR45 binds androgens via LBD and translocates to the nucleus. It can homo-dimerize with other AR45 receptors or hetero-dimerize with classical AR. AR45 can act as a negative modulator of AR activity via competitive inhibition of AREs by homodimers or by interfering with co-activator recruitment necessary for AR activity [20, 43, 110]. AR45 is expressed in multiple tissues, such as muscle, lung, heart, breast, uterus, and prostate [43, 47, 111]. Although AR45 protein expression was not observed in total brain homogenate [43], a recent study found low RNA expression of AR45 in human brain tissue that was commercially obtained from an aged population [48]. Neuronal function of AR45 is unknown.

To determine whether the putative mAR could be AR45 in the SN brain region, we measured protein expression of AR using antibodies targeting the CTD and NTD of the AR. Since neuronal mAR has been associated with intracellular calcium signaling [51, 52, 54, 60, 62, 65], we examined if the mAR complexed with G-proteins.

2.4 MATERIALS AND METHODS

Reagents: Testosterone (A6950-000) was obtained from Steraloids. Goat anti-Rabbit (sc-2004), androgen receptor C-19 (sc-815), androgen receptor N-20 (sc-816), Gαq (sc-393), Gαs (sc-823), Gαo (sc-393874), $G\alpha i_1$ (sc-391), $G\alpha i_2$ (sc-13534), and $G\alpha i_3$ (sc-262) antibodies were obtained from Santa Cruz. Flotillin (3253) and Caveolin-1 (3267) antibodies were obtained from

Cell Signaling. Alexa Fluor 594 antibody was purchased from Jackson ImmunoResearch Laboratories. GAPDH (GT239) antibody was obtained from GeneTex. Biotinylated Anti-Rabbit IgG (BA-1000) was purchased from Vector Laboratories. DMSO was purchased from VWR. RPMI 1640, Penicillin-streptomycin (PS), and trichloroacetic acid (BDH9310) were purchased from VWR. Fetal Bovine Serum (FBS) and phosphate buffer solution (PBS) were obtained from Corning. Charcoal-stripped Fetal Bovine Serum (CS-FBS) was purchased from Atlanta Biologicals. The mounting medium was obtained from Vector Laboratories (H-1200). SuperSignal West Pico/Femto chemiluminescent substrates, dithiothreitol (DTT), and Clean-Blot IP Detection Reagent (PI21230) were obtained from Thermo Fisher Scientific. Deoxycholic acid (D-6750), Inactin (T133), horse serum (H1138), and Triton X were purchased from Sigma. Tris, Any KD polyacrylamide gel, Tris-glycine buffer, and PVDF membranes were purchased from BioRad. Total testosterone ELISA (RTC001R) was purchased from BioVendor. Testosterone was made using an ethanol vehicle (final concentration of ethanol < 0.001%).

Animals: Experiments were conducted on adult male Sprague-Dawley rats (Charles River) at either young adult (3 months) or retired breeders (9-12 months). These retired breeders are considered middle age, but will be referred to as old in comparison to the young rats. Animals were either gonadally intact or were gonadectomized to remove circulating gonadal hormones. Rats were individually housed in a temperature-controlled environment on a 12:12 hour light dark cycle. All rats had *ad libitum* access to food and water. Animals were weighed at the time of surgery and at sacrifice. All experimental procedures were approved by UNT Health Science Center's IACUC in accordance with the guidelines of the Public Health Service, the American Physiological Society, and the Society for Neuroscience for animal care and use.

Gonadectomy: Under 2.5% isoflurane, a midline scrotal incision was made to expose the spermatic cord. The spermatic cord was tied off with sterile sutures, and the cord was cut distal to the thread to remove the testes. The incision was closed with sterile absorbable sutures [86].

Micropunch tissue dissection: One week following surgery, each rat was anesthetized with 2.5% isoflurane and decapitated. The brain was removed from the skull, rinsed in ice-cold PBS, and then placed into a brain matrix (Braintree Scientific) on ice. Using razor blades, the brain was cut into 1 mm coronal sections. The razor blades were placed on dry ice to freeze the freshly cut brain sections. Punches were obtained from the SN pars compacta (-4.80 to -6.04 mm from Bregma) using 1 ml syringes with a 20-gauge blunt needle. Samples were placed into microcentrifuge tubes, snap-frozen on dry ice, and stored at -80℃.

In vitro cell culture: The immortalized neuronal cell line $1RB3AN_{27}$ (N27) was derived from female fetal rat mesencephalic tissue. The N27 cell line is positive for tyrosine hydroxylase (TH+) expression, an enzyme marker for dopamine neurons [40, 112-114]. N27 cells were cultured and maintained at 37℃ in 5% CO_2. Media used was RPMI 1640 medium supplemented with 10% FBS and 1% PS. N27 cells were only used within passages 13-19 to avoid changes in morphology. The testosterone concentration used in this study was 100 nM, representing the high end of the normal testosterone range in men [115-118]. Prior to hormone treatment for whole cell lysate experiments, the media was switched to RPMI 1640 with charcoal-stripped FBS to avoid confounders due to the presence of hormones in the serum. Cells were exposed to testosterone or vehicle control overnight and then were collected for protein.

Whole cell lysates: For *in vitro* preparations, N27 cells were plated in 100x20 mm plates at a density of 8.0×10^4 cells per plate. Following treatments, cells were washed with PBS and lysed using NP40 and phosphatase inhibitors (1:100) cocktail on ice. For *in vivo* preparations, flash-

frozen SN micropunches were incubated with RIPA homogenization buffer with DTT (1uM), EDTA (1mM) protease and phosphatase inhibitors (1:200) for 30 minutes on ice, and then sonicated (QSonica) at 20% amplitude and pulsed 3 times for 3 seconds. Next, lysates were centrifuged at 4℃ for 20 minutes at 12,000 x g. Protein concentrations were determined by using the BCA assay (Thermo Fisher Scientific), according to the manufacturer's instructions.

Detergent-free cellular fractionation and sucrose density analysis of membrane lipid rafts: N27 cells were plated in 100x20 mm plates at a density of 8.0 x 10^4 cells per plate. Following treatments, cells were washed with PBS and lysed with hypotonic homogenization buffer and phosphatase inhibitors (1:100) cocktail on ice. Each sample (n) consisted of two 100x20 mm plates. For micropunches, a total of 0.025 g of SN tissue was homogenized in hypotonic homogenization using a sonicator to homogenize the tissue. Following homogenization, cellular fractionation followed by sucrose density analysis of membrane lipid rafts was performed [119, 120]. Cell lysate from either N27 cells or micropunches was centrifuged at 1000 x g for 5 min at 4℃ to separate the nuclei. The supernatant was centrifuged at 16,000 x g for 30 minutes 4℃ to separate the cytosolic proteins from the mitochondria, Golgi fragment, and the plasma membrane. The pellet was then re-suspended in homogenization buffer supplemented with 500 mM Na_2CO_3 [121]. The re-suspended membrane pellet was placed into a sucrose flotation-gradient fraction using a 5% / 35% / 45% discontinuous gradient that was spun at 175,000 x g for 18 hours at 4℃ in Optima ultracentrifuge Model LE-80K (Beckman Coulter) using a swing bucket rotor (SW 50.1, Beckman Coulter). Following the high-speed centrifugation, equal volume fractions were taken from the top layer of the gradient, resulting in 9 fractions (low-density proteins at the top gradient layers to high-density proteins at the bottom gradient layers). Protein was precipitated using the trichloroacetic acid (TCA) method [122]. The pellet was incubated in 0.15% deoxycholic acid and

then 72% trichloroacetic acid, followed by 16,000 x g for 30 minutes at room temperature. The pellet was re-suspended in RIPA lysis buffer, Laemmli loading buffer, and 2M Tris. The sample was loaded into polyacrylamide gels for electrophoresis and Western blot protein analysis.

Co-immunoprecipitation: Since C19+/N20- AR protein expression at 45 kDa was only observed in the membrane fraction (not the cytosol or nuclear fractions), whole cell lysates were used. Protein (25 ug) was incubated overnight at 4℃ in a cocktail containing RIPA lysis buffer and 1 ug of primary antibody (AR C-19 or Gαq). A sepharose bead slurry was coupled to each sample by incubating at 4℃ overnight. Samples were washed, eluted, and resuspended in 4X Laemmli buffer. To avoid IgG band interference, the blot was incubated with the Clean-Blot IP Detection Reagent HRP, per the manufacturer's instructions.

Western Blot: Equal amounts of (20ug protein) denatured whole cell lysates, micropunch tissues, cellular fractions, or lipid raft fractions were loaded into a Bio-Rad Any KD polyacrylamide gel, electrophoresed in a Tris-glycine buffer and then transferred onto a PVDF membrane. Membranes were blocked for 30 minutes with 5% nonfat milk in TBS-Tween at room temperature. Following blocking, the membranes were incubated with specific primary antibodies (AR C-19 1:1000, AR N-20 1:750, GAPDH 1: 10,000, Gαq 1:1000, Gαs 1: 10,000, $G\alpha i_{1\text{-}3}$ 1: 10,000, Gαo 1: 1,000, flotillin 1: 1,000, and caveolin-1 1: 10,000) in TBS-Tween with 1% nonfat milk for 2 hours or overnight in 4℃. Afterward, the membranes were washed every 10-minutes for 30 minutes and then incubated with secondary antibodies at 1:1,000 in TBS-Tween with 1% nonfat milk for 30 minutes at room temperature. Protein bands on the membrane were visualized using an enhanced chemiluminescence detection assay (Thermo Fisher Scientific). Protein band intensities were imaged using GeneSys software corresponding with the Syngene G-Box Chemi XRQ system. Protein band densities were quantified by NIH Image J densitometer software and

normalized to GAPDH for whole cell lysates, using the following equation (mean gray value for protein of interest / mean gray value of GAPDH) * 100.

Immunohistochemistry: One week after gonadectomy or sham surgery, each rat was anesthetized with Inactin (100 mg/kg, i.p. Sigma), transcardially flushed with 0.1 M PBS (100-200 mls), and then perfused with 4% paraformaldehyde in 0.1 M PBS (300-500 mls) [86, 123, 124]. The brain was removed from the skull and post-fixed with 4% paraformaldehyde in 0.1 M PBS for 24 hours. Next, brains were stored in vials containing 30% sucrose in PBS at 4^{o}C for 3-4 days prior to sectioning. Brains were cut into 3 separate sets of 40 μm coronal sections using a cryostat (CryoStar NX70, Thermo Fisher Scientific). Coronal sections containing the SN (-4.80 to -6.04 mm from Bregma) were blocked with 3% PBS diluent (3% horse serum in PBS with 0.25% Triton X) for 2 hours at room temperature followed by overnight incubation at 4℃ with primary antibody (AR C-19 or AR N-20 at 1:500) in 3% PBS diluent. Afterward, the sections were washed with PBS. Serial sections incubated with AR C-19 or AR N-20 antibodies were then incubated with secondary antibodies (Alexa Fluor donkey anti-goat 1:1000) at room temperature for 5 hours [125]. Afterward, sections were washed with PBS. Fluorescent images were mounted on slides and sealed with mounting medium. After sealing the slides, the slides were stored at 4℃. Images were captured from each section using an epifluorescent inverted microscope (VWR, USA) equipped with a digital camera (Photometrics Cool Snap Myo, Nikon) and imaging software (NIS Elements, Br 4.50.00, Nikon).

Bioassays: During the first two hours of the circadian light phase, each rat was anesthetized with 2.5% isoflurane and decapitated. Prostate and seminal vesicle wet tissue weights were measured. Trunk blood (5-7 mls) was obtained in EDTA coated tubes (13mm x 100mm, Covidien) on ice. The blood was centrifuged (Allegra X-30R, Beckman Coulter) at 2,000 x g for 10 min at

4℃, then separated plasma was placed in microcentrifuge tubes, and stored at -80℃ until ELISA analysis for total testosterone. Plasma testosterone levels were assayed, according to manufacturer's instructions. The intra-assay coefficient of variation was 8.54%, and the inter-assay coefficient of variation was 9.97%. The sensitivity of this assay was 0.066 ng/ml testosterone. The specificity of this assay is: 69.6% dihydrotestosterone (DHT), 7.4% dihydroxyandrosterone, and less than 0.1% for androstenedione, androsterone, epiandrosterone, dihydroandrosterone, estron, estradiol, estriol, cortisol, 11-deoxycortisol, progesterone, and 17OH-progesterone.

Statistical Analysis: Analysis was performed using IBM SPSS Statistics version 21 software. Data were expressed as mean $\pm$ SEM. Significance ($p \leq 0.05$) was determined by ANOVA.

2.5 RESULTS

Bioassays: Body weights, prostate and seminal vesicles weights, and total testosterone levels were quantified (Table 1). Hormone treatment did not have a significant impact on body weight, regardless of age. However, old rats (9-12 months) were significantly heavier than young rats (3 months) ($F_{1,45}$=233.284, $p < 0.05$). One week following gonadectomy (GDX) plasma testosterone levels were significantly decreased compared to gonadally intact rats, regardless of age ($F_{1,45}$=54.221, $p < 0.05$). No differences in testosterone levels between young and old gonadally intact rats were observed. Consistent with a decline in testosterone, GDX rats exhibited a significant decrease in weights for androgen sensitive accessory organs: prostate ($F_{1,45}$=74.117, $p < 0.05$) and seminal vesicles ($F_{1,45}$=33.088, $p < 0.05$). Old retired breeders, regardless of hormone condition, had significantly heavier prostate ($F_{1,45}$=23.577, $p < 0.05$) and seminal vesicle ($F_{1,45}$=42.929, $p < 0.05$) weights than young sexually naive rats. These results are consistent with prior studies showing sexual experience increases testosterone and androgen sensitive accessory organ weights [126-129]

Age	**Hormone**	**n**	**Body Weight** (g)	**Prostate** (g)	**Seminal Vesicles** (g)	**Total Testosterone** (ng/ml)
Young	GDX	13	325.25 ± 7.75	0.16 ± 0.08 #	0.40 ± 0.18 #	0.36 ± 0.63 #
Young	Intact	13	325.35 ± 27.75	0.46 ± 0.13	0.83 ± 0.19	5.61 ± 2.90
Old	GDX	12	444.75 ± 26.48 *	0.30 ± 0.11 *#	0.89 ± 0.20 *#	0.21 ± 0.18 #
Old	Intact	11	456.68 ± 43.96 *	0.75 ± 0.25 *	1.27 ± 0.39 *	4.34 ± 3.42

TABLE 1: Bioassays. Young rats are 3 months old, while old rats are 9-12 months old. Weights are expressed as mean grams ± SEM. Plasma testosterone levels are expressed as mean ng/ml ± SEM. GDX: Gonadectomy; Intact: Gonadally Intact. $P < 0.05$ * versus young, # versus Intact.

Androgen receptor expression in whole cell lysates: Androgen receptor expression was quantified in a dopaminergic N27 cell line and in substantia nigra (SN) tissue from young and old male rats that were either gonadally intact or GDX (Figure 1). Antibodies targeting either the N-terminal domain (NTD) or the C-terminal domain (CTD) of the AR were used to examine AR protein expression. Protein from testes was used as a positive control for AR expression. Full length AR (110 kDa) protein expression was found in testes using both NTD (AR N20) and CTD (AR C-19) AR antibodies (Figure 1A). However, no full-length AR expression was found in N27 cells or rat SN tissue, regardless of hormone treatment or age (Figure 1 A, C). Protein expression at 45 kDa was observed in N27 cells, SN tissue, and testes, which was unaffected by hormones and age (Figure 1 A-D). Interestingly, this 45 kDa band was only evident when using a CTD targeted antibody for the AR and not a NTD antibody, consistent with the AR splice variant AR45 that lacks a NTD. Similarly, CTD AR positive cells (and not NTD AR positive cells) were observed throughout the SN in young and old rats, irrespective of hormone status (Figure 2).

Expression profile of androgen receptors: N27 cells and SN brain tissue were split into membrane, cytosol, and nuclear fractions. The membrane portion of both N27 cells and SN brain tissue was further separated into 9 fractions using a sucrose gradient (Figures 3-5). Full length AR at 110 kDa was not observed in any membrane fraction. Although full length AR was not expressed, a 45 kDa protein corresponding to AR45 was observed in all samples. In N27 cells, regardless of testosterone exposure, AR45 expression was evident in fractions 3-6, which are lipid rafts as shown by caveolin-1 and flotillin immunoreactivity (Figure 3 A, B). Similarly, AR45 immunoreactivity was present in caveolin and flotillin enriched membrane lipid rafts in both young and old rats in all hormone groups (Figures 4-5). AR45 immunoreactivity was not found in any non-lipid raft portion of the membrane, nor was it observed in cytosolic or nuclear fractions.

Expression profile of GPCRs in membrane lipid rafts: Since neuronal mAR has been associated with intracellular calcium signaling [51, 52, 54, 60, 62, 65], we examined if G-proteins were present in lipid rafts. In both N27 cells and SN tissue the G-protein Gαq was expressed in the membrane fraction and in lipid rafts, but $G\alpha i_{1\text{-}3}$ and Gαs was not expressed in any of the membrane fractions. Interestingly, the G-protein Gαo was only expressed SN tissue and not in the N27 cell line. (Figures 3-5).

Androgen receptor variant association with GPCR subunits: To determine if AR45 interacts with G-proteins, co-immunoprecipitation was performed. Antibodies targeting the CTD of the AR or G-proteins were used to pull down proteins. After electrophoresis, membranes were probed for AR45 (C-19 antibody) or G-protein immunoreactivity. In N27 cells, AR45 interacted with Gαq, regardless of testosterone exposure (Figure 6A). Furthermore, in SN tissue AR45 was associated with Gαq and Gαo in all hormone states and ages (Figure 6B).

2.6 DISCUSSION

This study found membrane AR protein expression at 45 kDa molecular weight in N27 cells and SN brain tissue from young and old rats with and without sex hormones. The 45 kDa membrane protein was only evident using a CTD targeted antibody for AR, consistent with the AR45 splice variant. Little to no protein expression of full length classical AR was found in N27 cells and SN tissue. Since classical ARs are not present, it is possible that AR45 is mediating androgen's actions by acting as a mAR.

Men have an increased risk for PD than women [130], suggesting that steroid hormones play a role. Steroid hormone receptors (e.g. classical estrogen receptors and estrogen receptor variants) have been found in membrane lipid rafts [77, 131-134]. However, the mAR has not been

found, even though numerous studies support its presence. Functionally, previous studies indicate mAR is involved in mediating cell viability and calcium signaling in dopaminergic cells [41, 52], which are processes affected in PD.

Evidence is building that plasma membrane signaling via lipid rafts is involved in motor impairment and toxic protein accumulation in neurodegenerative disorders, such as PD [135-139]. Indeed, many proteins associated with PD, such as parkin and α-synuclein, have been found in lipid rafts [139-141]. Lipid rafts are low density microdomains, enriched with cholesterol and lipids, and insoluble in non-ionic detergents [142, 143]. Numerous signaling proteins, such as tyrosine kinase receptors, GPCRs, and G proteins, reside in lipid rafts [144-150]. Proteins within the lipid rafts can rapidly increase or even decrease cellular signaling, resulting in the lipid rafts playing a pivotal role in signal transduction [143, 151].

The proteins, flotillin and caveolin, are integral components of lipid rafts. Neuronal lipid rafts generally are planar and composed of flotillin, unlike non-neuronal cells that contain rafts with invaginations composed of caveolin [152-154]. However, studies have shown that caveolin-1 can be present in neurons under certain conditions, such as oxidative stress and aging [155-157]. One of the brain regions with the highest expression of caveolin-1 is the SN [158], which is composed mainly of dopaminergic neurons that have increased oxidative stress from dopamine metabolism [159, 160]. Interestingly, elevated oxidative stress is a key characteristic of PD [161-163], and upregulated caveolin expression has been linked with PD [136].

Our data showed that AR45 is present in membrane fractions from N27 cells and SN tissue. Specifically, AR45 was present only in caveolin and flotillin enriched lipid rafts, indicating localization to lipid rafts that contain invaginations. Prior studies have shown that Gαq mainly localizes in caveolae lipid rafts, unlike Gαs and Gαi proteins [64, 164, 165]. Indeed, the results

from this study show that Gαq and Gαo proteins are present in lipid rafts, along with AR45. Furthermore, AR45 co-immunoprecipitates with Gαq and Gαo proteins, indicating that AR45 interacts with G-proteins in lipid rafts in dopaminergic cells.

Gαq has been well established as an activator of intracellular calcium release from the endoplasmic reticulum [166, 167], which are dysregulated in PD [168]. Much less is known about the function of Gαo proteins that is highly present in frontal cortex, cerebellum, hypothalamus, hippocampus, and SN [169]. Gαo can couple to receptors that decrease intracellular calcium release [72, 170], and in the brain Gαo is predominantly coupled to inhibitory D2 dopamine receptors [171]. Interestingly, this association with D2 receptors may explain the lack of Gαo expression in the N27 cells, as D2 receptors are not expressed in this cell line [172]. Further supporting the role of Gαo in SN dopaminergic neuronal involvement, Gαo knockout mice exhibit poor motor coordination [73]. Although data about Gαo is sparse and its involvement with PD is unknown, Gαo is linked with motor function and calcium signaling.

Interestingly, androgens can increase intracellular calcium release in cells via a non-genomic mechanism [52, 59, 60, 63]. Many times androgens non-genomic actions are unaltered by antiandrogens [52, 55, 173]. Several studies have shown that androgen's non-genomic actions on calcium signaling can be mediated by pertussis toxin-sensitive G-proteins [60, 63, 67, 174], further supporting that the mAR may be interacting with a G protein.

This is the first study to show the presence of a putative AR splice variant protein in the brain. Specifically, our results show AR45 localizes in the membrane lipid rafts from N27 cells and SN dopaminergic neurons. Furthermore, AR45 interacts with Gαq and Gαo G-proteins, which can impact calcium signaling. More research needs to be conducted to further determine the function and role of this AR splice variant in dopaminergic neuronal function and dysfunction.

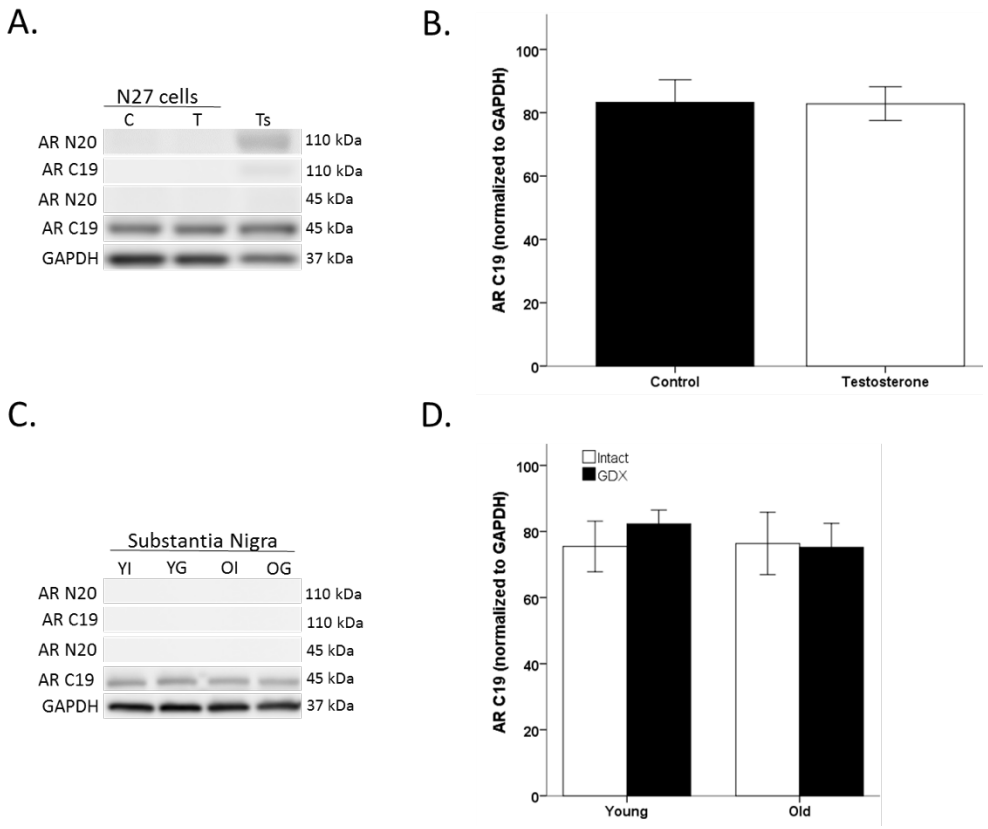

FIGURE 2. Androgen Receptor Expression in Whole Cell Lysate.

N27 cells were treated with vehicle control (n=8) or 100 nM testosterone (n=8) for 18 hours. Protein from testes was used as a positive control (A, B). Micropunches were obtained from young and old male rats that were either gonadectomized (n=3) or gonadally intact (n=3) (C, D). Equal amounts of (20ug) protein were loaded into the gel. Full length androgen receptor expression was only observed in testes (A), using antibodies targeting the c-terminus domain (AR C-19) and n-terminus domain (AR N-20). A CTD antibody revealed a 45 kDa molecular weight protein in all tissues probed, which is consistent with AR45 (A, C). Testosterone did not affect AR45 expression in N27 cells (A, B). Neither did hormones nor age affect AR45 expression in substantia nigra tissue (C, D). Abbreviations. C: control, T: testosterone, Ts: testes, YI: young gonadally intact male rats, YG: young GDX male rats, OI: old gonadally intact male rats, OG: old GDX male rats.

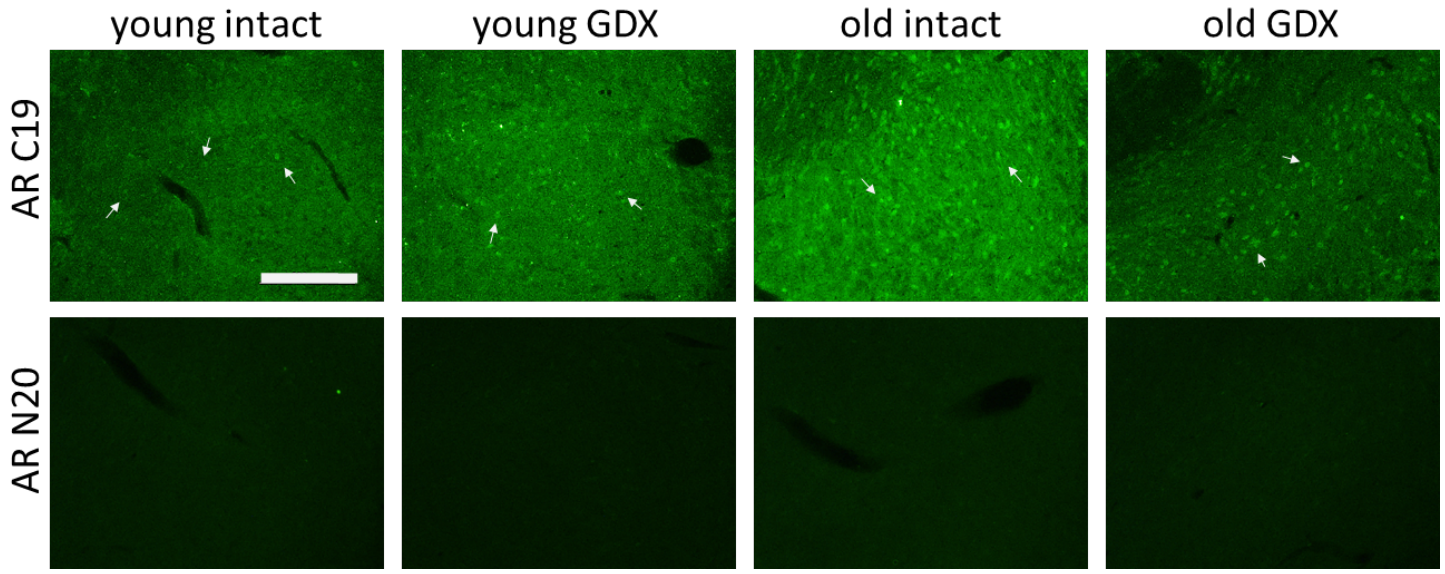

FIGURE 3. Widespread AR45 Distribution in the Substantia Nigra

AR immunoreactivity using a CTD targeted antibody (AR C19) was observed throughout the substantia nigra in all age and hormone groups. No AR immunoreactivity was observed using a NTD targeted antibody (data not shown). Arrows indicate AR positive cells. Scale bar = 500μm.

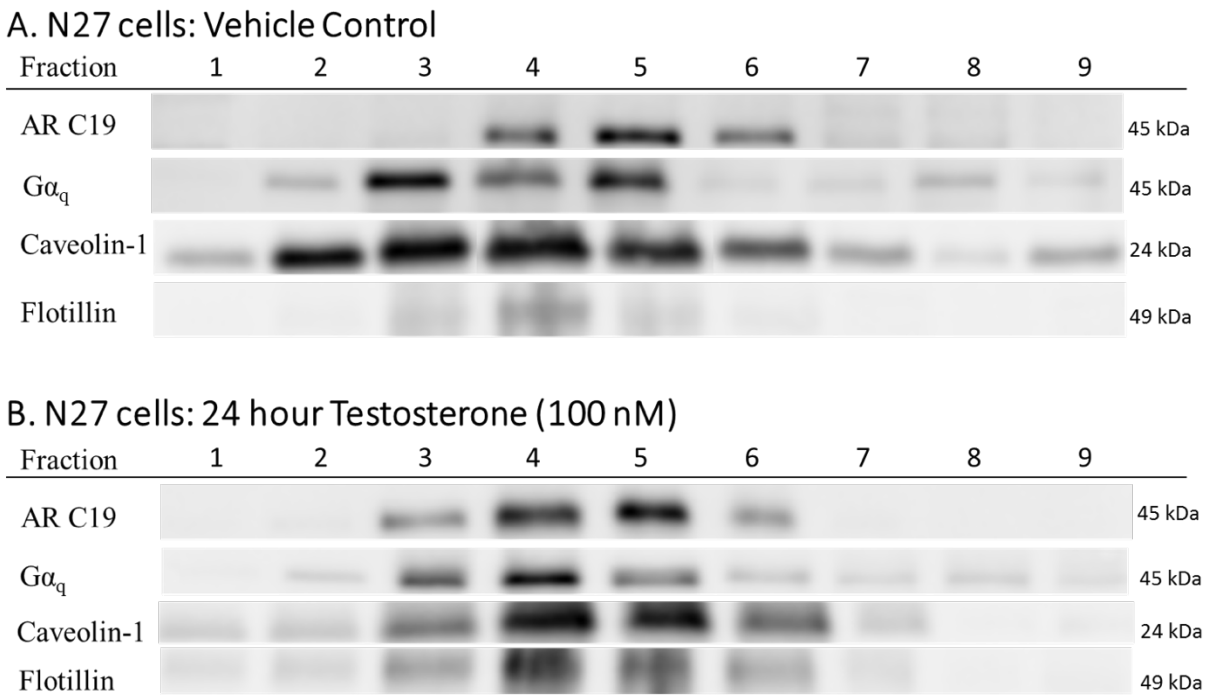

FIGURE 4. N27 Cells Express AR45 Protein in Membrane Lipid Rafts.

N27 cells were treated with either vehicle control (A) or 100 nM testosterone for 24 hours (B). The membrane portion of the cells were further separated into 9 fractions using a sucrose gradient and ultracentrifugation to examine lipid rafts. Equal amounts of (20ug) protein were loaded into the gel. Primary antibodies targeting AR45 (AR-C19 antibody), G-protein Gαq, and lipid raft markers (caveolin-1 and flotillin) were used. AR45 and Gαq expression was only observed in lipid raft fractions, as evidenced by caveolin-1 and flotillin expression. Hormone treatment did not alter AR45 and Gαq expression in lipid rafts. Full length 110 kDa AR, Gαo, $G\alpha i_{1\text{-}3}$, and Gαs G-proteins were not observed in the membranes of N27 cells (data not shown). N=3 per treatment group.

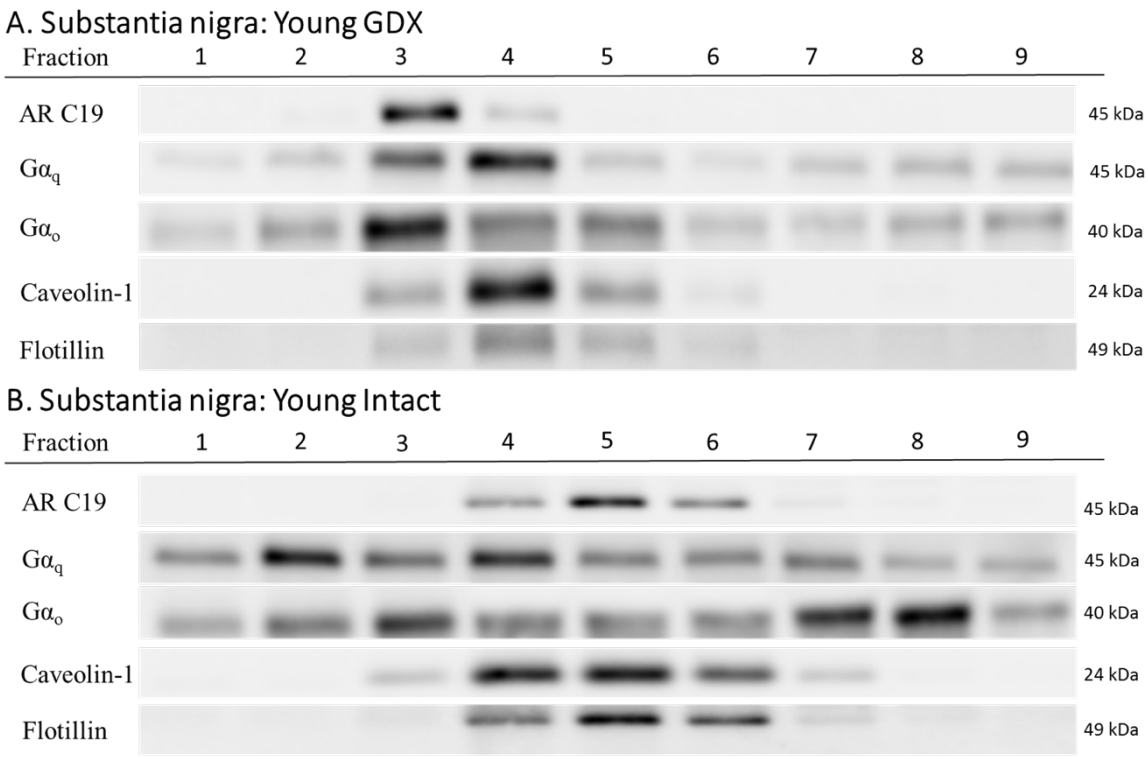

FIGURE 5. Young Male Rats Express AR45 Protein in Membrane Lipid Rafts.

Young rats (3 months old) were either gonadectomized (A) or gonadally intact (B). Micropunches of substantia nigra tissue was collected, and the membrane was isolated and then separated into 9 fractions using a sucrose gradient to examine lipid rafts. Equal amounts of (20ug) protein were loaded into the gel. Primary antibodies targeting AR45 (AR-C19 antibody), G-proteins Gαq and Gαo, and lipid raft markers (caveolin-1 and flotillin) were used. AR45 expression was only observed in lipid raft fractions, as evidenced by caveolin-1 and flotillin expression. Hormone status did not alter AR45 expression in lipid rafts. Gαq and Gαo was observed throughout the membrane, regardless of hormone status. No protein expression of $G\alpha i_{1\text{-}3}$ and Gαs G-proteins or full length 100 kDa AR were observed in the substantia nigral membranes of young male rats (data not shown). Abbreviations. GDX: Gonadectomy; Intact: Gonadally Intact. N=3 per treatment group.

FIGURE 6. Aged Male Rats Express AR45 Protein in Membrane Lipid Rafts.

Aged rats (9-12-month-old retired breeders) were either gonadectomized (A) or gonadally intact (B). Membrane portion of the substantia nigra micropunch tissue was isolated, and then separated into 9 fractions using a sucrose gradient. Equal amounts of (20ug) protein were loaded into the gel. AR45 (AR-C19 antibody), Gαq and Gαo, and lipid raft markers (caveolin-1 and flotillin) were examined. AR45 expression was only observed in lipid raft fractions, regardless of hormone status. Gαq and Gαo was observed throughout the membrane in both gonadectomized and gonadally intact males. Protein expression of $G\alpha i_{1\text{-}3}$, Gαs, or full length 110 kDa AR were not observed in the substantia nigral membranes of aged male rats (data not shown). Abbreviations. GDX: Gonadectomy; Intact: Gonadally Intact. N=3 per treatment group.

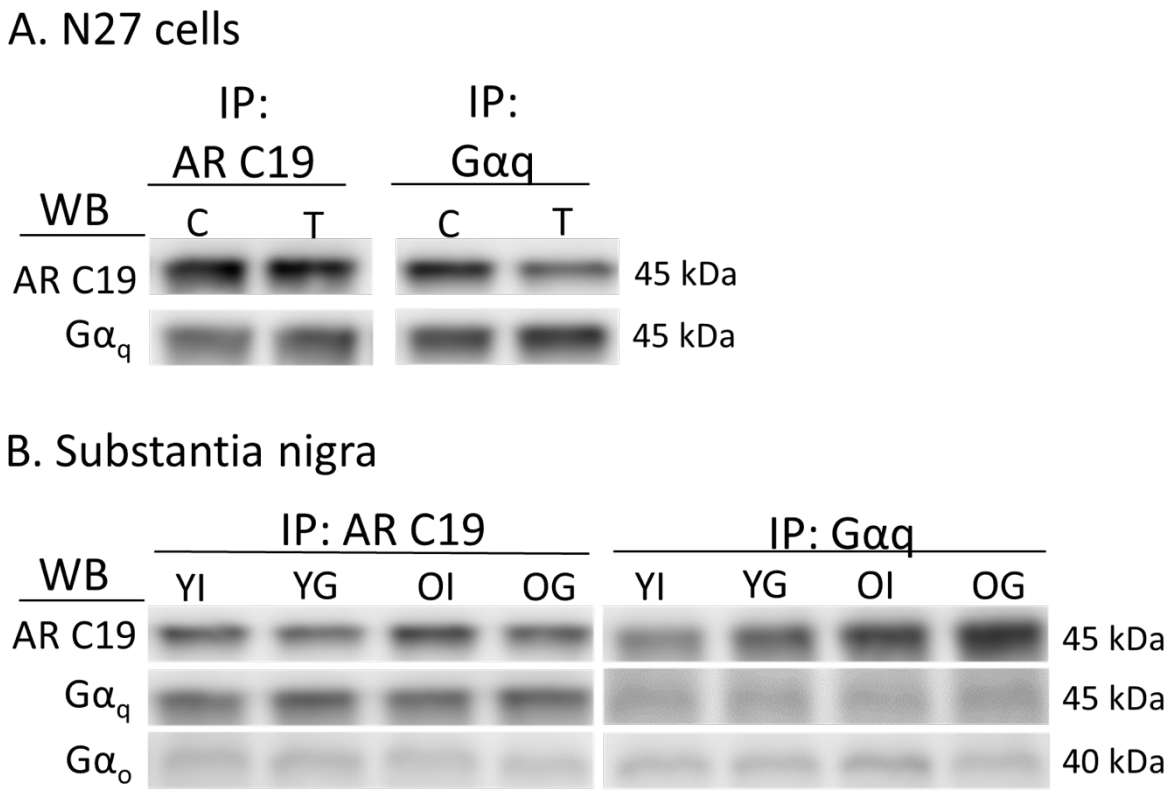

FIGURE 7. Co-Immunoprecipitation (IP) of the AR45-G-Protein Complex.

Whole cell lysates of N27 cells treated with either vehicle or 100 nM testosterone (A) and substantia nigral tissue from young and aged male rats that were either gonadectomized or gonadally (B) were used. Equal amounts of (25ug) AR CTD-containing AR45 proteins were immunoprecipitated and then immunoblotted (WB) for Gαq and Gαo. In a reciprocal fashion, Gαq-containing proteins were immunoprecipitated and then immunoblotted for AR45. In N27 cells, bands corresponding to AR45 (AR-C19) and Gαq were detectable. In substantia nigral tissue, bands corresponding to AR45 (AR-C19), Gαq, and Gαo were observed. Abbreviations. C: control, T: testosterone, YI: young gonadally intact male rats, YG: young GDX male rats, OI: old gonadally intact male rats, OG: old GDX male rats. N=3 per treatment group.

CHAPTER III

DISCUSSION

3.1 DISCUSSION

Men over the age of 65 are twice as likely to be diagnosed with PD [83]. The male sex hormone, testosterone, has been shown to mobilize calcium and under conditions of oxidative stress lead to neurodegeneration [52]. This study sought to characterize a putative membrane androgen receptor and its interaction with a calcium mobilizing protein which may be involved in the progression of PD.

We characterized the expression of AR, G proteins, and AR/G protein-protein interactions in dopaminergic neurons. A high abundance of AR splice variant (AR45) was found isolated to the plasma membrane in caveolae. Based on our data, protein expression of AR45 was not affected by testosterone or age as evidenced by lack of effects in aged rats with and without testosterone. Furthermore, AR45 was found to complex with G-proteins Gαq and Gαo, which are involved in calcium signaling (Table 2).

One of the unique features of the SN is very low AR expression when using an N-terminal antibody [40]. Interestingly, immunocytochemistry studies of dopaminergic neurons show high AR expression with the use of a C-terminal antibody [41]. These differences in expression are possibly due to an AR variant lacking the N-terminal region. This characteristic allowed us to easily identify and characterize this unconventional AR. The immunoblot studies show high AR protein expression at 45 kDa in both the N27 and SN cells. This is assumed to be the AR variant

AR45 due to similarity in molecular weight with the known AR variant AR45 as identified in LNCaP a human prostate cancer cell line [43]. In addition, this protein was detected using a C-terminal antibody and not an N-terminal antibody. In our immunohistochemistry study, we show high immunoreactivity with the C-terminal antibody and not the N-terminal antibody in the SN. This is the first study to identify the AR45 variant in brain tissue.

Ahrens-Fath et al., analyzed AR45 mRNA in human brain and found no expression [43]. However, Hu et al. found AR45 mRNA expression in their human brain samples [48]. Possible explanations for the discrepancies between our data and those studies may be due to several possibilities: 1.) In the Ahrens-Fath and Hu studies they purchased their brain samples from vendors and did not state what region of the brain these samples were derived from, 2.)we used freshly processed samples directly isolated from the SN of rat brain [43, 48], and 3.) we analyzed whole cell lysate in addition to the membrane fraction, whereas the other studies analyzed whole cell lysates [43, 48]. Our studies analyzed a very specific region of the brain and a very specific portion of the cell. Low FL-AR expression was observed in the testes and N27 cells. However, no FL-AR was found in the SN tissue (see Limitations). In addition, AR45 protein expression was not affected by hormone or age. This ligand independent expression of FL-AR and AR variants is not uncommon and has been observed in other tissues [48, 105, 108, 175-177].

Indeed, AR45 protein was present in the whole cell lysate of dopaminergic neurons. However, further characterization of AR45 was necessary to determine its localization to the membrane. Furthermore, expression of various G-protein subunits was analyzed to determine the signaling pathways that may be present in these dopaminergic neurons. The plasma membrane is made up of many proteins that function in support, signaling, transport and enzymatic activity [178]. Lipid rafts are signaling microdomains within the plasma membrane, rich in lipophilic

proteins and signaling proteins such as GPCR's [179]. GPCRs signal via activation of G-protein subunits, such as Gαq, Gαs, Gαo and Gαi. Gαq and Gαs are stimulatory proteins that act on different pathways, whereas Gαo and Gαi are inhibitory proteins. Gαq is ubiquitously expressed and activates phospholipase C (PLC), which is involved in calcium release via endoplasmic reticulum (Figure 8) [74]. Gαs and Gαi are also ubiquitously expressed and function in regulating adenylyl cyclase activity [74, 180]. In the rodent striatum, G-protein subunits Gαq and Gαs has been linked to excitatory D1-like receptors [181]. In the substantia nigra, Gαo is localized to striatal terminals [169] and linked to inhibitory D2-like receptors [171]. Gαo has been shown to mediate calcium mobilization in neurons [72, 73, 76]

Our results show that AR45 is present in the same membrane region as caveolin and flotillin. Flotillin is a support protein located within the planar lipid rafts and caveolae [182]. Caveolae are flask shaped invaginations made up of caveolin and flotillin that function similar to lipid rafts by organizing signaling complexes [183]. Previous studies have shown increased caveolin expression in the SN and in PD brains [136, 158]. In addition, AR has been shown to associate with caveolin in lipid rafts [184]. Interestingly, caveolin is upregulated in neurons affected by oxidative stress and aging [155, 156].

Androgens can induce intracellular calcium release in multiple cell types [51, 52, 54, 56, 59, 60, 62, 65]. These actions are unaltered by antiandrogens such as flutamide [52, 55, 173]. Additionally, the nongenomic actions induced by androgens can be mediated by pertussis toxin sensitive G-proteins [60, 63, 67]. These studies suggest a nongenomic mechanism of action via a calcium regulating G-protein. One signaling cascade that is initiated by the plasma membrane is calcium release via Gαq. An increase in intracellular calcium has been associated with increased

oxidative stress and neurodegeneration [71, 185, 186]. Another G-protein that mediates calcium is Gαo which has been shown to couple to receptors that decrease calcium.

We analyzed multiple G-protein subunits to determine which pathway may be involved with the mAR. We show no expression of Gαs or Gαi. Therefore, the adenylyl cyclase pathway is most likely not mediated by the mAR. In the SN, Gαo is present but in the N27 cell line Gαo expression was non-existent. One possible reason for this discrepancy is that N27 cells do not express D2 receptors [171]. This lack of D2 receptors may underlie the differences in Gαo expression between SN and N27 cells. Gαo is associated with calcium signaling and motor function. Poor motor coordination has been observed in Gαo knockout mice [72, 73, 76].

In the N27 cell line and the SN, our studies show Gαq protein expression. Furthermore, we show that Gαq and Gαo proteins are associated with the AR45 protein in the plasma membrane. A previous study has observed an AR splice variant (AR8) acting via a nongenomic mechanism complexed to a transmembrane protein in the plasma membrane [109]. Since AR45 interacts with Gαq, a subunit involved in calcium signaling, it is likely that AR45 can mediate intracellular calcium regulation (Figure 9). Interestingly, previous studies have shown that testosterone, via mAR, can increase calcium release in dopaminergic neurons, further supporting that the mAR may be AR45 complexing with Gαq [52]. Also, it is possible the loss of Gαo following mAR/Gαq mediated apoptosis leads to poor motor function as seen in PD patients.

3.2 CONCLUSION

In conclusion, AR45 was found in dopaminergic neurons within the SN and N27 cells, specifically isolated to membrane caveolae. To our knowledge, this is the first study showing

AR45 protein expression in the brain. Furthermore, G-proteins linked with intracellular calcium signaling were found to interact with AR45. Once the AR45 sequence is known, drugs can be manufactured to target this protein, which may modulate calcium dysregulation and slow the progression of PD in men, thus decreasing the male sex-bias that is observed in PD.

Protein	Testes	N27 Cells		Substantia Nigra tissue	
	Lysate	Lysate	Membrane	Lysate	Membrane
FL-AR	+	↓	—	—	—
AR45	+	+	+	+	+
Gαq	N/A	N/A	+	N/A	+
Gαo	N/A	N/A	—	N/A	+

TABLE 2. Protein Localization and Expression Profile.

KEY: (+) positive expression; (↓) low expression; (—) negative expression.

Positive expression was observed regardless of age and hormone treatment.

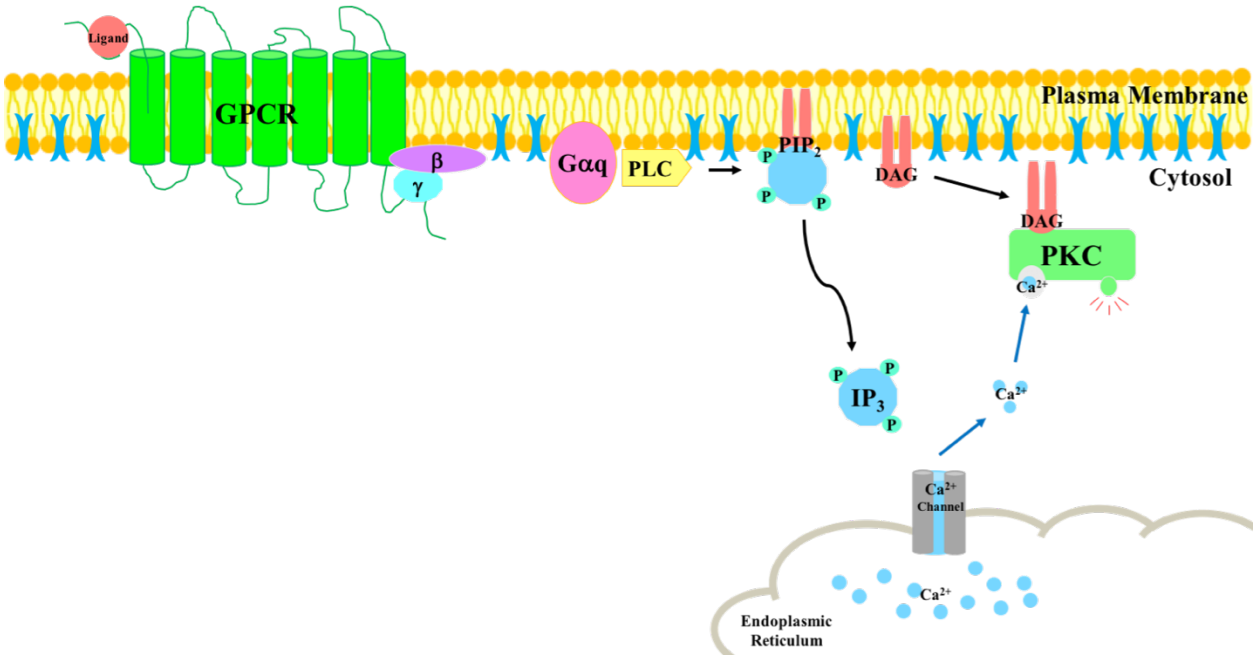

FIGURE 8. GPCR Signaling via G-Protein Subunit Gαq.

Lipid rafts are signaling microdomains within the plasma membrane, rich in lipophilic proteins and signaling proteins such as GPCR's. Ligand binding to a GPCR stimulates a conformational change and release of G-protein subunit Gαq. Gαq activates phospholipase C (PLC). In turn, PLC cleaves PIP_2 into IP_3 and DAG. Activated IP_3 binds to membrane receptors on the endoplasmic reticulum resulting in an intracellular release of calcium. Flotillin (blue) is a support protein located within the planar lipid rafts and caveolae. Adapted from Alberts et al. [187].

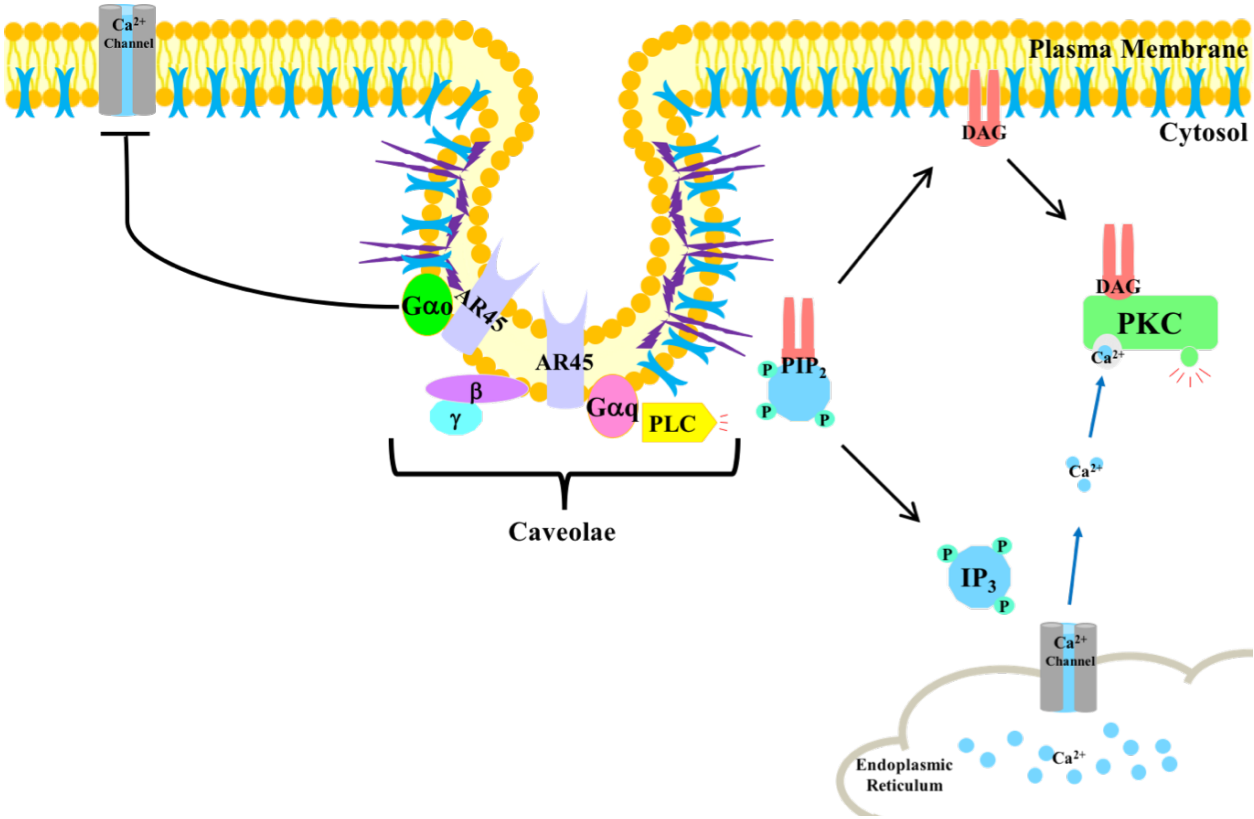

FIGURE 9. Nongenomic Androgen Action

Caveolae are flask shaped invaginations made up of caveolin (purple) and flotillin (blue) that function similar to lipid rafts by organizing signaling complexes Androgen receptor splice variant AR45 is localized to caveolae within the plasma membrane. Furthermore, Gαq and Gαo is associated with AR45. Adapted from Alberts et al. [187] and Martinez-Outschoorn [188].

3.3 LIMITATIONS

Previous studies have suggested AR is a labile protein susceptible to extraction methods and sample storage. These studies have optimized AR stabilization and decreased proteolysis using the following techniques: 1.) DHT stabilization of AR: Cells were extracted in ice cold PBS on ice in RIPA buffer containing DHT. DHT added to the extraction media resulted in an increase of FL-AR and a decrease in AR fragments. 2.) Protease inhibitors: Standard protease inhibitors were not sufficient in preventing AR proteolysis when stored and later analyzed. 3.) Storage temperature: Samples stored for 1 day at -20° resulted in an increase in AR fragments. Analysis of samples stored for three weeks during which they were thawed and frozen five times resulted in an increase in AR fragments and no FL-AR [189, 190].

In this study, we did not add DHT to our samples upon extraction. Our samples were immediately stored in -80°C but were not processed until weeks later. In addition, the samples were thawed and frozen more than five times throughout the experimental process. These differences between our processing may be the confounding factor in our low FL-AR expression in the rat testes, a FL-AR positive tissue. The loss of protein due to freeze/thaw cycles have been seen in human samples. For example, post-mortem brain tissue needs to be processed quickly and immediately frozen at – 80°C to maintain the integrity of the proteins [191-194]. It is possible that examining AR45 expression in human brain tissue may be difficult due to temperature and time constraints.

Another limitation of this study is the N27 cell line. N27 cells are derived from female rat mesencephalic tissues. Under *in vivo* conditions, cells interact through a network of various cell types. However, cell lines are isolated and deprived of afferent and efferent signals. Cell lines

devoid of neural networks may influence results. Therefore, cell lines are an imperfect model of biological functions.

3.4 FUTURE STUDIES

Our future studies include examining other brain regions for AR45. Based on prior studies that found cell impermeable testosterone induced fast non-genomic effects in primary cortical neurons and a glioma cell line [55, 57], it is probable that AR45 may also be present in these cell lines. Therefore, we would like to examine AR45 and FL-AR expression in those cell lines.

In addition to examining protein, we plan to determine the rat AR45 sequence of the RNA transcript via rapid amplification of cDNA ends (RACE) and reverse transcription polymerase chain reaction (RT-PCR). The cDNA product will then be cloned and sequenced. Upon sequencing, we can determine function by transfection and knock-down of AR45 using siRNA. Sequencing will also allow us to create site specific RNA primers and antibodies for AR45.

Postmenopausal women have higher oxidative stress levels and are at a greater risk for PD than premenopausal women [195-199]. Also, menopause results in a decline in estrogen, yet the ovaries continue to secrete androgens [200]. This leads to an androgenic state in which the estrogen to androgen ratio is weighted toward androgens. The androgenic state and increased oxidative stress in postmenopausal women may be contributing factors to the increased risk for PD. To determine whether the effects of androgens on neurodegenerative disease is due to the genetic sex or the hormones we will study young and old androgenic female rats.

REFERENCES

1. Nussey, S. and S. Whitehead, *Principles of Endocrinology*, in *Endocrinology: An Integrated Approach*. 2001, Oxford: BIOS Scientific Publishers: St. George's Hospital Medical School, London, UK.
2. Nuclear Receptors Nomenclature, C., *A unified nomenclature system for the nuclear receptor superfamily.* Cell, 1999. **97**(2): p. 161-3.
3. Handelsman, D.J., *Androgen Physiology, Pharmacology and Abuse*, in *Endotext*, L.J. De Groot, et al., Editors. 2016: South Dartmouth (MA).
4. Rey, R.A. and R.P. Grinspon, *Normal male sexual differentiation and aetiology of disorders of sex development.* Best Pract Res Clin Endocrinol Metab, 2011. **25**(2): p. 221-38.
5. Fiebitz, A., et al., *High-throughput mammalian two-hybrid screening for protein-protein interactions using transfected cell arrays.* BMC Genomics, 2008. **9**: p. 68.
6. Sinclair, A.H., et al., *A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif.* Nature, 1990. **346**(6281): p. 240-4.
7. Siiteri, P.K. and J.D. Wilson, *Testosterone formation and metabolism during male sexual differentiation in the human embryo.* J Clin Endocrinol Metab, 1974. **38**(1): p. 113-25.
8. Nussey, S. and S. Whitehead, *The Gonad*, in *Endocrinology: An Integrated Approach*. 2001, Oxford: BIOS Scientific Publishers: St. George's Hospital Medical School, London, UK.
9. O' Donnell, L., P. Stanton, and D.M. de Krester, *Endocrinology of the Male Reproductive System and Spermatogenesis*, in *Endotext*, L.J. De Groot, et al., Editors. 2017: South Dartmouth (MA).

10. Pagana, K., T.J. Pagana, and T.N. Pagana, *Mosby's Diagnostic and Laboratory Test Reference*, in *Testosterone*. 2015, Elsevier. p. 879.

11. Corradi, P.F., R.B. Corradi, and L.W. Greene, *Physiology of the Hypothalamic Pituitary Gonadal Axis in the Male.* Urol Clin North Am, 2016. **43**(2): p. 151-62.

12. Grino, P.B., J.E. Griffin, and J.D. Wilson, *Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone.* Endocrinology, 1990. **126**(2): p. 1165-72.

13. Attramadal, A., et al., *Androgen binding and metabolism in the human prostate.* Vitam Horm, 1975. **33**: p. 247-64.

14. Naess, O., et al., *Characterization of the androgen receptor in the anterior pituitary of the rat.* Endocrinology, 1975. **97**(6): p. 1355-63.

15. McPhaul, M.J. and M. Young, *Complexities of androgen action.* J Am Acad Dermatol, 2001. **45**(3 Suppl): p. S87-94.

16. Lubahn, D.B., et al., *Cloning of human androgen receptor complementary DNA and localization to the X chromosome.* Science, 1988. **240**(4850): p. 327-30.

17. Chang, C.S., J. Kokontis, and S.T. Liao, *Molecular cloning of human and rat complementary DNA encoding androgen receptors.* Science, 1988. **240**(4850): p. 324-6.

18. Brinkmann, A.O., et al., *Structure and function of the androgen receptor.* Urol Res, 1989. **17**(2): p. 87-93.

19. Trapman, J., et al., *Cloning, structure and expression of a cDNA encoding the human androgen receptor.* Biochem Biophys Res Commun, 1988. **153**(1): p. 241-8.

20. Jenster, G., et al., *Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization.* Mol Endocrinol, 1991. **5**(10): p. 1396-404.

21. Edwards, J. and J.M. Bartlett, *The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 2: Androgen-receptor cofactors and bypass pathways.* BJU Int, 2005. **95**(9): p. 1327-35.

22. Brinkmann, A.O., *Steroid Horomone Receptors: Activators of Gene Transcription* Steroid Hormone Receptors, 1994. **7**(4): p. 275-282.

23. Claessens, F., et al., *Diverse roles of androgen receptor (AR) domains in AR-mediated signaling.* Nucl Recept Signal, 2008. **6**: p. e008.

24. McEwan, I. and A.O. Brinkmann, *Androgen Physiology: Receptor and Metabolic Disorders*, in *Endotext*, L.J. De Groot, et al., Editors. 2000.

25. Brown, T.R., et al., *Deletion of the steroid-binding domain of the human androgen receptor gene in one family with complete androgen insensitivity syndrome: evidence for further genetic heterogeneity in this syndrome.* Proc Natl Acad Sci U S A, 1988. **85**(21): p. 8151-5.

26. Jenster, G., et al., *Functional domains of the human androgen receptor.* J Steroid Biochem Mol Biol, 1992. **41**(3-8): p. 671-5.

27. Simental, J.A., et al., *Transcriptional activation and nuclear targeting signals of the human androgen receptor.* J Biol Chem, 1991. **266**(1): p. 510-8.

28. Hiipakka, R.A. and S. Liao, *Molecular mechanism of androgen action.* Trends Endocrinol Metab, 1998. **9**(8): p. 317-24.

29. Dehm, S.M. and D.J. Tindall, *Alternatively spliced androgen receptor variants.* Endocr Relat Cancer, 2011. **18**(5): p. R183-96.

30. Ding, D., et al., *Effect of a short CAG (glutamine) repeat on human androgen receptor function.* Prostate, 2004. **58**(1): p. 23-32.

31. Ding, D., et al., *Effect of GGC (glycine) repeat length polymorphism in the human androgen receptor on androgen action.* Prostate, 2005. **62**(2): p. 133-9.

32. Ferro, P., et al., *The androgen receptor CAG repeat: a modifier of carcinogenesis?* Mol Cell Endocrinol, 2002. **193**(1-2): p. 109-20.

33. Quigley, C.A., et al., *Androgen receptor defects: historical, clinical, and molecular perspectives.* Endocr Rev, 1995. **16**(3): p. 271-321.

34. Wang, Y., et al., *Androgen receptor coregulators NOCR1, TIF2, and ARA70 may account for the hydroxyflutamide insensitivity of prostate cancer cells.* Ir J Med Sci, 2011. **180**(4): p. 865-72.

35. Edwards, J. and J.M. Bartlett, *The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 1: Modifications to the androgen receptor.* BJU Int, 2005. **95**(9): p. 1320-6.

36. Bennett, N.C., et al., *Molecular cell biology of androgen receptor signalling.* Int J Biochem Cell Biol, 2010. **42**(6): p. 813-27.

37. Gioeli, D. and B.M. Paschal, *Post-translational modification of the androgen receptor.* Mol Cell Endocrinol, 2012. **352**(1-2): p. 70-8.

38. Lee, D.K. and C. Chang, *Endocrine mechanisms of disease: Expression and degradation of androgen receptor: mechanism and clinical implication.* J Clin Endocrinol Metab, 2003. **88**(9): p. 4043-54.

39. Brock, O., C. De Mees, and J. Bakker, *Hypothalamic expression of oestrogen receptor alpha and androgen receptor is sex-, age- and region-dependent in mice.* J Neuroendocrinol, 2015. **27**(4): p. 264-76.

40. Kritzer, M.F., *Selective colocalization of immunoreactivity for intracellular gonadal hormone receptors and tyrosine hydroxylase in the ventral tegmental area, substantia nigra, and retrorubral fields in the rat.* J Comp Neurol, 1997. **379**(2): p. 247-60.

41. Holmes, S., et al., *Effects of Oxidative Stress and Testosterone on Pro-Inflammatory Signaling in a Female Rat Dopaminergic Neuronal Cell Line.* Endocrinology, 2016. **157**(7): p. 2824-35.

42. Galani, A., et al., *Androgen insensitivity syndrome: clinical features and molecular defects.* Hormones (Athens), 2008. **7**(3): p. 217-29.

43. Ahrens-Fath, I., et al., *Androgen receptor function is modulated by the tissue-specific AR45 variant.* FEBS J, 2005. **272**(1): p. 74-84.

44. Chen, J. and W.A. Weiss, *Alternative splicing in cancer: implications for biology and therapy.* Oncogene, 2015. **34**(1): p. 1-14.

45. Haendler, B., *Androgens and pharmacology.* Andrologia, 2005. **37**(6): p. 213-5.

46. Barnett-Norris, J., D. Lynch, and P.H. Reggio, *Lipids, lipid rafts and caveolae: their importance for GPCR signaling and their centrality to the endocannabinoid system.* Life Sci, 2005. **77**(14): p. 1625-39.

47. Weiss, B., H. Faus, and B. Haendler, *Phylogenetic conservation of the androgen receptor AR45 variant form in placental mammals.* Gene, 2007. **399**(2): p. 105-11.

48. Hu, D.G., et al., *Identification of androgen receptor splice variant transcripts in breast cancer cell lines and human tissues.* Horm Cancer, 2014. **5**(2): p. 61-71.

49. Guo, Z. and Y. Qiu, *A new trick of an old molecule: androgen receptor splice variants taking the stage?!* Int J Biol Sci, 2011. **7**(6): p. 815-22.

50. Pelekanou, V., et al., *Androgen receptors in early and castration resistant prostate cancer: friend or foe?* Hormones (Athens), 2013. **12**(2): p. 224-35.

51. Benten, W.P., et al., *Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages.* Mol Biol Cell, 1999. **10**(10): p. 3113-23.

52. Holmes, S., et al., *Oxidative stress defines the neuroprotective or neurotoxic properties of androgens in immortalized female rat dopaminergic neuronal cells.* Endocrinology, 2013. **154**(11): p. 4281-92.

53. Neri, R., E. Peets, and A. Watnick, *Anti-androgenicity of flutamide and its metabolite Sch 16423.* Biochem Soc Trans, 1979. **7**(3): p. 565-9.

54. Estrada, M., et al., *Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells.* Endocrinology, 2003. **144**(8): p. 3586-97.

55. Gatson, J.W. and M. Singh, *Activation of a membrane-associated androgen receptor promotes cell death in primary cortical astrocytes.* Endocrinology, 2007. **148**(5): p. 2458-64.

56. Benten, W.P., et al., *Rapid effects of androgens in macrophages.* Steroids, 2004. **69**(8-9): p. 585-90.

57. Gatson, J.W., P. Kaur, and M. Singh, *Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells.* Endocrinology, 2006. **147**(4): p. 2028-34.

58. Hatzoglou, A., et al., *Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo.* J Clin Endocrinol Metab, 2005. **90**(2): p. 893-903.

59. Vicencio, J.M., et al., *Testosterone induces an intracellular calcium increase by a nongenomic mechanism in cultured rat cardiac myocytes.* Endocrinology, 2006. **147**(3): p. 1386-95.

60. Estrada, M., P. Uhlen, and B.E. Ehrlich, *Ca2+ oscillations induced by testosterone enhance neurite outgrowth.* J Cell Sci, 2006. **119**(Pt 4): p. 733-43.

61. Estrada, M., A. Varshney, and B.E. Ehrlich, *Elevated testosterone induces apoptosis in neuronal cells.* J Biol Chem, 2006. **281**(35): p. 25492-501.

62. Gorczynska, E. and D.J. Handelsman, *Androgens rapidly increase the cytosolic calcium concentration in Sertoli cells.* Endocrinology, 1995. **136**(5): p. 2052-9.

63. Lieberherr, M. and B. Grosse, *Androgens increase intracellular calcium concentration and inositol 1,4,5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein.* J Biol Chem, 1994. **269**(10): p. 7217-23.

64. Sengupta, P., F. Philip, and S. Scarlata, *Caveolin-1 alters Ca(2+) signal duration through specific interaction with the G alpha q family of G proteins.* J Cell Sci, 2008. **121**(Pt 9): p. 1363-72.

65. Steinsapir, J., R. Socci, and P. Reinach, *Effects of androgen on intracellular calcium of LNCaP cells.* Biochem Biophys Res Commun, 1991. **179**(1): p. 90-96.

66. Strubing, C., et al., *Development of G protein-mediated Ca2+ channel regulation in mouse embryonic stem cell-derived neurons.* Eur J Neurosci, 1997. **9**(4): p. 824-32.

67. Sun, Y.H., et al., *Androgens induce increases in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells.* J Androl, 2006. **27**(5): p. 671-8.

68. Surmeier, D.J., J.N. Guzman, and J. Sanchez-Padilla, *Calcium, cellular aging, and selective neuronal vulnerability in Parkinson's disease.* Cell Calcium, 2010. **47**(2): p. 175-82.

69. Surmeier, D.J., et al., *The origins of oxidant stress in Parkinson's disease and therapeutic strategies.* Antioxid Redox Signal, 2011. **14**(7): p. 1289-301.

70. Berridge, M.J., *Neuronal calcium signaling.* Neuron, 1998. **21**(1): p. 13-26.

71. Guzman, J.N., et al., *Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1.* Nature, 2010. **468**(7324): p. 696-700.

72. Hescheler, J., et al., *The GTP-binding protein, Go, regulates neuronal calcium channels.* Nature, 1987. **325**(6103): p. 445-7.

73. Jiang, M., et al., *Multiple neurological abnormalities in mice deficient in the G protein Go.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3269-74.

74. Kamato, D., et al., *Structure, Function, Pharmacology, and Therapeutic Potential of the G Protein, Galpha/q,11.* Front Cardiovasc Med, 2015. **2**: p. 14.

75. Weerth, S.H., L.A. Holtzclaw, and J.T. Russell, *Signaling proteins in raft-like microdomains are essential for Ca2+ wave propagation in glial cells.* Cell Calcium, 2007. **41**(2): p. 155-67.

76. Campbell, V., et al., *Inhibition of the interaction of G protein Go with calcium channels by the calcium channel B-subunit in rat neurones.* Journal of Physiology, 1995. **485**(2): p. 365-372.

77. Pedram, A., et al., *A conserved mechanism for steroid receptor translocation to the plasma membrane.* J Biol Chem, 2007. **282**(31): p. 22278-88.

78. Chini, B. and M. Parenti, *G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there?* Journal of Molecular Endrocrinology, 2004. **32**: p. 325-338.

79. Hiol, A., et al., *Palmitoylation regulates regulators of G-protein signaling (RGS) 16 function. I. Mutation of amino-terminal cysteine residues on RGS16 prevents its targeting*

to lipid rafts and palmitoylation of an internal cysteine residue. J Biol Chem, 2003. **278**(21): p. 19301-8.

80. Ostrom, R.S. and P.A. Insel, *The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology.* Br J Pharmacol, 2004. **143**(2): p. 235-45.

81. Villar, V.A., et al., *Localization and signaling of GPCRs in lipid rafts.* Methods Cell Biol, 2016. **132**: p. 3-23.

82. Cacabelos, R., *Parkinson's Disease: From Pathogenesis to Pharmacogenomics.* Int J Mol Sci, 2017. **18**(3).

83. Kowal, S.L., et al., *The current and projected economic burden of Parkinson's disease in the United States.* Mov Disord, 2013. **28**(3): p. 311-8.

84. Schrag, A., Y. Ben-Shlomo, and N.P. Quinn, *Cross sectional prevalence survey of idiopathic Parkinson's disease and Parkinsonism in London.* BMJ, 2000. **321**(7252): p. 21-2.

85. Gillies, G.E., et al., *Sex dimorphisms in the neuroprotective effects of estrogen in an animal model of Parkinson's disease.* Pharmacol Biochem Behav, 2004. **78**(3): p. 513-22.

86. Cunningham, R.L., et al., *Androgens exacerbate motor asymmetry in male rats with unilateral 6-hydroxydopamine lesion.* Horm Behav, 2011. **60**(5): p. 617-24.

87. Lu, J.P., et al., *Androgens induce oxidative stress and radiation resistance in prostate cancer cells though NADPH oxidase.* Prostate Cancer Prostatic Dis, 2010. **13**(1): p. 39-46.

88. Chignalia, A.Z., et al., *Testosterone induces vascular smooth muscle cell migration by NADPH oxidase and c-Src-dependent pathways.* Hypertension, 2012. **59**(6): p. 1263-71.

89. Cunningham, R.L., A. Giuffrida, and J.L. Roberts, *Androgens induce dopaminergic neurotoxicity via caspase-3-dependent activation of protein kinase Cdelta.* Endocrinology, 2009. **150**(12): p. 5539-48.

90. Pike, C.J., et al., *Protective actions of sex steroid hormones in Alzheimer's disease.* Frontiers in neuroendocrinology, 2009. **30**(2): p. 239-58.

91. Pike, C.J., E.R. Rosario, and T.V. Nguyen, *Androgens, aging, and Alzheimer's disease.* Endocrine, 2006. **29**(2): p. 233-41.

92. Rosario, E.R., et al., *Brain levels of sex steroid hormones in men and women during normal aging and in Alzheimer's disease.* Neurobiology of aging, 2011. **32**(4): p. 604-13.

93. Verdile, G., et al., *Associations between gonadotropins, testosterone and beta amyloid in men at risk of Alzheimer's disease.* Molecular psychiatry, 2014. **19**(1): p. 69-75.

94. Kritzer, M.F. and L.M. Creutz, *Region and sex differences in constituent dopamine neurons and immunoreactivity for intracellular estrogen and androgen receptors in mesocortical projections in rats.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2008. **28**(38): p. 9525-35.

95. McEwen, B.S., *Binding and metabolism of sex steroids by the hypothalamic-pituitary unit: Physiological implications.* Ann Rev Physiol, 1980. **42**: p. 97-110.

96. Song, C.S., et al., *Androgen receptor messenger ribonucleic acid (mRNA) in the rat liver: changes in mRNA levels during maturation, aging, and calorie restriction.* Endocrinology, 1991. **128**(1): p. 349-56.

97. Ozanne, D.M., et al., *Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin.* Mol Endocrinol, 2000. **14**(10): p. 1618-26.

98. Green, S.M., E.A. Mostaghel, and P.S. Nelson, *Androgen action and metabolism in prostate cancer.* Mol Cell Endocrinol, 2012. **360**(1-2): p. 3-13.

99. Smith, D.F. and D.O. Toft, *Minireview: the intersection of steroid receptors with molecular chaperones: observations and questions.* Mol Endocrinol, 2008. **22**(10): p. 2229-40.

100. Callewaert, L., N. Van Tilborgh, and F. Claessens, *Interplay between two hormone-independent activation domains in the androgen receptor.* Cancer Res, 2006. **66**(1): p. 543-53.

101. Foradori, C.D., M.J. Weiser, and R.J. Handa, *Non-genomic actions of androgens.* Front Neuroendocrinol, 2008. **29**(2): p. 169-81.

102. Modrek, B. and C. Lee, *A genomic view of alternative splicing.* Nat Genet, 2002. **30**(1): p. 13-9.

103. Roberts, G.C. and C.W. Smith, *Alternative splicing: combinatorial output from the genome.* Curr Opin Chem Biol, 2002. **6**(3): p. 375-83.

104. Stamm, S., *Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome.* Hum Mol Genet, 2002. **11**(20): p. 2409-2416.

105. Hu, R., et al., *Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer.* Cancer Res, 2012. **72**(14): p. 3457-62.

106. Jagla, M., et al., *A splicing variant of the androgen receptor detected in a metastatic prostate cancer exhibits exclusively cytoplasmic actions.* Endocrinology, 2007. **148**(9): p. 4334-43.

107. Sun, S., et al., *Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant.* J Clin Invest, 2010. **120**(8): p. 2715-30.

108. Watson, P.A., et al., *Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor.* Proc Natl Acad Sci U S A, 2010. **107**(39): p. 16759-65.

109. Yang, X., et al., *Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival responses in prostate cancer cells.* J Biol Chem, 2011. **286**(41): p. 36152-60.

110. Ikonen, T., J.J. Palvimo, and O.A. Janne, *Heterodimerization is mainly responsible for the dominant negative activity of amino-terminally truncated rat androgen receptor forms.* FEBS Lett, 1998. **430**(3): p. 393-6.

111. Wu, Z.Y., et al., *Stimulation of N-terminal truncated isoform of androgen receptor stabilizes human ether-a-go-go-related gene-encoded potassium channel protein via activation of extracellular signal regulated kinase 1/2.* Endocrinology, 2008. **149**(10): p. 5061-9.

112. Anantharam, V., et al., *Microarray analysis of oxidative stress regulated genes in mesencephalic dopaminergic neuronal cells: relevance to oxidative damage in Parkinson's disease.* Neurochemistry international, 2007. **50**(6): p. 834-47.

113. Carvour, M., et al., *Chronic low-dose oxidative stress induces caspase-3-dependent PKCdelta proteolytic activation and apoptosis in a cell culture model of dopaminergic neurodegeneration.* Annals of the New York Academy of Sciences, 2008. **1139**: p. 197-205.

114. Clarkson, E.D., et al., *Immortalized dopamine neurons: A model to study neurotoxicity and neuroprotection.* Proc Soc Exp Biol Med, 1999. **222**(2): p. 157-63.

115. Smith, K.W., H.A. Feldman, and J.B. McKinlay, *Construction and field validation of a self-administered screener for testosterone deficiency (hypogonadism) in ageing men.* Clin Endocrinol (Oxf), 2000. **53**(6): p. 703-11.

116. Zitzmann, M., M. Brune, and E. Nieschlag, *Vascular reactivity in hypogonadal men is reduced by androgen substitution.* J Clin Endocrinol Metab, 2002. **87**(11): p. 5030-7.

117. Kelly, S.J., N.L. Ostrowski, and M.A. Wilson, *Gender differences in brain and behavior: hormonal and neural bases.* Pharmacol Biochem Behav, 1999. **64**(4): p. 655-64.

118. Mooradian, A.D., J.E. Morley, and S.G. Korenman, *Biological actions of androgens.* Endocr. Rev., 1987. **8**(1): p. 1-28.

119. Jeske, N.A., M.J. Glucksman, and J.L. Roberts, *EP24.15 is associated with lipid rafts.* J.Neurosci.Res., 2003. **74**(3): p. 468-473.

120. Jeske, N.A., M.J. Glucksman, and J.L. Roberts, *Metalloendopeptidase EC3.4.24.15 is constitutively released from the exofacial leaflet of lipid rafts in GT1-7 cells.* J.Neurochem., 2004. **90**(4): p. 819-828.

121. Song, K.S., et al., *Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains.* J.Biol.Chem., 1996. **271**(16): p. 9690-9697.

122. Link, A.J. and J. LaBaer, *Trichloroacetic acid (TCA) precipitation of proteins.* Cold Spring Harb Protoc, 2011. **2011**(8): p. 993-4.

123. Gottlieb, H.B., et al., *Differential effects of water and saline intake on water deprivation-induced c-Fos staining in the rat.* Am.J.Physiol Regul.Integr.Comp Physiol, 2006. **290**(5): p. R1251-R1261.

124. Ji, L.L., et al., *Differential effects of water deprivation and rehydration on Fos and FosB/DeltaFosB staining in the rat brainstem.* Exp.Neurol., 2007. **203**(2): p. 445-456.

125. Nedungadi, T.P., et al., *Expression and distribution of TRPV2 in rat brain.* Experimental neurology, 2012. **237**(1): p. 223-37.

126. Cummings, J.A., et al., *Male rats that differ in novelty exploration demonstrate distinct patterns of sexual behavior.* Behav Neurosci, 2013. **127**(1): p. 47-58.

127. Aumuller, G., et al., *Effects of sexual rest or sexual activity on the structure and function of the ventral prostate of the rat.* Anat Rec, 1985. **212**(4): p. 345-52.

128. Drori, D. and Y. Folman, *Effects of Cohabitation on the Reproductive System, Kidneys and Body Composition of Male Rats.* J Reprod Fertil, 1964. **8**: p. 351-9.

129. Thomas, T.R. and C.N. Neiman, *Aspects of copulatory behavior preventing atrophy in male rats' reproductive system.* Endocrinology, 1968. **83**(3): p. 633-5.

130. Baldereschi, M., et al., *Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. ILSA Working Group. Italian Longitudinal Study on Aging.* Neurology, 2000. **55**(9): p. 1358-63.

131. Marin, R., et al., *An oestrogen membrane receptor participates in estradiol actions for the prevention of amyloid-beta peptide1-40-induced toxicity in septal-derived cholinergic SN56 cells.* J Neurochem, 2003. **85**(5): p. 1180-9.

132. Razandi, M., et al., *ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions.* Mol Endocrinol, 2002. **16**(1): p. 100-15.

133. Kim, H.P., et al., *Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae.* Biochem Biophys Res Commun, 1999. **263**(1): p. 257-62.

134. Kelly, M.J. and E.J. Wagner, *Estrogen Modulation of G-protein-coupled Receptors.* Trends Endocrinol Metab, 1999. **10**(9): p. 369-374.

135. Trushina, E., et al., *Neurological abnormalities in caveolin-1 knock out mice.* Behav Brain Res, 2006. **172**(1): p. 24-32.

136. Hashimoto, M., et al., *Alpha-synuclein up-regulates expression of caveolin-1 and down-regulates extracellular signal-regulated kinase activity in B103 neuroblastoma cells: role in the pathogenesis of Parkinson's disease.* J Neurochem, 2003. **85**(6): p. 1468-79.

137. Schengrund, C.L., *Lipid rafts: keys to neurodegeneration.* Brain Res Bull, 2010. **82**(1-2): p. 7-17.

138. Sonnino, S., et al., *Lipid rafts in neurodegeneration and neuroprotection.* Mol Neurobiol, 2014. **50**(1): p. 130-48.

139. Cha, S.H., et al., *Loss of parkin promotes lipid rafts-dependent endocytosis through accumulating caveolin-1: implications for Parkinson's disease.* Mol Neurodegener, 2015. **10**: p. 63.

140. Fallon, L., et al., *Parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain.* J Biol Chem, 2002. **277**(1): p. 486-91.

141. Fortin, D.L., et al., *Lipid rafts mediate the synaptic localization of alpha-synuclein.* J Neurosci, 2004. **24**(30): p. 6715-23.

142. Brown, D.A. and J.K. Rose, *Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface.* Cell, 1992. **68**(3): p. 533-44.

143. Brown, D.A. and E. London, *Structure and function of sphingolipid- and cholesterol-rich membrane rafts.* J Biol Chem, 2000. **275**(23): p. 17221-4.

144. Mineo, C., G.N. Gill, and R.G. Anderson, *Regulated migration of epidermal growth factor receptor from caveolae.* J Biol Chem, 1999. **274**(43): p. 30636-43.

145. Rybin, V.O., et al., *Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway.* J Biol Chem, 2000. **275**(52): p. 41447-57.

146. Lasley, R.D., et al., *Activated cardiac adenosine A(1) receptors translocate out of caveolae.* J Biol Chem, 2000. **275**(6): p. 4417-21.

147. Pucadyil, T.J. and A. Chattopadhyay, *Cholesterol modulates ligand binding and G-protein coupling to serotonin(1A) receptors from bovine hippocampus.* Biochim Biophys Acta, 2004. **1663**(1-2): p. 188-200.

148. Xu, W., et al., *Localization of the kappa opioid receptor in lipid rafts.* J Pharmacol Exp Ther, 2006. **317**(3): p. 1295-306.

149. Allen, J.A., et al., *Beta-adrenergic receptor stimulation promotes G alpha s internalization through lipid rafts: a study in living cells.* Mol Pharmacol, 2005. **67**(5): p. 1493-504.

150. Toki, S., R.J. Donati, and M.M. Rasenick, *Treatment of C6 glioma cells and rats with antidepressant drugs increases the detergent extraction of G(s alpha) from plasma membrane.* J Neurochem, 1999. **73**(3): p. 1114-20.

151. Rebois, R.V. and T.E. Hebert, *Protein complexes involved in heptahelical receptor-mediated signal transduction.* Receptors Channels, 2003. **9**(3): p. 169-94.

152. Lang, D.M., et al., *Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons.* J Neurobiol, 1998. **37**(4): p. 502-23.

153. Murata, M., et al., *VIP21/caveolin is a cholesterol-binding protein.* Proc Natl Acad Sci U S A, 1995. **92**(22): p. 10339-43.

154. Bickel, P.E., et al., *Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins.* J Biol Chem, 1997. **272**(21): p. 13793-802.

155. Kang, M.J., et al., *Caveolin-1 upregulation in senescent neurons alters amyloid precursor protein processing.* Exp Mol Med, 2006. **38**(2): p. 126-33.

156. Marquet-de Rouge, P., et al., *Citrulline diet supplementation improves specific age-related raft changes in wild-type rodent hippocampus.* Age (Dordr), 2013. **35**(5): p. 1589-606.

157. Volonte, D., et al., *Cellular stress induces the tyrosine phosphorylation of caveolin-1 (Tyr(14)) via activation of p38 mitogen-activated protein kinase and c-Src kinase. Evidence for caveolae, the actin cytoskeleton, and focal adhesions as mechanical sensors of osmotic stress.* J Biol Chem, 2001. **276**(11): p. 8094-103.

158. Galbiati, F., et al., *Expression of caveolin-1 and -2 in differentiating PC12 cells and dorsal root ganglion neurons: caveolin-2 is up-regulated in response to cell injury.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10257-62.

159. Lotharius, J., et al., *Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line.* J Biol Chem, 2002. **277**(41): p. 38884-94.

160. Hastings, T.G., *The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease.* J Bioenerg Biomembr, 2009. **41**(6): p. 469-72.

161. Jenner, P., et al., *Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group.* Ann Neurol, 1992. **32 Suppl**: p. S82-7.

162. Dickson, D.W., *Linking selective vulnerability to cell death mechanisms in Parkinson's disease.* The American journal of pathology, 2007. **170**(1): p. 16-9.

163. Sherer, T.B., et al., *An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(16): p. 7006-15.

164. Oh, P. and J.E. Schnitzer, *Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default.* Mol Biol Cell, 2001. **12**(3): p. 685-98.

165. Murthy, K.S. and G.M. Makhlouf, *Heterologous desensitization mediated by G protein-specific binding to caveolin.* J Biol Chem, 2000. **275**(39): p. 30211-9.

166. Shi, C.S. and J.H. Kehrl, *PYK2 links G(q)alpha and G(13)alpha signaling to NF-kappa B activation.* J Biol Chem, 2001. **276**(34): p. 31845-50.

167. Rhee, S.G., *Regulation of phosphoinositide-specific phospholipase C.* Annu Rev Biochem, 2001. **70**: p. 281-312.

168. Surmeier, D.J., et al., *Physiological phenotype and vulnerability in Parkinson's disease.* Cold Spring Harb Perspect Med, 2012. **2**(7): p. a009290.

169. Worley, P.F., et al., *Go, a guanine nucleotide-binding protein: immunohistochemical localization in rat brain resembles distribution of second messenger systems.* Proc Natl Acad Sci U S A, 1986. **83**(12): p. 4561-5.

170. Lewis, D.L., F.F. Weight, and A. Luini, *A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary cell line.* Proc Natl Acad Sci U S A, 1986. **83**(23): p. 9035-9.

171. Jiang, M., et al., *Most central nervous system D2 dopamine receptors are coupled to their effectors by Go.* Proc Natl Acad Sci U S A, 2001. **98**(6): p. 3577-82.

172. Urban, J.D. and R.B. Mailman. *Characterization of the N27 dopaminergic cell line as a potential model for elucidating the mechanisms of action of functionally selective dopamienrgic ligands*. in *Society for Neuroscience*. 2005. Washington DC: Society for Neuroscience.

173. Falkenstein, E., et al., *Multiple actions of steroid hormones--a focus on rapid, nongenomic effects.* Pharmacol Rev, 2000. **52**(4): p. 513-56.

174. Kampa, M., et al., *Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis.* Experimental cell research, 2005. **307**(1): p. 41-51.

175. Young, W.J. and C. Chang, *Ontogeny and autoregulation of androgen receptor mRNA expression in the nervous system.* Endocrine, 1998. **9**(1): p. 79-88.

176. Chen, K., X. Dai, and J. Wu, *Alternative splicing: An important mechanism in stem cell biology.* World J Stem Cells, 2015. **7**(1): p. 1-10.

177. Lu, C. and J. Luo, *Decoding the androgen receptor splice variants.* Transl Androl Urol, 2013. **2**(3): p. 178-186.

178. Head, B.P., H.H. Patel, and P.A. Insel, *Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling.* Biochim Biophys Acta, 2014. **1838**(2): p. 532-45.

179. Pike, L.J. and L. Casey, *Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains.* J Biol Chem, 1996. **271**(43): p. 26453-6.

180. Birnbaumer, L., *The discovery of signal transduction by G proteins: a personal account and an overview of the initial findings and contributions that led to our present understanding.* Biochim Biophys Acta, 2007. **1768**(4): p. 756-71.

181. Wang, H.Y., A.S. Undie, and E. Friedman, *Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: possible role in dopamine-mediated inositol phosphate formation.* Mol Pharmacol, 1995. **48**(6): p. 988-94.

182. Otto, G.P. and B.J. Nichols, *The roles of flotillin microdomains--endocytosis and beyond.* J Cell Sci, 2011. **124**(Pt 23): p. 3933-40.

183. Ariotti, N. and R.G. Parton, *SnapShot: caveolae, caveolins, and cavins.* Cell, 2013. **154**(3): p. 704-704 e1.

184. Pronsato, L., R. Boland, and L. Milanesi, *Non-classical localization of androgen receptor in the C2C12 skeletal muscle cell line.* Arch Biochem Biophys, 2013. **530**(1): p. 13-22.

185. Gandhi, S., et al., *PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death.* Mol Cell, 2009. **33**(5): p. 627-38.

186. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.

187. Alberts, B., A. Johnson, and J. Lewis, *Signaling through G-Protein-Linked Cell-Surface Receptors*, in *Molecular Biology of the Cell*. 2002, Garland Science: New York.

188. Martinez-Outschoorn, U.E., F. Sotgia, and M.P. Lisanti, *Caveolae and signalling in cancer.* Nat Rev Cancer, 2015. **15**(4): p. 225-37.

189. Gregory, C.W., B. He, and E.M. Wilson, *The putative androgen receptor-A form results from in vitro proteolysis.* J Mol Endocrinol, 2001. **27**(3): p. 309-19.

190. Kemppainen, J.A., et al., *Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones.* J Biol Chem, 1992. **267**(2): p. 968-74.

191. Samarasekera, N., et al., *Brain banking for neurological disorders.* Lancet Neurol, 2013. **12**(11): p. 1096-105.

192. Hulette, C.M., *Brain banking in the United States.* J Neuropathol Exp Neurol, 2003. **62**(7): p. 715-22.

193. Ravid, R. and I. Ferrer, *Brain banks as key part of biochemical and molecular studies on cerebral cortex involvement in Parkinson's disease.* FEBS J, 2012. **279**(7): p. 1167-76.

194. Ravid, R. and K. Ikemoto, *Pitfalls and practicalities in collecting and banking human brain tissues for research on psychiatric and neulogical disorders.* Fukushima J Med Sci, 2012. **58**(1): p. 82-7.

195. Currie, L.J., et al., *Postmenopausal estrogen use affects risk for Parkinson disease.* Arch Neurol, 2004. **61**(6): p. 886-8.

196. Ragonese, P., et al., *Multiple sclerosis survival: a population-based study in Sicily.* Eur J Neurol, 2010. **17**(3): p. 391-7.

197. Ragonese, P., et al., *Age at menopause predicts age at onset of Parkinson's disease.* Mov Disord, 2006. **21**(12): p. 2211-4.

198. Ragonese, P., M. D'Amelio, and G. Savettieri, *Implications for estrogens in Parkinson's disease: an epidemiological approach.* Ann N Y Acad Sci, 2006. **1089**: p. 373-82.

199. Signorelli, S.S., et al., *Behaviour of some indicators of oxidative stress in postmenopausal and fertile women.* Maturitas, 2006. **53**(1): p. 77-82.

200. Longcope, C., *Adrenal and gonadal androgen secretion in normal females.* Clin Endocrinol Metab, 1986. **15**(2): p. 213-28.