EFFECTS OF AGE ON MESOCORTICOLIMBIC TESTOSTERONE LEVELS AND ANDROGEN RECEPTORS IN MALE RATS

by

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B.Sc., The University of British Columbia, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2016

Abstract

Androgens, such as testosterone (T), are steroid hormones that exert effects on several tissues, including the brain, through interaction with androgen receptors (ARs). In the brain, androgens are traditionally known for modulation of reproductive behaviors mediated by classical regions rich in ARs. However, there is growing recognition of androgen involvement in higher-order cognitive processes, such as executive functions, which are mediated by non-classical brain regions like the prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA), which are part of the mesocorticolimbic system. In males, executive functions and serum T levels decline with age, but it is unclear how age impacts mesocorticolimbic ARs, and also mesocorticolimbic T levels. In these regions, ARs are present, but often at lower abundances per cell, and are difficult to detect immunochemically. Given the lack of information about mesocorticolimbic ARs and T, and how both may be altered by age, the main goals of this thesis were to: (1) improve immunochemical visualization of ARs, (2) phenotype prefrontal AR-expressing cells, and (3) examine how aging affects levels of ARs and neural T. In brief, we use a male rat model to demonstrate superior detection of ARs through application of tyramide signal amplification (TSA), confirm that prefrontal AR-expressing cells are neuronal and not glial, and show region-dependent reductions in ARs and neural T levels with age. More specifically, we show an age-associated decline in serum T and neural T, but an increase in the ratio of neural T: serum T, suggesting partially compensatory T production may occur in the aging brain. We also show an age-associated decrease in the amount of ARs in the PFC, but not the NAc or VTA. We conclude that the observed declines in T and AR levels may contribute to age-related impairment in executive functions. Furthermore, our results also contribute to improved visualization and examination of mesocorticolimbic ARs, and ultimately, a better understanding of the role they play in cognitive processes.

Preface

All animal research was conducted in accordance with Canadian Council on Animal Care (CCAC) and institutional guidelines. Research ethics approval was obtained from the UBC Animal Care Committee, project title “Modeling Deficits in Cognition in Rats: Behavioural and Electrophysiological Analyses”, Certificate Numbers A10-0197 and A14-0210.

Chapter 1 was researched and written by Katelyn L. Low.

Chapter 2 is based on work conducted by Katelyn L. Low, Chunqi Ma, Jennifer K. Ferris, Matthew D. Taves, Ryan J. Tomm, Maric T. Tse, Madison M. Grist, and Kiran K. Soma. KKS, CM, and KLL designed the experiments. KLL and CM developed the protocols. JKF, MDT, RJT, MTT, and MMG performed the tissue collection, and CM performed the brain sectioning. KLL performed the immunohistochemistry piloting, immunohistochemistry experiments, and microscopy; analyzed data; and wrote the manuscript.

Chapter 3 is based on work conducted by Katelyn L. Low, Ryan J. Tomm, Chunqi Ma, Madison M. Grist, Maric T. Tse, Anastasia Korol, and Kiran K. Soma. KKS designed the experiments. RJT, MTT, MMG, and KLL collected tissue. CM, MMG, and AK performed the steroid measurements. KLL developed the immunohistochemistry protocol and performed immunohistochemical staining, microscopy, and quantification. KLL analyzed data and wrote the manuscript.

Chapter 4 was researched and written by Katelyn L. Low.

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List of Abbreviations

AD	Alzheimer's Disease
AR	androgen receptor
BT	biotin tyramide
cVTA	caudal portion of the VTA
DA	dopamine
DAB	3,3'-diaminobenzidine
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
ER	estrogen receptor
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERK	extracellular signal-regulated kinases
F344/BN	Fischer 344 x Brown Norway
GDX	gonadectomy
GnRH	gonadotropin-releasing hormone
HPG	hypothalamic-pituitary-gonadal
HPLC	high-performance liquid chromatography
HRT	hormone replacement therapy
IHC	immunohistochemistry
IL	infralimbic subregion of the mPFC
IP	intraperitoneal
ir	immunoreactivity
LH	luteinizing hormone
LOFC	lateral orbitofrontal cortex
MAPK	mitogen-activated protein kinases
MCI	mild cognitive impairment
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
mRNA	messenger RNA
MTL	medial temporal lobe
NAc	nucleus accumbens
NAcC	nucleus accumbens core
NAcS	nucleus accumbens shell
OFC	orbitofrontal cortex
PADAM	partial androgen deficiency in aging men
PBP	parabrachial portion of the VTA
PBS	phosphate-buffered saline
PBS-GT	phosphate-buffered saline with gelatin and Triton X-100
PFC	prefrontal cortex
PN	paranigral portion of the VTA
PR	progesterone receptor
PrL	prelimbic subregion of the mPFC
pVTA	parabrachial and paranigral portion of the VTA
qPCR	quantitative (real-time) polymerase chain reaction
RIA	radioimmunoassay
ROI	region of interest
SPE	solid phase extraction
T	testosterone

TBS	Tris-buffered saline
TH	tyrosine hydroxylase
TSA	tyramide signal amplification
VTA	ventral tegmental area

Acknowledgments

The decision to pursue this Masters project was not an easy decision for me to make, but I am profoundly grateful that I decided to do it. From beginning to end, I have had the support of many people, without whom this project would not have been possible. I would like to express my sincerest and most heartfelt thanks to those individuals. This work was also supported by a CIHR grant to KS, and in part, by a Quinn Psychology Award to KL.

Firstly, thank you to my supervisor, Dr. Kiran Soma. Thank you for giving me opportunities as an undergraduate student, and for your patience and mentorship as I progressed through the challenges and successes of my project. Thank you for pushing me to express my opinions and ideas, and for teaching me to be a critical thinker and a skeptical (but never cynical) scientist.

Thank you to my committee members, Dr. Stan Floresco and Dr. Michael Gordon for your engagement with my project, support for my quick timeline, and valuable feedback.

Thank you to Dr. Cathy Ma, for sharing your expertise in chemistry and lab techniques, for assisting me with protocol optimization, and for patiently answering countless questions. Thank you to Soma Lab members, past and present: Drs. Nora Prior, Matthew Taves, Kim Schmidt, and Daniel Tobiansky; grad students Ryan Tomm and Jordan Hamden, and undergraduates Madison Grist, Anastasia Korol, Sara Marjanovic, and George Kachkovski. Thanks for making the lab a great place to work, and for providing training, motivation, advice, and assistance. Thank you to Drs. Dwayne Hamson, Jason Snyder, Sarah Heimovics, and also Jenn Ferris, Maric Tse, and Grace Truong, for technical advice, assistance, and generous use of lab equipment.

Thank you to my undergraduate research mentors and supervisors, Yvonne Krüger, Dr. Sarah Woolley, and Barbara Koot. Thanks for giving me some of my first opportunities to do research, allowing me the opportunity to learn from you, and inspiring interest in pursuing science.

On a more personal note, thank you to my parents, Albert and Debbie, my brother, Matthew, and my sister, Kimberly. You have always been my biggest supporters, and I am so grateful for your love and encouragement.

Chapter 1: Introduction

1.1 Gonadal Hormones and Brain Aging

During the aging process, there is a decline in the function of many key endocrine systems (Lamberts et al., 1997). These systems, including those involving the thyroid, pancreas, adrenals, and gonads, are responsible for production and regulation of hormones involved in a wide array of physiological processes, and as a result, their decline has significant impacts on health during aging (Lamberts et al., 1997). In particular, declines in gonadal hormones such as androgens and estrogens have important effects on the brain, and therefore important implications in age-associated neurocognitive disorders (Veiga et al., 2004; Pike et al., 2009). Gonadal hormones are known primarily for their effects in sexual differentiation and development, but have more recently become known to play a role in cognition, learning, and memory (Patchev et al., 2004). Consequently, their respective declines over the lifespan -- menopause in females, and andropause in males – are associated with symptoms of psychological decline. The relationship between menopause, cognition, and the aging brain in females has been well studied over the past few decades (Luine, 2014; McEwen et al., 1997), although parallel issues in males have received comparatively less attention.

In males, age-associated decreases in circulating androgen levels, such as testosterone (T) result in a number of brain changes, including impaired myelination and loss of synaptic density (Janowsky, 2006). Androgen declines are also related to a number of neuropsychiatric disorders, such as depression and Alzheimer's Disease (AD). Depression is associated with lower levels of bioavailable T (Barrett-Connor et al., 1991; Baulieu, 2002), while men with AD have been observed to have lower endogenous T levels (Moffatt et al., 2004), and there is strong evidence for neuroprotective effects of T in AD (Pike et al., 2009). In light of these

findings, the study of gonadal steroids is important to our understanding of neurocognitive pathologies of aging, and offers promising therapeutic potential to support healthful aging mentally and physically (Schumacher et al., 2003).

1.2 Andropause

Andropause refers to the gradual decrease in the levels of androgen hormones such as T and its metabolite dihydrotestosterone (DHT) that occurs during male aging (Morley and Perry, 1999; Vermeulen, 1991). Declines are observed in both bioavailable and total levels of serum T, as well as increases in sex hormone binding globulin, a carrier protein that binds androgens in the bloodstream (Swerdloff and Wang, 1993; Vermeulen et al., 1996; Baulieu, 2002), beginning around the third and fourth decade of life (Pike et al., 2009). From age 30-80, free T declines by as much as 50% (Beauchet, 2006; Lamberts et al., 1997), corresponding to about 100 ng/dl per decade (Sternbach, 1998; Bhasin et al., 2010; Harman et al., 2001), or 3-4% per year (Pike et al., 2009). Other sex hormones, such as estrogens, do not usually show decline in aged men (Janowsky et al., 1994; Swerdloff and Wang, 1993). This progressive decline in serum T was first reported in the 1970's, and has since been supported by a number of cross-sectional (see Vermeulen, 1991 for review) and longitudinal studies (Morley et al., 1997; Harman et al., 2001; Feldman et al., 2002; Kaufman and Vermeulen, 2005).

In contrast to menopause in females, andropause features a gradual decrease in hormone levels, and does not signal a complete and irreversible cessation of reproductive ability (Kaufman and Vermeulen, 2005). Furthermore, the decrease in androgen levels is not considered a universal characteristic of male aging, and shows great individual variation (Kaufman and Vermeulen, 2005). Some elderly men maintain testosterone levels similar to those of young men, while others do not (Vermeulen, 2000). It is estimated that one in five men over age 60 (Beauchet, 2006; Harman et al., 2001; Seidman and Walsh, 1999), and 50% of men aged over 80 have hypogonadal levels of serum T (Stanworth and Jones, 2008). Of these

hypogonadal men, only a subset will be symptomatic (Borst and Mulligan, 2007; Seidman and Weiser, 2013). For those reasons, terms like “late onset hypogonadism”, “partial androgen deficiency in aging men (PADAM)” (Baulieu, 2002; Seidman and Weiser, 2013; Pike et al., 2009), or “low-testosterone syndrome” are sometimes preferred over terms such as “andropause” or “male climacteric” that imply direct parallels to menopause (Sternbach, 1998).

Symptoms of andropause range from somatic to sexual to psychological in nature (Lund et al., 1999), and include frailty, reduced muscle mass and bone density, increased body fat, changes in emotion and mood, decreased libido and erectile dysfunction, sleep disturbances, and reduced memory and cognition (Swerdloff and Wang, 1993; Baulieu, 2002; Sternbach, 1998).

Aged men experiencing deficient androgen levels and symptoms of andropause can receive treatment with hormone replacement therapy (HRT) in the form of injections, pills, buccal tablets, gels, and transdermal patches (Bhasin et al., 2010). HRT can offer significant benefits, but is also accompanied by poorly understood potential risks, such as increasing the likelihood of prostate cancer (Bhasin et al., 2010; Sternbach, 1998; Schumacher et al., 2003; Borst and Mulligan, 2007).

It is important to note that in addition to aging, several other factors can influence androgen levels, including lifestyle, genetics, obesity, chronic diseases, and medication (Seidman and Weiser, 2013; Bhasin et al., 2010; Vermeulen, 2000).

1.3 Androgens

Androgens are a major subclass of steroid hormones, chiefly known for their contribution to differentiation, development, and maintenance of male phenotypic traits (Luu-The et al., 2008). Like all steroid hormones, they are small, lipophilic molecules derived from a cholesterol precursor, and therefore share a common four-ring carbon structure, and can easily cross the blood brain barrier and diffuse through cell membranes (Sotomayor-Zarate et al., 2014).

Androgens exert physiologic effects on a number of body systems (Seidman and Walsh, 1999), including the musculoskeletal, cardiovascular, reproductive, and nervous systems (Bhasin et al., 2001). In the nervous system, they play an important role in modulating a range of behaviours including sexual behaviours, aggression, territoriality, social dominance and submission, visual acuity, stress responses, spatial learning and memory, and other aspects of cognition (DonCarlos et al., 2006; Seidman and Walsh, 1999). Androgens also contribute to cellular processes in the nervous system such as growth and survival of neurons, expression of neurotransmitters, myelination, and adult neurogenesis (Don Carlos et al., 2006; Schumacher et al., 2003). During aging, androgens can perform neuroprotective actions, including protecting against oxidative stress and apoptosis, reducing β-amyloid secretion, inhibiting phosphorylation of tau, and helping neurons recovery after injury (Beauchet, 2006).

1.3.1 Systemic Androgen Production

In males, the majority of androgens are produced and secreted by Leydig cells of the testes, which are considered the primary provider of serum T (Luu-The et al., 2008). Androgen production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which involves negative feedback between the hypothalamus, anterior pituitary gland, and testes through their associated hormones. In general, T synthesis and secretion is stimulated by release of luteinizing hormone (LH) from the anterior pituitary gland. LH is released upon receiving stimulation from gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus. T secretion follows a diurnal rhythm, with its peak in the morning, and declining levels in the evening (Seidman and Walsh, 1999). Androgens can also be produced to a lesser extent by the adrenal glands in the form of dehydroepiandrosterone (DHEA), which can be metabolized to T in other tissues, including the brain**.** Notably, while DHEA is produced by the adrenals in humans, it is not produced in significant amounts by the adrenals of rats (Baulieu

and Robel, 1998). Age-associated declines in T are mainly attributed to decline in Leydig cell function, as well as dysfunction of the HPG axis (Swerdloff and Wang, 1993; Seidman and Walsh, 1999; Chen et al., 1994).

1.3.2 Androgen Metabolism

Major androgens include T, dihydrotestosterone (DHT), and their respective metabolites, which are produced via stepwise conversions from cholesterol. These conversions are dependent on a number of steroidogenic enzymes which maintain cell- and tissue-specific expression and concentrations (Mellon and Griffin, 2002; Seidman and Walsh, 1999). The principal androgen, T, is the most abundant, and can be metabolized in multiple ways to other biologically active steroids, including reduction via 5α-reductase to 5α-DHT, a more potent androgen with higher affinity for the androgen receptor (AR), and aromatization via cytochrome p450 aromatase to 17β-estradiol, an active estrogen which interacts with estrogen receptors (ERs) (Beyer et al., 1971; Lieberburg and McEwen, 1977).

1.3.3 Neural Androgen Production

According to the traditional endocrine understanding of androgen action, androgens are produced by endocrine organs such as the testes, and subsequently transported through the bloodstream to influence target tissues, such as muscle, bone, or the brain (Schmidt et al., 2008; Mukai et al., 2006; Baulieu and Robel, 1998). In this view, androgens act in a systemic manner to influence physiology or behavior, and the brain is primarily a regulator and target of hormones. However, more recent evidence also suggests that the brain itself is capable of producing androgens and other steroids that can interact locally with their intracellular receptors (Zwain and Yen, 1998; Gooren, 2007; Do Rego et al., 2009). Described by the term

"neurosteroid" (Corpechot et al., 1981), these brain-derived steroids could be produced via *de novo* synthesis from cholesterol, or conversion from precursors or metabolites (Schimdt et al., 2008; Schumacher et al., 2003). Expression of enzymes required for T and other sex steroids has been demonstrated in the human and rat brain (Zwain and Yen, 1998; Schumacher et al., 2003; Hojo et al., 2009; Hojo et al., 2004).

Importantly, brain and circulating levels of steroids are not equivalent counterparts, and blood levels are not always indicative of brain concentrations (Schumacher et al., 2003). As a result, the age-associated decline in systemic androgens may not be fully reflected in neural androgens (Schumacher et al., 2003). It has been suggested, but not rigorously explored, that neurosteroid synthesis may compensate for the decrease in systemic steroids in aging (Schumacher et al., 2003; Wu et al., 2009).

1.4 Androgen Receptor

Androgens can exert biological effects through interaction with their receptor, AR (Arnold and Gorski, 1984). A member of the nuclear steroid hormone receptor NR3 superfamily that also includes ERs, progesterone receptors (PRs), mineralocorticoid receptors (MRs), and glucocorticoid receptors, AR acts as a ligand-dependent transcription factor to regulate gene expression (Evans, 1988). It interacts primarily with T and DHT for which it has high affinity, and is not known to significantly bind other androgens (Kaufman and Vermeulen, 2005). Upon ligand binding in the cytosol, AR dissociates from chaperone proteins, dimerizes, and translocates to the nucleus, where it binds to androgen-response elements of target genes (Pascal and Wang, 2014).

Along with the well-established genomic effects of AR, there is growing evidence for rapid non-classical capabilities of nuclear ARs, including inducing of signaling cascades such as the MAPK/ERK pathway, and increasing intracellular calcium levels (Heinlein and Chang, 2002;

Rahman and Christian, 2007). In contrast to genomic actions, which take hours to days for effect, these actions are rapid, occurring on the time scale of seconds to minutes (Rahman and Christian, 2007). It is thought that these actions occur via association of nuclear ARs with the plasma membrane and membrane proteins such as the c-Src kinase, and may ultimately serve to activate gene expression, albeit a potentially wider array of genes than are affected by traditional AR action (Rahman and Christian, 2007).

Finally, in addition to the classical nuclear AR, recent work has uncovered evidence of a novel membrane-bound AR (Thomas et al., 2014). Existence of such a receptor had long been suspected, due to experiments showing rapid effects of androgens, and androgen binding to the plasma membrane (Sato et al., 2010), as well as the fact that several other steroid receptors such as ER, PR, and MR have corresponding membrane receptors (Heinlein and Chang, 2002).

Classical nuclear ARs are broadly but selectively distributed throughout the mammalian body and brain (Seidman and Walsh, 1999). In humans, there have been relatively few studies describing AR distribution in the central nervous system, so the bulk of knowledge on AR's brain distribution has originated from studies of primate and rodent species (Simerly et al., 1990; Clancy et al., 1992; Wood and Newman, 1999). In primates and rodents, AR is widespread, found in cortical regions, midbrain nuclei, limbic structures, and the cerebellum (Beyenburg et al., 2000). It is most highly concentrated in regions associated with control of reproductive behavior such as the paraventricular, periventricular, arcuate, ventromedial, and medial preoptic nuclei of the hypothalamus, as well as other hypothalamus-associated areas like the medial amygdala, lateral septum, and bed nucleus of the stria terminalis (BNST) (Don Carlos et al., 2006; Simerly et al., 1990). AR expression is regulated in part by circulating androgen levels; however, whether ARs are up- or down-regulation is not always clear, and may depend on many factors (Menard and Harlan, 1993).

1.5 The Relationship between Androgen Levels and Cognition

The relationship between androgen levels and cognitive abilities like learning and memory has been studied since the late 1920's. The earliest studies in animals focused on rats and tested if castration altered the ability to learn a maze (Tuttle and Dykshorn, 1928). Additional studies in species such as goldfish and deer mice examined the effects of seasonal changes in androgen levels on learning abilities (Agranoff and Davis, 1974; Galea et al., 1994). In humans, early research centered largely on investigating the connection between spatial abilities and androgens, since spatial ability is one cognitive domain for which it is widely acknowledged that males outperform females (Linn and Petersen, 1985).

More recently, over the past few decades, a substantial number of the studies that examine the relationship between androgen levels and cognitive abilities have focused specifically on aged men. Aged men form an appropriate study population, since aging is accompanied by declines in both androgens ("andropause") and in cognitive ability ("cognitive aging"). The studies that have been performed include those that are observational, assessing how endogenous hormone levels are associated with cognitive ability, and those that are experimental, investigating the effects of exogenous hormone administration on cognition. Observational findings have included positive (Yaffe et al., 2002; Moffat et al., 2002; Thilers et al., 2006), negative (Martin et al., 2007), and curvilinear (Barrett-Connor et al., 1999) relationships between androgen levels and various measures of cognitive abilities, in addition to complete lack of a significant relationship (Fonda et al., 2005), while experimental findings have shown that androgen administration can improve (Cherrier et al., 2001; Janowsky et al., 1994), impair (Wolf et al., 2000; Maki et al., 2007), or have no significant impact on cognition (Emmelot-Vonk et al., 2008; Vaughan et al., 2007).

While the results are inconsistent, it is worthwhile to note that among the studies cited, there were many methodological differences that could account for the discordance. In addition,

delineating the relationship of cognition and androgens is a complex undertaking. It is likely that androgens exert influence on cognition, but that the relationship is more nuanced, and contingent on optimal hormone levels specific to different cognitive domains and age ranges (Holland et al., 2011).

1.6 Cognitive Aging

Cognitive aging is broadly defined as a general deterioration in brain function and cognition with age. Defined operationally, it refers to the age-associated decrease in performance on cognitive tasks, for example, tests of IQ (Lindeboom and Weinstein, 2004). It is a dynamic and variable process, and shows considerable differences in onset and progression across individuals (Buckner, 2004; Lindeboom and Weinstein, 2004). Although not a clinically-defined disease, "normal" cognitive aging can progress to one, for example, mild cognitive impairment (MCI), which describes cognitive decline that is greater than expected for an individual's age and education level, but is not severe enough to significantly interfere with activities of daily life (Gauthier et al., 2006). MCI is considered a risk state for dementias such as AD, as more than half of MCI cases are expected to progress to dementia within five years (Gauthier et al., 2006).

Cognitive aging does not manifest as uniform decline across all brain structures and functions; rather, it has selective effects. Crystallized abilities such as vocabulary are not greatly affected (Lindeboom and Weinstein, 2004), while the greatest decline occurs in two primary areas: (1) in learning and declarative (long-term) memory, governed by medial temporal lobe (MTL) structures like the hippocampus and parahippocampal regions (Squire, 2004), and (2) executive function, governed by frontal cortical structures like the prefrontal cortex (PFC) (Beas et al., 2013; Janowksy, 2006; Gallagher et al., 2011). Significant attention has been devoted to understanding age-related impairments in long-term memory and MTL function, (Gallagher et al., 2011); however, comparatively less is known about impairment in executive function (Beas et al., 2013).

1.7 Executive Function

Executive function is a term that describes a group of cognitive processes involved in coordinating complex behaviors and goal-directed actions (Miller and Wallis, 2009). It encompasses several "higher-order" cognitive functions such as attention, planning, decision-making, judgment, behavior inhibition, problem-solving, working memory, task switching, and behavioral flexibility (Miller and Cohen, 2001; Beas et al., 2013; Robbins et al., 1996; Dalley et al., 2004; Kesner and Churchwell, 2011). In contrast to automatic, reflexive "bottom-up" cognitive processing based on reactions to environmental cues, executive function involves "top-down" integration and organization of big-picture information and current goals to manage behaviour (Miller and Wallis, 2009). Decline in executive function is observed in cases of "normal/healthy" cognitive aging (Alexander et al., 2012; Janowsky and Chavez, 2000) as well as in pathological aging disorders such as AD (Lindeboom and Weinstein, 2004; Rapp and Reischies, 2005; Perry and Hodges, 1999). Impairments in executive function are also observed in other neuropsychiatric disorders like schizophrenia, attention deficit hyperactivity disorder, autism, obsessive compulsive disorder (Hill, 2004), and Parkinson's disease (Zgaldarjic et al., 2006).

1.8 The Prefrontal Cortex

The part of the brain responsible for controlling executive function is the PFC. A highly interconnected portion of the frontal cortex, the PFC receives inputs from sensory areas, motor systems, and limbic and midbrain structures (Dalley et al., 2004; Miller and Wallis, 2009). As a result, it is well situated to coordinate complex behaviors on the basis of diverse information received from multiple internal and external sources (Miller and Wallis, 2009).

In the rat, two subregions of the PFC critical for executive function are the medial PFC (mPFC) and orbitofrontal region of the PFC, termed the orbitofrontal cortex (OFC). Both subregions are associated with distinct types of behavioural flexibility (Ghods-Sharifi et al., 2008). The mPFC, consisting of the prelimbic (PrL) and infralimbic (IL) cortices is involved in modulating higher-order extradimensional attentional shifts, while the OFC is involved in a simpler form of behavioural flexibility, called reversal learning (Dalley et al., 2004; Gallagher et al., 2011). The PFC also governs working memory, a form of short-term memory involving maintenance of information and management of the maintained information (Goldman-Rakic, 1995; Funahashi, 2006).

The PFC is one of the brain regions most sensitive to the detrimental effects of aging, such as loss of gray and white matter (Reuter-Lorenz and Lustig, 2005), which may serve to increase its vulnerability to development of neuropsychiatric disorders (Mizoguchi et al., 2010).

1.9 The Mesocorticolimbic Dopamine Pathway

The PFC receives dopaminergic innervation as part of the mesocortical dopamine (DA) pathway (Mizoguchi et al., 2009; Fuxe et al., 1974). This pathway, consisting of midbrain DAergic cell bodies originating in the ventral tegmental area (VTA) and projecting to prefrontal areas including the mPFC, OFC, and anterior cingulate cortex, is associated with executive control of goal-motivated behavior (Le Moal and Simon, 1991; Floresco and Magyar, 2006; Kritzer, 1997).

DAergic cell bodies originating in the VTA also form a second pathway referred to as the mesolimbic DA pathway. The mesolimbic DA pathway is heavily implicated in reward and motivation, and consists of projections to the nucleus accumbens (NAc), hippocampus, and other limbic areas (Fibiger and Phillips, 1988). Together, the mesocortical and mesolimbic pathways are often referred to as the mesocorticolimbic DA circuit.

The primary mechanism through which androgens are thought to influence cognition is through modulation of prefrontal DA signaling (Kritzer et al., 2007). In adult rats, experimental alterations in androgen levels result in changes to mesocorticolimbic DAergic cells and circuitry (Kritzer, 2000; 1999). For example, gonadectomy (GDX) increases both axon density of cells with tyrosine hydroxylase, a DA-synthesizing enzyme (Adler et al., 1999; Kritzer et al., 2000), and extracellular DA levels in the PFC but not other frontal regions (Aubele and Kritzer, 2010). In many cases, the effects of GDX are attenuated or restored by androgen but not estrogen replacement (Kritzer, 2000). The fact that prefrontally-projecting DAergic midbrain neurons in males are enriched in AR, but not ERα or ERβ also suggests that DAergic circuits are sensitive to androgens but not estrogens (Kritzer and Creutz, 2008).

Androgens also affect the prefrontal behaviours critically dependent on mesocorticolimbic-DA innervation (Kritzer et al., 2007). Animal studies using hormone manipulations coupled with cognitive testing have revealed that GDX impairs prefrontal DA-dependent behaviours such as spatial working memory and behavioral flexibility (Aubele et al., 2008; Kritzer et al., 2001; Gibbs, 2005), and T administration can restore and/or improve performance on such tasks (Bimonte-Nelson et al., 2003). In addition, chronic high doses of anabolic-androgenic steroids impair reversal learning and set-shifting (Wallin and Wood, 2015).

1.10 Thesis Objectives and Structure

In light of the interrelationships between androgens, cognition, and aging, the overall objective of my thesis was to investigate how the systemic declines in T levels that accompany aging can impact androgen signaling in mesocorticolimbic brain regions involved in executive function. More specifically, I examined the effects of age on endogenous levels of serum T, neural T, and ARs. A secondary objective of my thesis was to improve the immunochemical visualization of ARs in mesocorticolimbic regions.

My thesis contains four chapters: a general introduction (Chapter 1), analysis of biotin tyramide signal amplification in chromogenic and immunofluorescent immunohistochemistry of androgen receptors in the rat brain (Chapter 2), analysis of the effects of aging on testosterone and androgen receptors in mesocorticolimbic brain regions of male rats (Chapter 3), and a general conclusion (Chapter 4). Chapters including original data (Chapters 2 and 3) are written in the style of manuscripts to submit to peer-reviewed journals.

Chapter 2: Analysis of biotin tyramide signal amplification in chromogenic and fluorescent immunohistochemistry of androgen receptors in the rat brain

2.1 Summary

Research on neural androgen receptors (ARs) has traditionally centered on "classical" brain regions associated with control of reproductive behaviors. Although ARs are also present in "non-classical" regions such as the prefrontal cortex (PFC), their abundance per cell is much lower, such that immunostaining is often poor or hard to detect above background. In this study, we demonstrate that biotin tyramide signal amplification (TSA), a method of improving weak immunostaining, dramatically improves AR immunoreactivity in the rat brain in regions of low AR per-cell abundance like the medial PFC (mPFC) and lateral orbitofrontal cortex (LOFC). We show that TSA is critical in permitting AR detection in both chromogenic and immunofluorescent immunohistochemistry. We also use double-labeling experiments to confirm that the dominant phenotype of prefrontal cortical AR-expressing cells is neuronal and not glial. Finally, we show sex differences in the expression of prefrontal ARs. In light of the emerging recognition of androgen involvement in higher-order brain functions, these results may help advance understanding of the distribution and roles of ARs in non-classical brain regions.

2.2 Introduction

Immunohistochemical studies examining androgen receptor (AR) in the rodent brain have traditionally focused on "classical" regions such the medial preoptic area (MPOA), ventromedial hypothalamus (VMH), medial amygdala (MeA), lateral septum (LS), and bed nucleus of the stria terminalis (BNST) where androgen effects are well-established and ARs per cell are abundant (Simerly et al., 1990; Chambers et al., 1990; Menard and Harlan, 1993). However, recent studies have started to examine ARs in non-classical regions where they may

have lower abundance per cell. In such regions, like the medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), and the isocortex (Don Carlos et al., 2006), AR staining per cell can be weak and difficult to visualize and subsequently quantify.

Many factors can influence the quality of steroid receptor immunostaining. For example, tissue fixation can contribute to substantial differences in signal strength. Butler et al. (1999) found that ERα immunoreactivity in rat cerebral cortex could be increased considerably by fixing tissue with acrolein or a paraformaldehyde (PFA) and glutaraldehyde mixture, rather than just PFA alone. In addition, injecting animals with T prior to tissue collection can improve immunostaining when using the anti-AR antibody, PG-21 (Smith et al., 1996; Soma et al., 1999). For weak signal that is due to masked antigens, antigen retrieval methods are often successful for improving signal visibility (Shi et al., 1991; Kim et al., 2003). For weak signal that is due to low abundance of antigen, tyramide signal amplification (TSA) methods are often highly effective (Toda et al., 1999; Ramos-Vara, 2005).

First described by Bobrow et al. (1989), TSA relies on the rapid deposition of a reporter molecule, biotinylated tyramine, at the site of the antigen and adjacent proteins to achieve amplification of signal (Adams, 1992; Bobrow et al., 1992). TSA methodology can be applied to both immunofluorescent and chromogenic detection systems of immunohistochemistry, and typically results in a 10-100-fold increase in staining efficiency (Hunyady et al., 1996).

TSA has been used in immunostaining for ERα and ERβ (Kallo et al., 2001; Mitra et al., 2003; Garcia-Ovejero et al., 2002; Vida et al., 2008; Quesada et al., 2007; Tapia-Gonzalez et al., 2008; Koibuchi and Hayashi, 2007; Thammacharoen et al., 2013; Asarian and Geary, 2013; VanderHorst et al., 2005), glucocorticoid receptor (Ostrander et al., 2003), and progesterone receptor (Bonkhoff et al., 2001) in both neural and non-neural tissues of humans and rodents. TSA has also been used with AR immunostaining in a variety of tissue types, including muscle (Kadi et al., 2000; Li et al., 1998), testis (Suarez-Quian et al., 1999; Li et al., 1998; Hazra et al.,

2012), prostate (Litvinov et al., 2006), blood vessels (Sader et al., 2004), kidney, spleen, heart, and intestine (Li et al., 1998), and neural tissue including the spinal cord (Li et al., 1998) and brain (Fernandez-Guasti et al., 2000; Bao et al., 2006; García-Ovejero et al., 2002; Li et al., 1998).

However, to our knowledge, TSA has never been applied to brain regions that have low per-cell abundances of ARs. Although such regions have often been overlooked in favour of classical reproductive regions, there is strong evidence that androgens exert important effects on higher-order cognitive functions mediated by these areas (DonCarlos et al., 2006; Janowsky, 2006; Kritzer et al., 2007). Therefore, we evaluated the effect of TSA on AR immunostaining in the rat brain in low-AR-per-cell regions like the PFC, as well as high-AR-per-cell regions. Our first study assessed the effect of implementing TSA in a chromogenic IHC protocol in select male rat brain regions. Our second study assessed the effect of TSA applied to double-label immunofluorescent IHC to characterize AR+ cell types (neuronal vs. glial) in male and female rats.

2.3 Methods

2.3.1 Study 1: Tyramide Signal Amplification Applied to Chromogenic Immunohistochemistry of AR

Study 1 assessed the effect of TSA applied to chromogenic IHC in the male rat brain in areas of high and low AR abundance.

2.3.1.1 Animals

Subjects were male Fischer 344 x Brown Norway F1 hybrid rats (F344/BN) (National Institute of Aging, Taconic Farms) that were young (5 months, n=12) and aged (22 months, n=12). Rats were grouped-housed with rats of similar age for the first week after arrival and

given *ad libitum* access to food (Rat Diet 5012; LabDiet, St. Louis, MO) and water. All cages contained Aspen chip bedding (Nepco, Warrensburg, NY), paper towel, and a PVC pipe for environmental enrichment. Rats were kept on a 12 hr light:dark cycle at an average temperature of 21°C and relative humidity of 40-50%. All animal procedures complied with the Canadian Council on Animal Care and the University of British Columbia guidelines for animal care.

2.3.1.2 Tissue Fixation

Rats were euthanized by isoflurane inhalation and rapid decapitation. The brains were removed and cut in half along the sagittal plane. One half of each brain was immersion fixed in 4% PFA in phosphate-buffered saline (PBS) for 4 h at room temperature, and then cryoprotected in 30% sucrose solution at 4°C for 48 h or until they had sunk. Brains were stored at -80°C until sectioning. Brains were serially sectioned on a cryostat into five series of 40µm coronal sections. Free-floating sections were stored in anti-freeze solution at -20°C until immunohistochemical processing.

2.3.1.3 Immunohistochemistry

Of the five tissue series, two series were used here. Each series underwent immunohistochemical processing separately. The first series, hereafter referred to as TSA^-, was stained for AR using a protocol that did not include a TSA step. Nine months later, the second series, hereafter referred to as TSA^+, was stained for AR for using a similar protocol featuring the addition of TSA. Because a TSA step was introduced, a number of minor adjustments to the protocol were required in order to prevent increased background. For both series, the primary antibody was a monoclonal anti-AR antibody [EPR1535(2)] raised in rabbit (ab133273; Abcam, Inc., San Francisco, CA), previously validated for specificity by Western blot and use in testicular feminization mutant rat tissue lacking fully functional ARs (Hamson et al., 2013), and

the secondary antibody was a biotin-SP-conjugated affinity-purified donkey anti-rabbit secondary antibody (711-065-152; Jackson ImmunoResearch Laboratories, West Grove, PA).

TSA^- (Control) Series: Sections were washed for 20 min in 0.1M Tris-buffered saline (TBS), then incubated in 0.5% hydrogen peroxide (H_2O_2) solution for 30 min to quench endogenous peroxidase activity. Sections were then washed for 25 min in 0.1M TBS, then for 5 min in 0.1M PBS with 0.3% Triton X-100 and 0.1% gelatin (hereafter referred to as PBS-GT). Next, sections were incubated in 10% normal donkey serum (017-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 2 h to block nonspecific staining. Directly following the block, sections were incubated in primary antibody solution (1:200 in PBS-GT) for 1 h at room temperature (RT) then for 3 nights at 4°C. The following day, sections were washed for 45 min in PBS-GT, then incubated in secondary antibody solution (1:500 in PBS-GT) for 1 h at RT, and then overnight at 4°C. Following the secondary antibody incubation, sections were washed for 45 min in PBS-GT, then incubated in AB solution in TBS-T (VectaStain ABC Kit; PK-6100 Standard; Vector Laboratories) for 1 h at room temperature, then washed in PBS-GT for 20 min and TBS for 10 min. Sections were incubated in DAB in H_2O_2 (Vector Labs Peroxidase Substrate Kit SK-4100) with nickel for 10 minutes at room temperature. Lastly, sections were mounted onto gelatin-subbed slides, dehydrated, and cover-slipped.

TSA^+ Series: This series followed a similar protocol, with the following differences. Sections were incubated in 2% hydrogen peroxide (H_2O_2) solution, and sections were blocked in 8% tryptone (J859; Amresco, Inc., Solon, OH, USA) in PBS instead of normal donkey serum. The primary antibody incubation was shortened to 24 hr at room temperature, and the secondary antibody was used at a concentration of 1:2000 in 0.8% tryptone in PBS. Sections also underwent a modified AB incubation schedule to accommodate addition of TSA. Sections were incubated in AB (in 0.8% tryptone in PBS) for 30 min at room temperature, washed in PBS-GT

for 15 min, and then incubated in biotin tyramide solution ("BT"; made in-lab as described by Adams, 1992; 15 ml PBS with 45 µL BT and 5 µL 30% H_2O_2) for 10 min, then washed in PS-GT for 15 min, incubated in AB solution for 30 min, and washed in PBS-GT for 15 min. Lastly, the DAB incubation time was extended to 20 min.

2.3.1.4 Measurement of Immunoreactivity

Photomicrographs were taken using a Nikon Digital Sight DS-U1 camera and Nikon Eclipse 90i microscope (at the 20X objective) using NIS-Elements Basic Research software (Nikon Canada, Inc., Richmond, BC, Canada). For each image, a background mean intensity value (BMI) was calculated by averaging six intensity measurements taken from random locations within each brain area where specific staining was not present. The BMI for each image was used to generate a threshold value which was 1.25 or 1.5 times as dark as the BMI. Only staining that was at or above threshold was quantified. Thresholds were applied to a defined region of interest (ROI) superimposed onto each photomicrograph within the limits of each brain region, and the program calculated the percentage of the ROI that was above threshold (% AR-ir). AR-ir values were measured for two areas of low per-cell AR abundance (mPFC and LOFC) and one area of high per-cell AR abundance (MeA).

2.3.1.5 Statistical Analysis

Data were analyzed using SPSS Statistics software (Version 23.0 for Mac OS X; Chicago, IL). Pearson correlations between TSA^+ and TSA^- AR-ir values were computed for the LOFC and MeA. The paired samples t-test was used to compare means of TSA^+ and TSA^- AR-ir in the PrL, LOFC, and MeA.

2.3.2 Study 2: Tyramide Signal Amplification Applied to Fluorescent Immunohistochemistry of AR Co-labeled with NeuN or GFAP

Study 2 assessed the effect of TSA applied to fluorescent IHC in the male and female rat mPFC, LOFC, and CA1 of the hippocampus. Study 2 also aimed to characterize the phenotype of AR-expressing cells by double-labeling cells for (1) AR, and (2) one of the following: NeuN, a marker for adult neurons, or GFAP, a marker for glial fibrillary acidic protein that is expressed by glial cells.

2.3.2.1 Animals

Subjects were male and female adult (9-10 weeks old) Long-Evans rats (Charles River, Saint-Constant, Quebec, Canada; n=3 per sex) (Ferris et al., 2015). Rats were separated by sex for housing in two colony rooms, and group-housed in clear cages with aspen chip bedding. Rats were given *ad libitum* access to standard lab chow (Rat Diet 5012; LabDiet, St. Louis, MO) and water, and kept on a 12 hr light:dark cycle. Rats were handled daily for 1 week prior to start of experiment.

2.3.2.2 Perfusion and Tissue Fixation

Subjects were euthanized via overdose of chloral hydrate (140 mg/kg), given by IP injection, and then transcardially perfused with 0.9% saline (60ml) and then 4% PFA (120ml). Following perfusion, brains were extracted and post-fixed in 4% PFA for 4 h at room temperature, then cryoprotected in 30% sucrose solution at 4°C for 72 h or until they had sunk. Brains were then flash frozen on powdered dry ice and stored at -80°C. Brains were sectioned on a cryostat into 40µm coronal sections, and free-floating sections were stored in anti-freeze solution at -20°C until immunohistochemical processing.

2.3.2.3 Double-Label Immunofluorescence

Brain tissue from 6 animals (n=3 female, n=3 male) was processed in one IHC run to fluorescently co-label AR/NeuN with and without TSA, and AR/GFAP with and without TSA.

In all conditions, sections were washed in PBS for 15 min, then incubated in 0.5% H_2O_2 in PBS for 30min. Unless otherwise stated, all subsequent washes were in PBS. Sections were washed for 15min, then blocked in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS-T (PBS with 0.2% Triton X-100) for 2 hr. Sections were then incubated in the primary antibodies, anti-AR (1:200) and anti-NeuN (1:500) in PSB-T, or anti-AR (1:200) and anti-GFAP (1:500) in PBS-T. The anti-AR antibody was the same as used in Study 1. The anti-NeuN antibody (MAB377, Millipore, Temecula, CA) and anti-GFAP antibody (MAB360, Millipore) were both monoclonal. Following primary antibody incubation for 24 hr at RT, sections were washed for 15 min, then incubated in biotinylated donkey anti-rabbit secondary antibody (same as in Study 1) in PBS-T for 1 hr at RT, then overnight at 4°C. After incubation, sections were then washed in PBS for 15 min. For TSA^+ treatments only, sections were incubated in AB solution for 30min, washed in PBS for 15min, incubated in BT for 10min, and washed in PBS for 15min. For fluorophore labeling in all treatments, sections were incubated in streptavidin-conjugated Alexa Fluor 488 (1:300, S32354, Invitrogen, Carlsbad, CA) and Alexa Fluor 647 donkey anti-mouse IgG (1:500, A31571, Invitrogen) in PBS-T for 3 hr. Lastly, sections were washed in PBS for 15 min, and mounted onto gelatin-coated slides and coverslipped with ProLong Gold Mountant with DAPI (P36931, Invitrogen).

2.3.2.4 Confocal Scanning Laser Microscopy

A confocal laser scanning microscope (TCS SP8; Leica Microsystems, Wetzlar, Germany) was used to acquire Z-section images of tissue sections. High-magnification images (1024 x 1024 µm**)** were acquired using a multiline argon ion laser and water-immersion 25 X

objective. For AR signal, emitted fluorescence was detected at 488 nm in the green channel. For NeuN or GFAP signal, emitted fluorescence was detected at 647 nm in the far red channel. In order to avoid cross-talk from the two colour channels, a sequential scanning mode was used during image collection. Final images were prepared using ImageJ software. Images were adjusted for brightness and contrast. Approximately 200 cells in each region (the mPFC, LOFC, and CA1 of the hippocampus) were examined in each animal for colocalization of AR with NeuN or GFAP in a single cell. The percentage of cells with co-localization was calculated as a proportion of the total number of cells counted.

2.4 Results

2.4.1 Study 1 Results

The overall distribution of AR was consistent with previous reports of AR in rat brain, with immunostaining highly concentrated in hypothalamic and limbic regions (Simerly et al., 1990; Clancy et al., 1992). Regions regarded to be AR-negative such as the dentate gyrus of the hippocampus, the septofimbrial nucleus, and the septophippocampal nucleus (Simerly et al., 1990; Clancy et al., 1992) did not express immunoreactivity with or without TSA treatment (Figure 2.1). In addition, TSA^+ and TSA^- controls omitting primary antibody were performed, and showed absence of specific AR staining (data not shown).

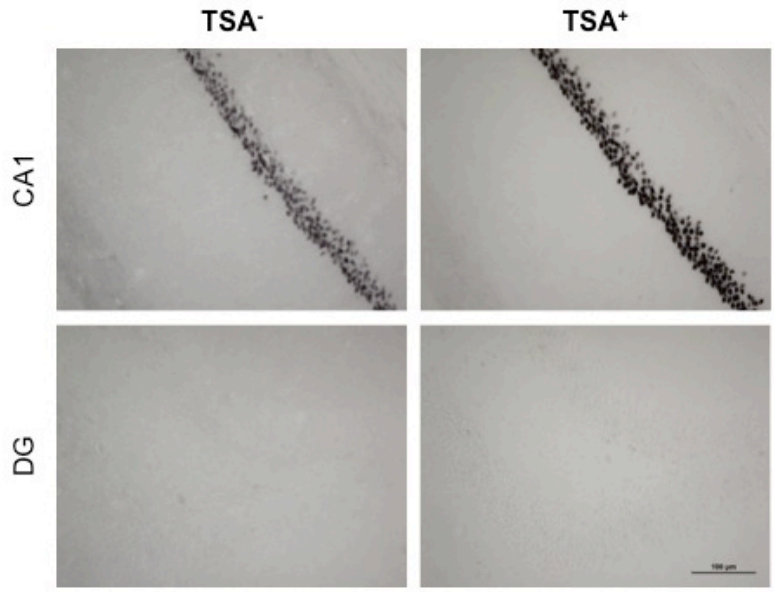

Figure 2.1. Within-subject AR-positive and AR-negative control regions. In both TSA^- and TSA^+ sections, area CA1 of the hippocampus, a known AR-positive region, showed strong AR-ir (top), with slightly stronger staining in the TSA^+ section (top right). In contrast, in the dentate gyrus (DG), a known AR-negative region, there was no AR-ir, with and without TSA (bottom). Subject is a male F344/BN rat. Scale bar represents 100 μm.

2.4.1.1 Effect of TSA in Classical Regions

Compared to control sections that did not undergo TSA, TSA^+ sections had a qualitatively and quantitatively greater intensity of immunostaining. TSA treatment increased the intensity of staining in areas of typically high per-cell AR abundance such as the MeA, LS, VMH, and BNST (Figure 2.2). For example, paired samples t-tests revealed that in the MeA, mean AR-ir increased from 6.56 $\pm$ 0.61% in TSA^- sections to 17.62 $\pm$ 1.65% in TSA^+ sections ($t(22) = -8.19$, $P < .0001$) (Figure 2.4). Across subjects in the MeA, the AR-ir values from TSA^+ and TSA^- series were positively correlated ($r = .64$, $P = .001$).

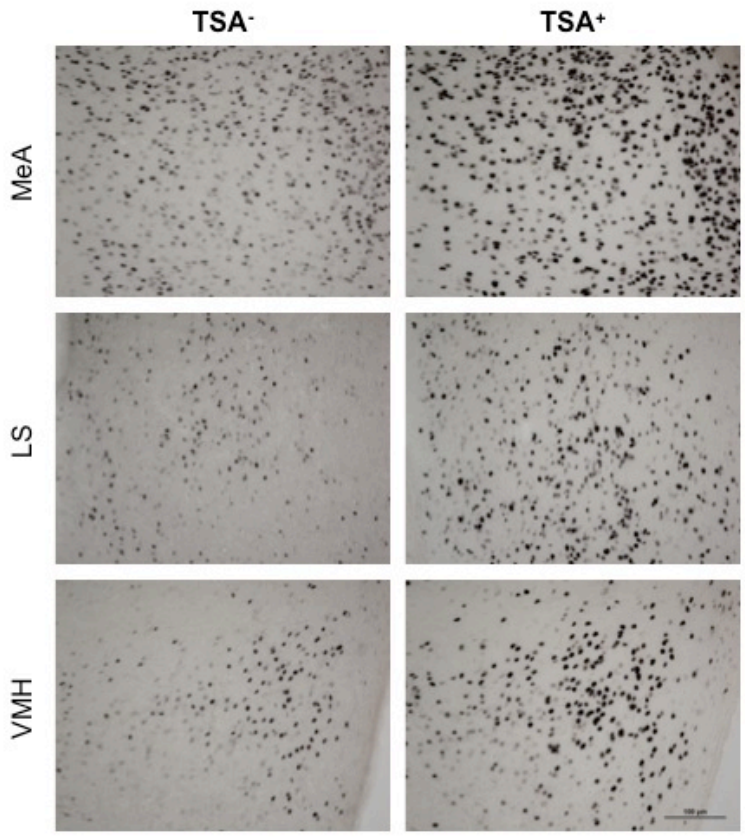

Figure 2.2. Within-subject TSA-induced increase in AR-ir in regions known to be rich in AR. TSA^- sections (left) and TSA^+ sections (right). From top to bottom: medial amygdala (MeA), lateral septum (LS), ventromedial hypothalamus (VMH). Subject is a male F344/BN rat. Scale bar represents 100 μm.

2.4.1.2 Effect of TSA in Non-Classical Regions

This effect of increased staining intensity was even more pronounced in areas of low per-cell AR abundance like the mPFC where, without TSA, staining intensity was so low that quantification of immunoreactivity was not possible using an intensity thresholding method (Figure 2.3, top row). The robust increase in staining intensity was also noticeable in other cortical regions like the LOFC (Figure 2.3, bottom row).

TSA-treated sections had higher AR-ir values. Paired samples t-tests revealed that mean AR-ir value for the mPFC significantly increased from 0% in TSA^- sections to 2.13 $\pm$ 0.38% in TSA^+ sections ($t(20) = -5.66$, $P < .0001$), and the LOFC increased from 0.76 $\pm$ 0.11%

to 3.54 ± 0.49% ($t(23) = -6.06$, $P < .0001$) (Figure 2.4). Across subjects, AR-ir values in the LOFC were showed a trend towards positive correlation (r = .39, $P = .06$).

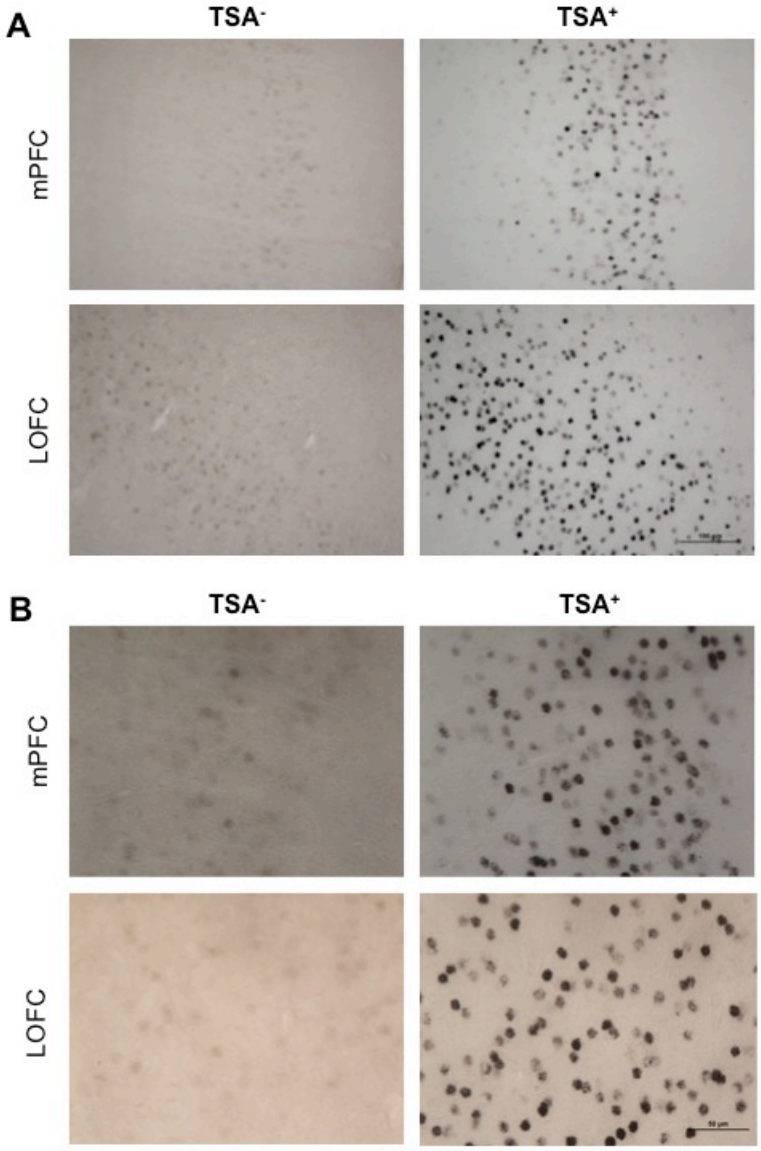

Figure 2.3. Within-subject TSA-induced increase in AR-ir in regions not traditionally considered to be rich in AR. TSA^- sections (left) and TSA^+ sections (right). (A) photomicrographs taken at 20X (B) photomicrographs taken at 40X. Regions include the medial prefrontal cortex (mPFC), and lateral orbitofrontal cortex (LOFC). Subjects are male F344/BN rats. Scale bar represents 100 µm in (A) and 50 µm in (B).

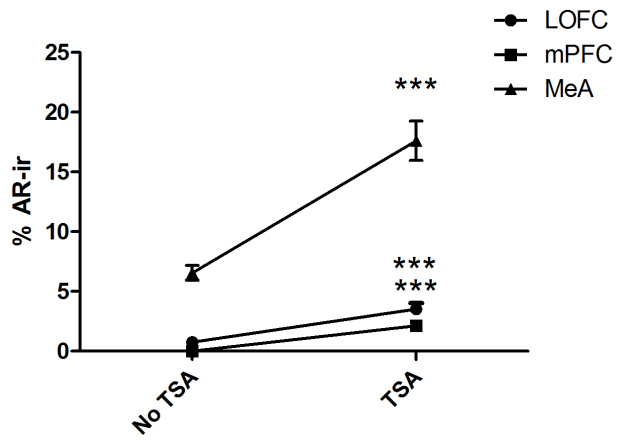

Figure 2.4. TSA-induced increases in AR-ir in the lateral orbitofrontal cortex (LOFC), medial prefrontal cortex (mPFC), and medial amygdala (MeA). TSA increased AR-ir in regions not traditionally considered rich in AR (LOFC and mPFC), as well regions known to contain abundant ARs (MeA). Data are means ± SEM, analyzed by the paired samples t-test, n = 21-24 F344/BN rats. ****P* < .0001.

2.4.2 Study 2 Results

The overall pattern of AR staining closely matched previous descriptions (Simerly et al., 1990), as well as the distribution of AR observed with chromogenic staining in Study 1.

2.4.2.1 Effect of TSA

Similar to Study 1 results, immunofluorescently-stained TSA^+ sections in Study 2 had a qualitatively greater intensity of AR immunostaining than TSA^- sections. Notably, in all TSA^- sections, AR signal was not visible, although some low background staining was present, e.g. at tissue edges (Figure 2.5). As expected, NeuN and GFAP signal appeared to be consistent across TSA^+ and TSA^- conditions and did not noticeably change in intensity (data not shown).

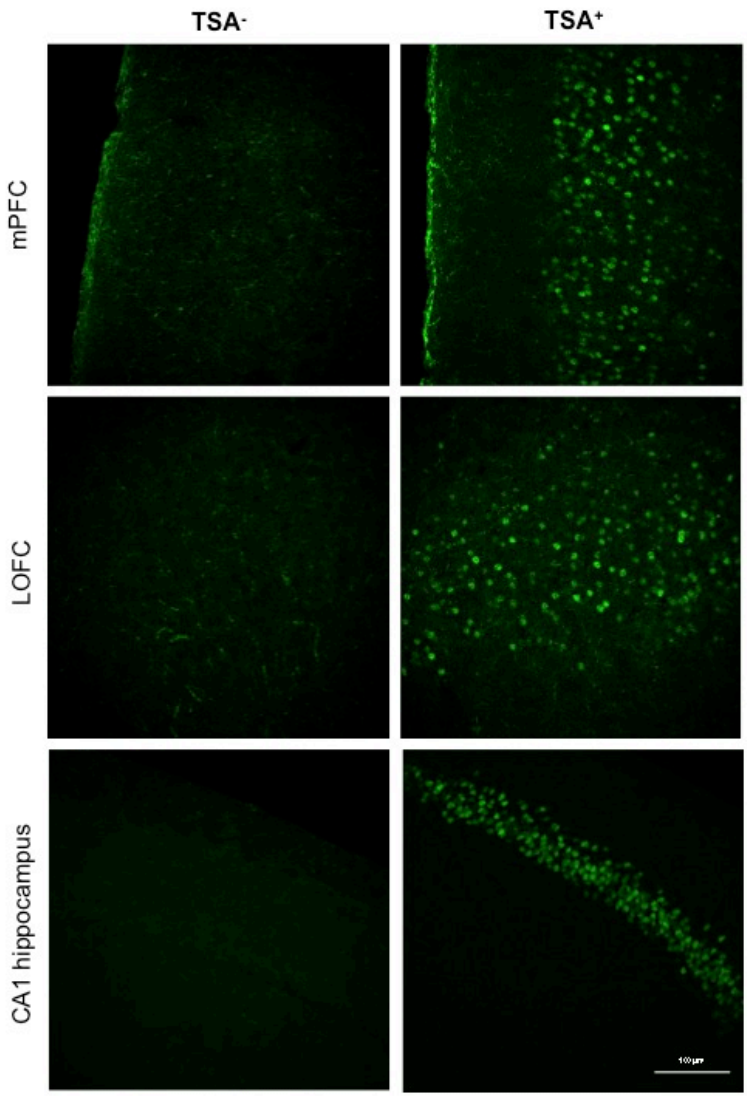

Figure 2.5. TSA-induced increase in AR-ir in regions not traditionally considered rich in AR. TSA^- sections (left) and TSA^+ sections (right). Regions include the medial prefrontal cortex (mPFC, top), lateral orbitofrontal cortex (LOFC, middle), and CA1 of the hippocampus (CA1, bottom). Without TSA, AR-ir was not detectable. Subjects are male Long-Evans rats. Scale bar represents 100 μm.

2.4.2.2 AR+ Cell Phenotype and Sex Differences

100% of AR+ cells in the mPFC, LOFC, and hippocampus were NeuN+, consistent with a neuronal phenotype (Figure 2.6A). Further, 0% of AR+ cells in the regions examined were GFAP+ (n=3; > 600 cells total) (Figure 2.6B). Not all NeuN+ cells expressed AR.

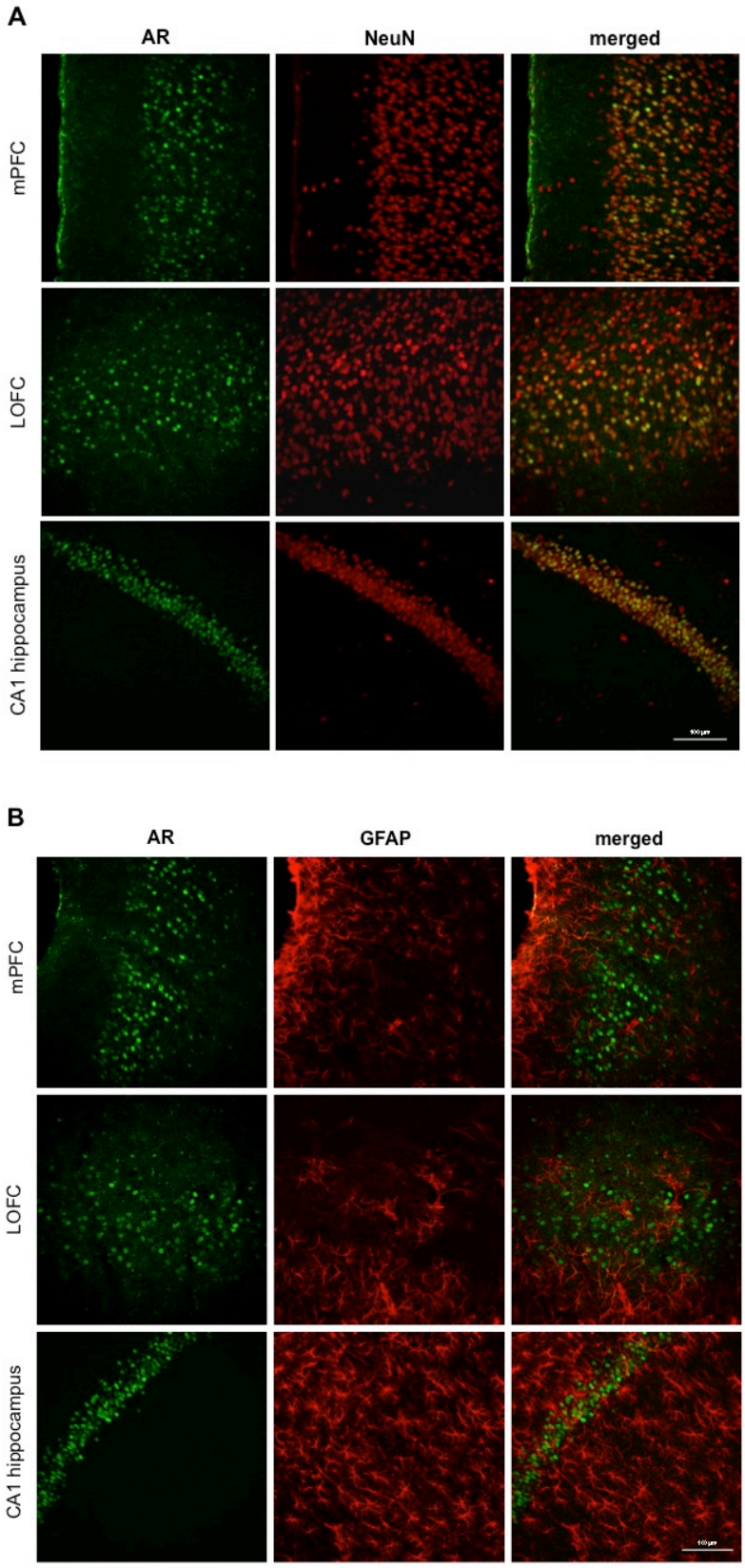
A
AR
NeuN
merged
mPFC
LOFC
CA1 hippocampus
B
AR
GFAP
merged
mPFC
LOFC
CA1 hippocampus

Figure 2.6. Phenotyping of AR+ cells via double-label immunofluorescence with TSA in the medial prefrontal cortex (mPFC, top row), lateral orbitofrontal cortex (LOFC, middle row), and CA1 of the hippocampus (CA1, bottom row). (A) AR-ir in green (left), NeuN-ir in red (middle), merged picture (right). In all three regions, there is 100% colocalization of AR with NeuN. (B) AR-ir in green (left), GFAP-ir in red (middle), merged picture (right). In all three regions, there is 0% colocalization of AR with GFAP. Subjects are male Long-Evans rats. Scale bar represents 100 μm.

In the mPFC and LOFC of females (n=3), AR staining was not detectable; however, AR-ir was present elsewhere. There was moderate to high intensity of staining in areas of relatively higher AR abundance such as CA1 of the hippocampus and MeA. In contrast, AR staining was present in the mPFC and LOFC of males (n=3) (Figure 2.7).

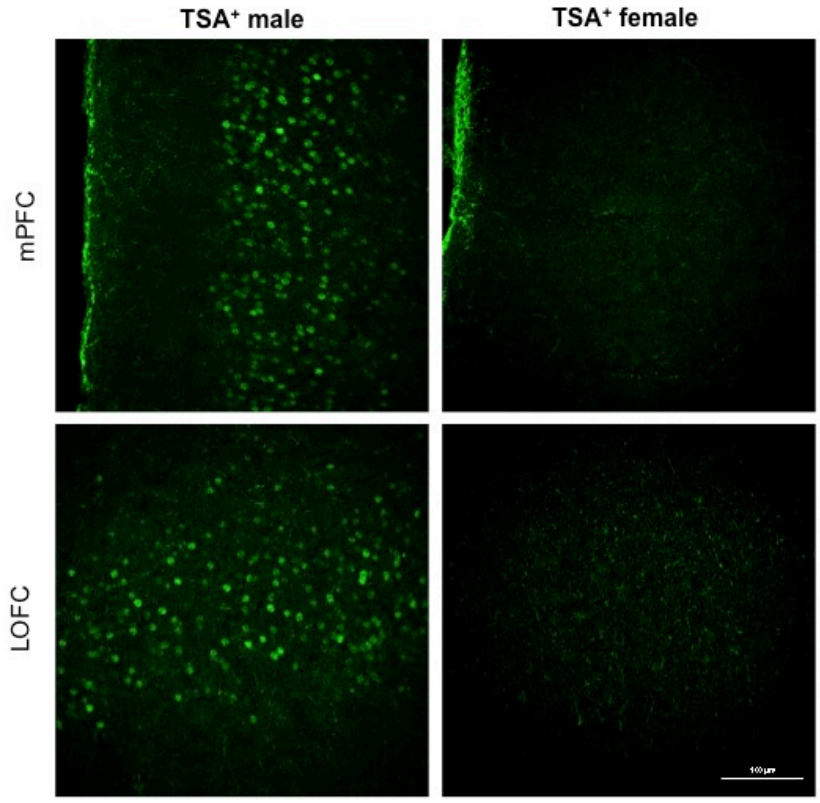

Figure 2.7. Sex differences in AR-ir in the medial prefrontal cortex (mPFC, top) and lateral orbitofrontal cortex (LOFC, bottom). Males (left) had detectable AR-ir, but females (right) did not. Subjects were Long-Evans rats, n = 6 (3 males, 3 females). Scale bar represents 100 μm.

2.5 Discussion

Here we evaluated the effect of TSA on AR immunostaining in the rat brain in two contexts: (1) with chromogenic immunohistochemistry in F344/BN males in low AR per-cell regions like the mPFC, as well as high AR per-cell regions like the MeA, and (2) with double-label immunofluorescent immunohistochemistry to phenotype AR+ cell types in Long-Evans males and females. TSA was found to significantly increase AR staining intensity across multiple contexts: in both chromogenic and fluorescent applications, in two different strains of rat, in young and old ages, and in males and females, suggesting wide applicability and impact.

2.5.1 TSA is Important for Visualizing ARs in Non-Classical Regions

Evidence for presence of ARs in the rat brain has been provided by a number of studies, employing a variety of detection techniques, including autoradiography and binding assays (Stumpf and Sar, 1976; Lischiotto and Morrell, 1994; Handa et al., 1987), in-situ hybridization (Simerly et al., 1990; Kerr et al., 1995; McAbee and DonCarlos, 1999), immunohistochemistry with polyclonal antibodies (Clancy et al., 1992; Don Carlos et al., 2003) and qPCR of selected regions (Munetomo et al., 2015). Such studies are in clear accordance on AR distribution in classical regions of AR action like the VMH, MPOA, BNST, and MeA; however, when it comes to forebrain cortical regions like the mPFC and LOFC, there is relatively less information available, although some studies do report AR in layers of the cerebral cortex (Simerly et al., 1990; Kritzer, 2004; Clancy, 1992; DonCarlos et al., 2006).

This lack of information could be due in part to greater difficulty visualizing ARs, as they are less abundant per cell, especially in comparison to the strong staining intensity seen in classical regions. Despite the fact that ARs per cell are less abundant, there is a large population of AR+ cells in cortical regions (DonCarlos et al., 2006), suggesting an important role for androgen modulation of cortical functions. Several studies have examined the role of

androgens in non-classical regions and on behaviours mediated by these regions, including executive functions like working memory and set-shifting (Kritzer et al., 2007; DonCarlos et al., 2006; Gibbs, 2005; Bimonte-Nelson et al., 2003; Wallin and Wood, 2015). Consequently, the ability to visualize (and quantify) ARs in these areas is critical. Development of an effective staining protocol is a key step in allowing examination of these ARs, and we have found that addition of a TSA step makes the difference from a complete lack of signal to one that is easily detectable.

2.5.2 Correlations Between TSA$^-$ and TSA$^+$ Series

Across animals, TSA$^-$ and TSA$^+$ series AR-ir values were moderately positively correlated, suggesting that TSA increases staining intensity in a consistent and proportional manner across animals. However, the strength of this correlation was not as strong as expected, especially in lower-abundance regions like the LOFC. This is more likely due to variation and error in measurement of the staining, rather than inconsistencies in the staining itself due to effects of TSA, since faint staining in the TSA$^-$ LOFC sections was more difficult to accurately quantify compared to darker staining in the TSA$^-$ and TSA$^+$ MeA sections.

2.5.3 AR-Expressing Cell Phenotype

Our results demonstrating that AR+ cells in the PFC and LOFC co-localize 100% with NeuN and 0% with GFAP, consistent with a neuronal phenotype, are in agreement with a number of studies observing neuronal AR in distinct regions of the rat brain including the cerebral cortex, forebrain, BNST, MeA, and CA1 of the hippocampus (Kritzer, 2004; Bingaman et al., 1994; Clancy et al., 1992; Zhou et al., 1994). Studies in primates also report similar findings (Finley and Kritzer, 1999) suggesting that in mammals, AR expression predominates in neurons (Lorenz et al., 2005).

However, it has been shown that AR can also be expressed by glial cells in specific contexts (Lorenz et al., 2005). In rats, this expression is age and location-dependent, such that AR+ glia have been observed in the cerebral cortex of postnatal day 10 rats, and in the arcuate nucleus of the hypothalamus and posterodorsal MeA of adults (Lorenz et al., 2005; Johnson et al., 2012). AR expression in microglia is observed after traumatic brain injury (Garcia-Ovejero et al., 2002), at extranuclear sites in astrocytic profiles in the hippocampus (Tabori et al., 2005), and in primary culture of rat astrocytic cells (Hosli et al., 2001; Jung-Testas et al., 1992). The lack of co-localization of AR and GFAP that we observed in the mPFC and LOFC is consistent with these findings.

2.5.4 Sex Differences in AR Expression

Studies of sex differences in neural AR expression have produced mixed results. Some studies have shown males to have higher levels of nuclear ARs than females in regions associated with reproductive behavior such as the BNST, VMH, LS, and preoptic area (Roselli, 1991; Lu et al., 1998); however, previous evaluations of the cerebral cortices have not detected any sexual dimorphisms in AR distribution or density (Simerly et al., 1990; Clancy et al., 1992; Kritzer, 2004). Our detection of sex differences in prefrontal AR staining diverges from these results, although strain and age differences may be responsible. In addition, several of the previous studies were whole-brain examinations of AR distribution, and as such, acknowledge that subtle sex differences may have been unnoticed (Simerly et al., 1990). Our results are also consistent with the idea that higher levels of ARs in males may be expected because of higher circulating T levels (Lu et al., 1998).

2.5.5 Potential Concerns Associated with TSA Use

TSA is not without some disadvantages, most notably increasing background staining (Mengel et al., 1999). The range of specific amplification achieved can also vary unpredictably (Mengel et al., 1999; Adams, 1992). Therefore, it is essential to optimize the primary antibody concentration and blocking conditions. In our experiments, a solution of 8% tryptone in PBS was effective in blocking nonspecific background staining and outperformed standard blocking agents like normal serum and skim milk. In addition, 0.8% tryptone was added to other protein-containing solutions, including the secondary antibody and AB solutions. Tryptone consists of partially-digested peptides from casein, and has been previously determined to generate strong blocking against background resulting from TSA-based immunohistochemistry (Kim et al., 2003). The combination of tryptone blocking and use of a highly-specific monoclonal antibody permitted use of TSA with minimal background staining.

Another concern with using a signal amplification technique like TSA is that the amplification observed is not specific. In this case, it is unlikely that TSA boosts all staining in general in a manner that is not exclusive to AR. Known AR-negative regions such the dentate gyrus of the HIP (Simerly et al., 1999; Clancy et al., 1992) did not express immunoreactivity with or without TSA treatment (Figure 1.7). In addition, the laminar staining pattern of AR in the PFC, observable only with TSA, is consistent with that described by Kritzer (2004). Other studies employing TSA with a wide range of antibodies also conclude that staining specificity is unaffected by amplification (Hunyady et al., 1996).

2.6 Conclusion

We conclude that the addition of TSA to an AR immunostaining protocol can dramatically increase staining intensity. This has particularly important implications in brain regions of low per-cell AR abundance, as it allows visualization and quantification of signal that

was previously unquantifiable, thereby promoting closer examination of AR distribution and better understanding of its action and regulation. These findings may prove useful as more studies investigate androgen effects on cognition, learning, and memory mediated by prefrontal cortical areas.

Chapter 3: Analysis of the effects of aging on testosterone and androgen receptors in mesocorticolimbic brain regions of male rats

3.1 Summary

Circulating levels of T, the primary male sex steroid, gradually decrease with age. Aging is also accompanied by decline in executive functions mediated by the PFC and dependent on DA innervation. It has been suggested that these declines are more than just temporally related; in fact, T may modulate prefrontal function through effects on mesocorticolimbic DA signaling, and this modulation may change with age. To characterize the potential effects of age on mesocorticolimbic androgen activity, we examined serum T levels, neural T levels, and AR levels in young (5 months) and aged (22 months) male Fischer 344 x Brown Norway rats. Serum T levels decreased with age, as did T levels in mesocorticolimbic brain regions like the mPFC, OFC, NAc, and VTA. However, aged rats had higher neural T relative to serum T, suggesting a mechanism of partially compensatory neural T production. Aged rats also had lower AR-immunoreactivity in cortical regions, but not mesolimbic regions. Altogether, these results show clear alterations in T and AR levels, and indicate that age-related declines in serum T, neural T, and cortical ARs could contribute to impaired mesocorticolimbic DA signaling underlying decline in executive functions.

3.2 Introduction

Across mammalian species such as humans, monkeys, and rodents, aging is associated with a decline in cognitive function, as well as significant changes in endocrine systems (Lamberts et al., 1997), including a gradual but marked decrease in systemic levels of androgen hormones such as T (Morley and Perry, 1999; Vermeulen, 1991; Kaler and Neaves, 1981;

Ghanadian et al., 1975). This decline in T is particularly relevant to study of cognitive aging in males, as androgens are known to play an important role in several aspects of cognition over the lifespan (Cherrier and Craft, 2003) and accordingly, their decline is accompanied by psychological in addition to somatic symptoms (Lund et al., 1999).

Cognitive aging is characterized in part by decline in executive functions, which are governed by prefrontal cortical structures like the mPFC and OFC (Beas et al., 2013; Janowksy, 2006; Gallagher et al., 2011). Executive functions encompass "higher-order" coordinative behaviors such as attention, decision-making, working memory, and behavioural flexibility (Miller and Cohen, 2001; Beas et al., 2013; Robbins et al., 1996; Dalley et al., 2004; Kesner and Churchwell, 2011). Decline in these functions is observed in cases of "normal/healthy" cognitive aging (Alexander et al., 2012; Janowsky et al., 2000) as well as in pathological aging disorders such as AD (Lindeboom and Weinstein, 2004; Rapp and Reischies, 2005; Perry and Hodges, 1999).

Several studies provide evidence for gonadal influence on prefrontal executive functions (Bimonte-Nelson et al., 2003; Aubele and Kritzer, 2011; van Haaren et al., 1990; Janowsky et al., 2000; Luine, 2008). T supplementation has been shown to improve performance on PFC-dependent tasks, such as those testing working memory (Janowsky et al., 2000; Kritzer et al., 2001), and T deprivation impairs performance (Daniel et al., 2003). One major mechanism through which androgens are thought to exert effects on executive function is regulation of mesocorticolimbic DA signaling. The mesocorticolimbic DA circuit, classically associated with reward, motivation, and higher-order cognitive functions (Wise, 2009; Kritzer, 1997; Fibiger and Phillips, 1988; Koob and Volkow, 2010) consists of midbrain dopaminergic cell bodies originating in the VTA, with limbic projections to the NAc, as well as cortical projections to the frontal regions including the mPFC and the OFC (Sato et al., 2008; Mizoguchi et al., 2009; Le Moal and Simon, 1991). Androgens could influence executive function through direct effects on

the mPFC and/or OFC to alter behavior, or indirect effects on regions downstream or upstream of the PFC such as the NAc and VTA.

While brain androgens are generally sourced from the testes and received through systemic circulation, there is also evidence for androgen production by the brain itself. Presence of steroidogenic enzymes for T and other sex steroids has been demonstrated in human and rat brain (Zwain and Yen, 1998; Schumacher et al., 2003; Hojo et al., 2009; Hojo et al., 2004). Such neuroandrogens may be produced in mesocorticolimbic regions, and have effects relevant for cognition.

Androgen effects on the brain are generally mediated through interaction with traditional ARs (Janowsky et al., 2000). ARs are nuclear receptors that act as ligand-dependent transcription factors to regulate gene expression (Evans, 1988) although there is growing evidence for non-genomic membrane-associated capabilities of traditional ARs (Heinlein and Chang, 2002; Rahman and Christian, 2007). ARs are broadly but selectively distributed throughout the brain, and their expression is regulated in part by circulating androgen levels (Menard and Harlan, 1993). Consequently, age-related declines in circulating T could impact AR expression. Although several studies have examined age-related changes in AR expression in the brain, these have largely focused on brain regions associated with control of reproductive and social behaviors such as the hypothalamus (Wu et al., 2009; Chambers et al., 1991), amygdala (Chambers et al., 1991), and cranial nerve nuclei (McGinnis and Yu, 1995). Fewer studies have examined the effects of age in mesocorticolimbic regions, although previous work has reported the presence of ARs in the PFC, NAc, and VTA, and that PFC-projecting and NAc-projecting dopaminergic cells from the VTA contain ARs (Simerly et al., 1991; Kritzer, 1997; Kritzer and Creutz, 2008; Creutz and Kritzer, 2004).

In this study, we aim to understand how the systemic declines in T levels that accompany aging can impact androgen-sensitive dopaminergic brain circuitry underlying

executive function. More specifically, we investigate the effect of age on endogenous levels of serum T, neural T, and ARs in mesocorticolimbic brain regions.

3.3 Materials and Methods

3.3.1 Subjects

Two cohorts of male Fischer 344 x Brown Norway F1 hybrid (F344/BN) rats (species: *Rattus norvegicus*) consisting of young (5 months old) and aged (22 months old) rats (n=12 per age, n=24 per cohort) were examined. Both cohorts were obtained from the National Institute on Aging (Taconic Farms), and were sexually naïve. Rats were group-housed with rats of similar age for the first week after arrival and given *ad libitum* access to food (Rat Diet 5012; LabDiet, St. Louis, MO) and water. In the remaining weeks, rats were single-housed and food-restricted to 85-90% of their free-feeding weight. All cages contained Aspen chip bedding (Nepco, Warrensburg, NY), paper towel, and a PVC pipe for environmental enrichment. Rats were kept on a 12 hr light:dark cycle at an average temperature of 21-22°C and relative humidity of 40-50%. Two weeks after arrival, rats were subjected to behavioral training (Tomm et al., 2015) for several weeks prior to sacrifice. Subjects were sacrificed at least three hours after completion of behavioral testing. The two cohorts underwent behavioral testing separately and were processed separately. The reason for performing analysis of two independent cohorts was primarily to replicate the results, and also because the two cohorts were tested on slightly different sets of behavioural tasks. All animal procedures complied with the Canadian Council on Animal Care and the University of British Columbia guidelines for animal care.

3.3.2 Tissue Collection and Fixation

Rats were euthanized by isoflurane inhalation and rapid decapitation. To minimize the stress levels of subjects, sacrifice duration was kept to under three minutes per rat. Trunk blood

was collected into a microcentrifuge tube and brains were removed. The microcentrifuge tube was centrifuged for 10 min at room temperature, and the serum was pipetted into a separate microcentrifuge tube. Serum samples were stored at -80 °C until processing for steroid measurement. Fresh brains were cut in half along the sagittal plane; one half (left or right) was immediately fixed for later immunohistochemical processing; the remaining half was frozen fresh on dry ice and stored at -80°C for measurement of steroids. Half-brains were immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h at room temperature then cryo-protected in 30% sucrose solution at 4°C for 48 h or until they had sunk. Brain samples were stored at -80°C for sectioning. Half-brains were serially sectioned on a cryostat into five series of 40µm coronal sections. Free-floating sections were stored in anti-freeze solution at -20°C until immunohistochemical processing.

3.3.3 T Measurement from Brain and Blood

3.3.3.1 Microdissection and Homogenization

To measure the amount of T in selected brain regions, microdissected tissue was first acquired from four brain regions of interest including the mPFC, OFC, NAc, and VTA. To do so, fresh frozen half-brains were sectioned into 300 µm coronal sections on a cryostat at -15° C. Sections were collected starting at the rostral end of the brain. From these sections, brain regions of interest were located using the rat brain atlas (Paxinos and Watson, 2009), and removed via the Palkovits punch technique as described previously (Taves et al., 2011; Charlier et al., 2010). Briefly, a stainless steel corer (id = 1.0 mm, od = 1.8 mm; Fine Science Tools, catalog #18035) was used to punch the tissue from 6-10 adjacent sections, depending on the region. One punch was collected per section. The correct placement of punches was verified by Nissl staining. Subjects with incorrectly placed punches were excluded from analysis. The

punched tissue was collected in microcentrifuge tubes, then homogenized with 1 mL 84% ice-cold methanol, using a bead homogenizer (Omni Bead Ruptor 24; #19-101).

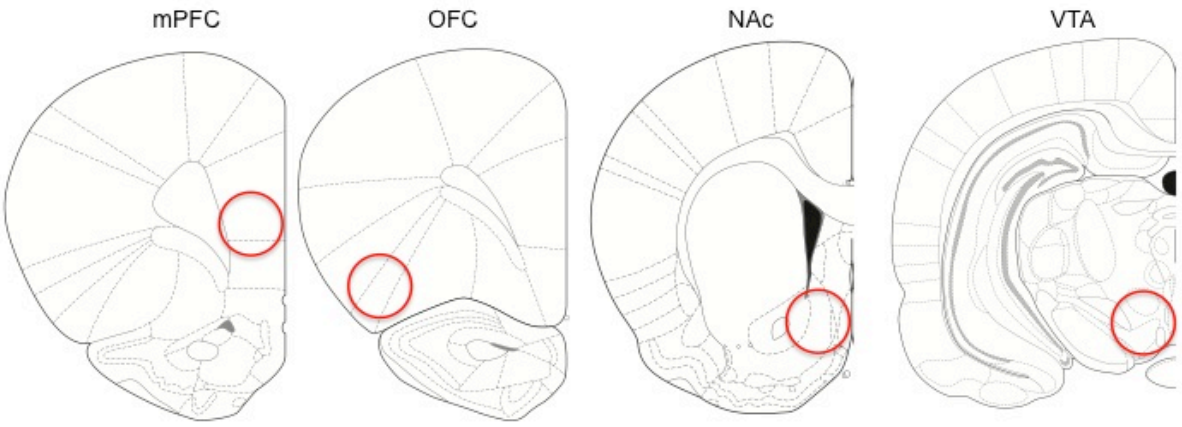

Figure 3.1. Approximate placement of brain tissue punches for neural T measurement. From left: medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), nucleus accumbens (NAc), ventral tegmental area (VTA). Punches were made with a corer with an internal diameter of 1.0 mm and an external diameter of 1.8 mm. Tissue was punched from 6-10 adjacent sections for each region.

3.3.3.2 Steroid Extraction

Homogenized tissue samples were left overnight at -20 °C. The following day, samples were centrifuged, and the supernatant was collected and diluted in 10 mL deionized water in preparation for steroid extraction. For T measurement from blood serum, frozen serum was thawed and mixed with 10 mL deionized water. Steroids were extracted from the sample using solid phase extraction (SPE) with C_{18} columns (Agilent Bond Elut LRC-C18 OH, #12113045) mounted on a glass vacuum manifold (UCT #VMF024GL). After one wash with 10mL of 40% methanol as described by Taves et al. (2010), steroids were eluted from columns with 5mL of 90% HPLC-grade methanol, and dried in a vacuum centrifuge (ThermoElectron SPD111V Speedvac) at 40 °C.

3.3.3.3 Radioimmunoassay Measurement

Extracted steroids were then measured using radioimmunoassay (RIA). T was measured using a commercial RIA kit (#07189102; MP Biomedicals, Solon, OH), with modifications described by Overk et al. (2013) and Ferris et al., (2015). Dried elutes were re-suspended in 100% ethanol to promote steroid solubility, and diluted with the buffer provided in RIA kit. The amount of ethanol in samples did not exceed 1%. 200 µL of primary antibody was added to the re-suspended sample and standards, and incubated for 4 h at room temperature. 200µL of tracer was then added and incubated overnight at room temperature. The following day, 50 µL of precipitant (secondary antibody) was added to all tubes and incubated for 1 h at 37°C in a hot water bath. Tubes were then centrifuged at 4°C and decanted. Lastly, tubes were counted for 2 min on a gamma counter. Final values were corrected for recovery. Recovery percentages were calculated for each sample type (serum or brain tissue) by comparing unspiked samples to samples spiked with a known amount of steroid. Recoveries were 88% for the serum, and 113% for the brain tissue.

3.3.4 Immunohistochemistry for AR

IHC for the two cohorts was conducted in two independent runs, the first run containing all the subjects from Cohort 1, and the second run containing all the subjects from Cohort 2. IHC was performed using free-floating tissue sections placed in 24-well plates, and age groups were counterbalanced such that each plate contained tissue from one young and one aged subject, so any plate-to-plate immunohistochemical variability would be evenly spread.

Sections were washed for 20 min in 0.1M Tris-buffered saline (TBS), then incubated in 2% hydrogen peroxide (H_2O_2) solution for 30 min to quench endogenous peroxidase activity. Sections were then washed for 20 min in 0.1M TBS, then for 5 min in 0.1M PBS with 0.03% Triton X-100 and 0.1% gelatin (hereafter referred to as PBS-GT). Next, sections were incubated

in 8% tryptone (J859; Amresco, Inc., Solon, OH, USA) in PBS for 2 h to block nonspecific staining. Directly following the block, sections were incubated in primary antibody solution (1:200 in PBS-GT) for 24 h at room temperature. The primary antibody was a monoclonal anti-AR antibody [EPR1535(2)] raised in rabbit (ab133273; Abcam, Inc., San Francisco, CA, USA), previously validated for specificity by Western Blot and use in testicular feminization mutant rat tissue lacking fully functional ARs (Hamson et al., 2013). It recognizes a region within the first 30 amino acids of the AR protein (as per the manufacturer), which is a similar epitope to the one recognized by the extensively used and validated polyclonal anti-AR antibody, PG-21 (Prins et al., 1991). The following day, sections were washed for 40 min in PBS-GT, then incubated in biotin-SP-conjugated affinity-purified donkey anti-rabbit secondary antibody solution (1:2000 in 0.8% tryptone in PBS; 711-065-152; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature, then overnight at 4°C. Following the secondary antibody incubation, sections were washed for 40 min in PBS-GT, incubated in avidin-biotin peroxidase (AB) solution (in 0.8% tryptone in PBS; VectaStain ABC Kit; PK-6100 Standard; Vector Laboratories) for 30 min at room temperature, then washed in PBS-GT for 15 min. Next, signal was amplified by incubation in biotin tyramide “BT” solution for 10 min. BT was made in-lab as described by Adams (1992), and solution contained 45 µL BT mixed with 5 µL 30% H_2O_2 and 15 mL PBS). Sections were then washed in PBS-GT for 15 min, incubated in AB solution for 30 min, and washed in PBS-GT for 15 min. Sections underwent one quick wash in TBS, and then were incubated in DAB in H_2O_2 (Vector Labs Peroxidase Substrate Kit SK-4100) with nickel for 20 minutes at room temperature. Lastly, sections were mounted onto charged slides (Fisher Colorfrost Plus; 12-550-19), dehydrated, and cover-slipped. Slides were recoded such that the age group of each subject was hidden from experimenters who were performing subsequent analysis.

3.3.5 Analysis of AR Immunoreactivity

3.3.4.1 Brain Regions

AR immunoreactivity (AR-ir) was assessed in eight brain regions total, consisting of four cortical regions and four mesolimbic regions. The cortical regions were: (1) the prelimbic portion (PrL) of the mPFC, (2) the infralimbic (IL) portion of the mPFC; (3) the lateral orbitofrontal cortex (LOFC), and (4) the medial orbitofrontal cortex (MOFC). The mesolimbic regions were: (1) the NAc shell (NAcS), (2) the NAc core (NAcC), (3) the parabrachial/paranigral (PBP/PN) regions of the VTA (pVTA), and (4) the caudal region of the VTA (cVTA). The eight regions were defined according to common landmarks and bregma coordinates consistent with the rat brain atlas (Paxinos and Watson, 2009).

3.3.4.2 Photomicroscopy

For each brain region, photomicrographs were taken using a Nikon Digital Sight DS-U1 camera and Nikon Eclipse 90i microscope (at the X 20 objective) interfaced with NIS-Elements Basic Research software (Nikon Canada, Inc., Richmond, BC, Canada). Images were taken from one to five serial sections, depending on the region sampled (Table 3.1). For some subjects, it was not possible to obtain the maximum number of images due to folded or ripped tissue, or poor staining. In those cases, subjects were still included in analysis, but the photos of compromised tissue were excluded. Subjects were completely excluded from analysis in cases where no images were possible, such as when the entire brain region was missing from the tissue section, e.g. due to a sagittal cut not centered on the midline of the brain, or when the staining quality was too poor for adequate quantification. All images were captured at identical brightness and contrast settings with the exception of the NAc, for which brightness and contrast was increased in all subjects. All images were captured at maximum resolution (2560 x 1920). All photomicroscopy and subsequent image analysis was conducted by an experimenter who was blind to the age of each subject.

Region	Number of brain sections examined	Number of photos taken per section	Total number of photos analyzed
PrL	4	1	4
IL	4	1	4
LOFC	5	1	5
MOFC	2	1	2
NAcS	5	1	5
NAcC	5	1	5
pVTA	3	2	6
cVTA	1	1	1

Table 3.1. Number of photomicrographs analyzed per region per subject for quantification of AR-ir.

3.3.4.3 Quantification of Immunoreactivity

For each image, a background mean intensity value was calculated by averaging six intensity measurements taken from random locations within each brain area where specific staining was not present. Next, the background intensity value for each image was used to generate a quantification threshold value. For the PrL, IL, MOFC, LOFC, and Cohort 1 NAc and VTA regions, the threshold was set to a value 1.25 times (1.25X) as dark as the background. Only staining that was at or above threshold was quantified. For the NAc and VTA in Cohort 2, the threshold was set to 1.5X the background in select images because of higher background staining. Using 1.5X allowed more accurate capture of true immunoreactivity and elimination of background capture while a large portion of background staining was captured using a 1.25X threshold. Once thresholds were calculated for all images, they were applied to a defined region of interest (ROI) with area 135,000 μm^2 for each brain region. In NIS-Elements, the ROI was superimposed onto each photomicrograph within the limits of each brain region, and the program generated a value (hereafter referred to as % AR-ir) indicating the percentage of pixel area within the ROI that was above threshold. For each image, adjustments were made to the clean function in NIS-Elements, and restrictions were applied based on object circularity and size in order to most accurately capture stain and exclude background.

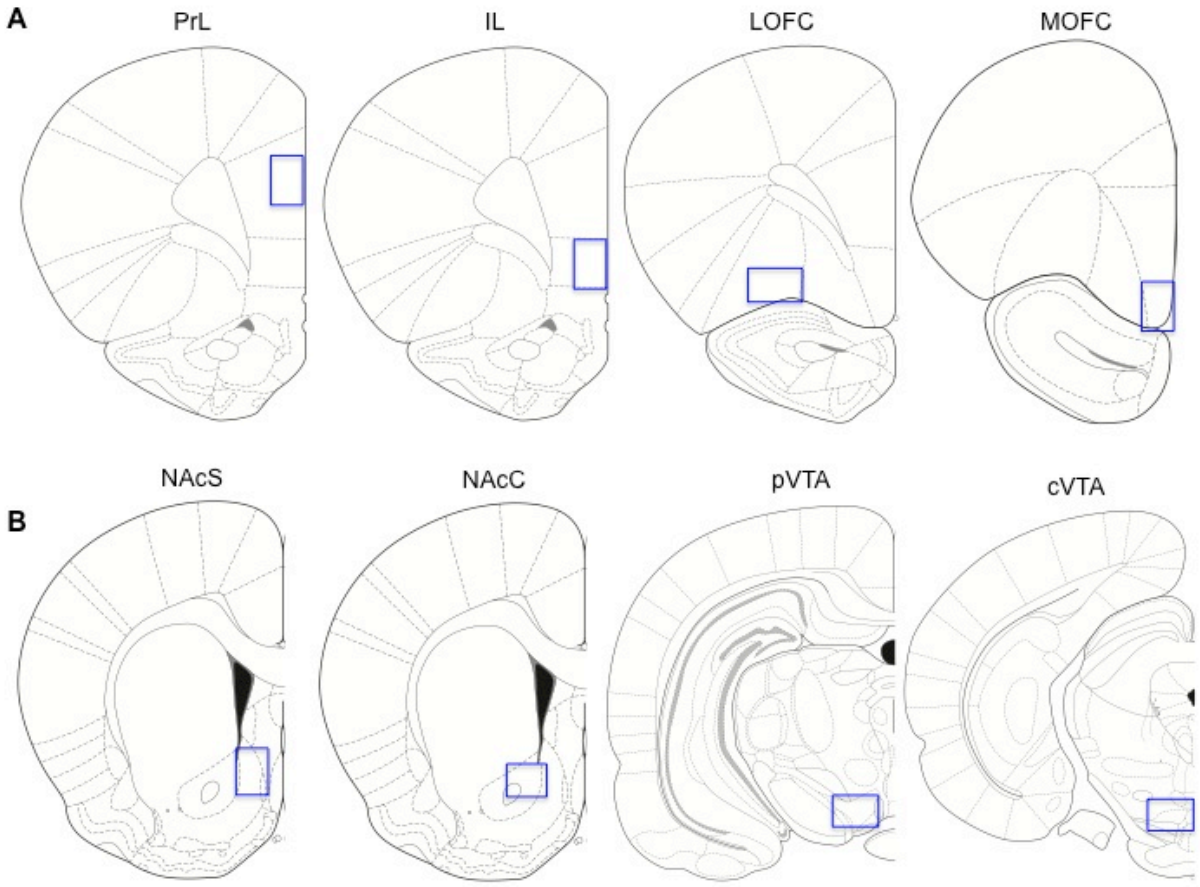

Figure 3.2. Approximate placement of ROI for quantification of AR-ir in (A) cortical regions, (B) mesolimbic regions. Top row from left: prelimbic (PrL), infralimbic (IL), lateral orbitofrontal cortex (LOFC), medial orbitofrontal cortex (MOFC); bottom row from left: nucleus accumbens shell (NAcS), nucleus accumbens core (NAcC), parabrachial and paranigral ventral tegmental area (pVTA), caudal ventral tegmental area (cVTA).

3.3.6 Statistical Analysis

Statistical analysis was conducted using SPSS Statistics software (Version 23.0 for Mac OS X; Chicago, IL). Effects of age were analyzed on the following endpoints: T levels (in serum and in mesocorticolimbic brain regions), a calculated Neural T: Serum T ratio, and AR-ir in mesocorticolimbic brain regions.

3.3.6.1 Analysis of Absolute Testosterone Levels

To analyze age differences and regional differences in absolute T levels, a Two-Way Mixed Model analysis of variance (ANOVA) was performed, using age as a between-subjects factor, and region as a within-subjects repeated measure. There were five regions analyzed: (1) serum, (2) mPFC, (3) OFC, (4) NAc, and (5) VTA. For this analysis, the mPFC, OFC, NAc, and VTA were not subdivided into their constituent regions of the PrL and IL, LOFC and MOFC, NAcS and NAcC, and pVTA and cVTA. Data from both cohorts was pooled and log-transformed in order to normalize the distributions. ANOVA analysis revealed a significant interaction, so simple main effects were subsequently analyzed via One-Way ANOVA and Repeated Measures ANOVA. For Repeated Measures ANOVAs, the Bonferroni correction for multiple comparisons was used. Analysis was conducted on pooled data, and also on data from each cohort individually.

3.3.6.2 Analysis of the Neural T: Serum T Ratio

Neural T: Serum T ratios were calculated for each brain region of each subject by dividing the absolute T level of the brain region by the serum T level. Age differences in the neural T: serum T ratios were analyzed as above for absolute T levels using a Two-Way Mixed Model ANOVA. Data were log-transformed because of non-normal distributions. Ratios were analyzed using pooled data from all subjects, and also within each cohort.

3.3.6.3 Analysis of AR-immunoreactivity

Analysis of the effects of age on AR-ir was performed separately for each of the eight brain regions, including the (1) PrL, (2) IL, (3) LOFC, (4) MOFC, (5) NAcS, (6) NAcC, (7) pVTA, and (8) cVTA. For each region, a Two-Factor ANOVA was performed with age and cohort as between-subjects factors. Cohort was included as a between-subjects factor because overall staining levels and AR-ir values were consistently higher in Cohort 2 than in Cohort 1 (Figure

3.12), so data from the two cohorts could not be pooled. Prior to ANOVA analysis, data sets for each brain region were square root transformed because of heterogeneous variances and non-normality.

Additionally, in order to facilitate combining data from both cohorts, data were normalized within each cohort, for each brain region separately. Normalization was achieved by dividing by all raw AR-ir scores by the young subjects group mean score and then multiplying by 100. Thus, all values became percentages of the young mean for a given brain region. Normalized values from Cohort 1 and 2 were pooled together and analyzed using the Independent Samples t-test. For one brain region (the NAcC), the assumption of homogeneity of variance was violated, so the t-test with Welch's correction was used.

3.3.6.4 Correlations Between T and AR-ir Levels

Pearson correlations were used to examine the relationship between T levels and AR-ir levels for a given brain region. In each cohort, T data was log-transformed and AR data was square-root transformed to normalize distributions.

3.4 Results

3.4.1 Absolute T Levels in the Serum and Brain

Two-Factor Mixed Model ANOVA revealed a significant effect of age on absolute T levels in the serum and brain ($F_{1,37} = 4.82$, $P < .05$), such that T levels were lower in aged rats relative to young (Figure 3.3). There was also significant effect of region ($F_{1.881,69.597} = 144.32$, $P < .001$), showing higher T levels in the brain than in the serum. There was a significant age x region interaction ($F_{1.881,69.597} = 7.71$, $P < .005$, indicating that age differences in T levels depended on the region.

Since there was a significant interaction, simple main effects were subsequently analyzed. For each region, except for the VTA ($F_{1,39}$ = 3.30, P = .08), there was a main effect of age, with T levels significantly lower in aged rats than in young in the serum ($F_{1,46}$ = 15.91, P < .0001), PFC ($F_{1,45}$ = 6.02, P < .05), OFC ($F_{1,46}$ = 6.32, P < .05), and NAc ($F_{1,43}$ = 5.21., P < .05). There was a main effect of region in young rats ($F_{1.699,\ 30.575}$ = 51.43, P > .0001) and aged rats ($F_{3.716,\ 35.901}$ = 96.97, P > .0001). Pairwise comparisons using the Bonferroni correction for multiple comparisons revealed that T levels in all brain regions were significantly higher than in serum in young (P < .005) and aged rats (P < .0001).

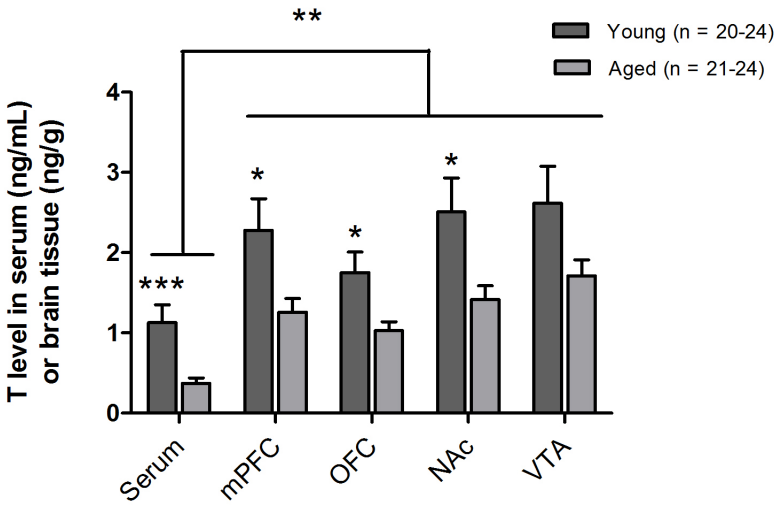

Figure 3.3. Absolute T levels in young (5 months old) and aged (22 months old) male rats from cohorts 1 and 2. There was a significant main effect of age (P < .05) in all regions except the VTA. Regardless of age, T levels were higher in mesocorticolimbic brain regions than in the serum (P < .005). *P < .05, **P <.01, ***P <.001.

When each cohort was analyzed separately, similar trends were seen. There was a significant age x region interaction (P < .05) in both Cohort 1 and 2, so simple main effects were analyzed. In both cohorts, there was a main effect of age on serum T levels, with higher T in young animals than aged (P < .05). In Cohort 2, there were significant age differences in all

mesocorticolimbic regions except the VTA ($P < .05$), while in Cohort 1 these differences were not statistically significant. Brain levels of T were also significantly higher than serum levels in young and aged subjects of Cohort 2 ($P < .05$), and in all regions in Cohort 1 except for the OFC.

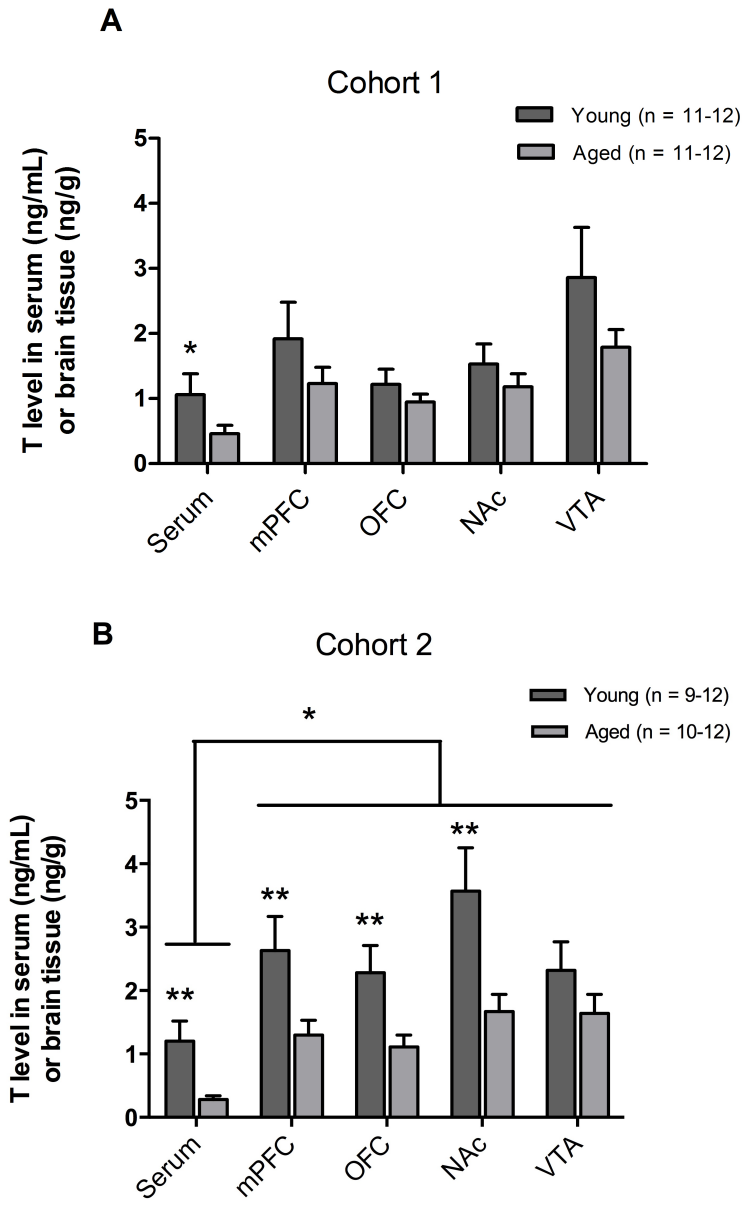

Figure 3.4. Absolute T levels in young (5 months old) and aged (22 months old) male rats, separated by cohort. (A) Cohort 1, (B) Cohort 2. Regardless of age, T levels were higher in mesocorticolimbic brain regions than in the serum in Cohort 1 (except for the OFC), and in Cohort 2. *$P < .05$, **$P < .01$.

3.4.2 Neural T: Serum T Ratio

There was a significant main effect of age on the Neural T: Serum T ratio. Although aged rats had lower absolute levels of serum T and neural T, their neural T: serum T ratio was consistently higher than that of young rats in all mesocorticolimbic regions examined ($F_{1,37}$ = 12.12, P < .005) (Figure 3.5). There was also a significant main effect of region ($F_{3,111}$ = 38.90, P < .0001), with the highest ratio in the VTA, followed by the NAc, PFC, and OFC. There was no significant age x region interaction ($F_{3,111}$ = .32, P > .80).

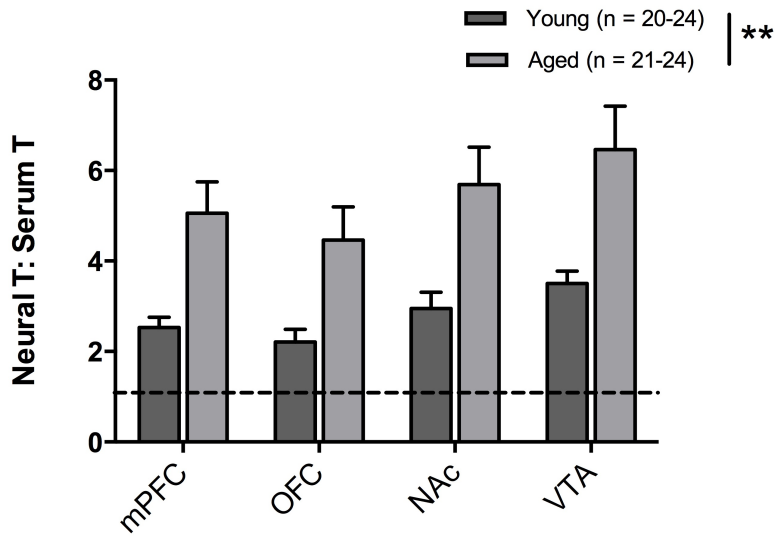

Figure 3.5. Neural T: Serum T ratio in young and aged male rats from cohorts 1 and 2. Although there were lower absolute T levels in aged rats, the neural T: serum T ratio in mesocorticolimbic regions was higher than in young. Horizontal line represents a ratio of 1. ***P*<.001

When each cohort was analyzed separately, similar results were seen, with a significant main effect of age (P < .05 in Cohort 1 and 2), and region (P < .0001 in Cohort 1 and 2) (Figure 3.6). In both cohorts, there was no age x region interaction (P > .29).

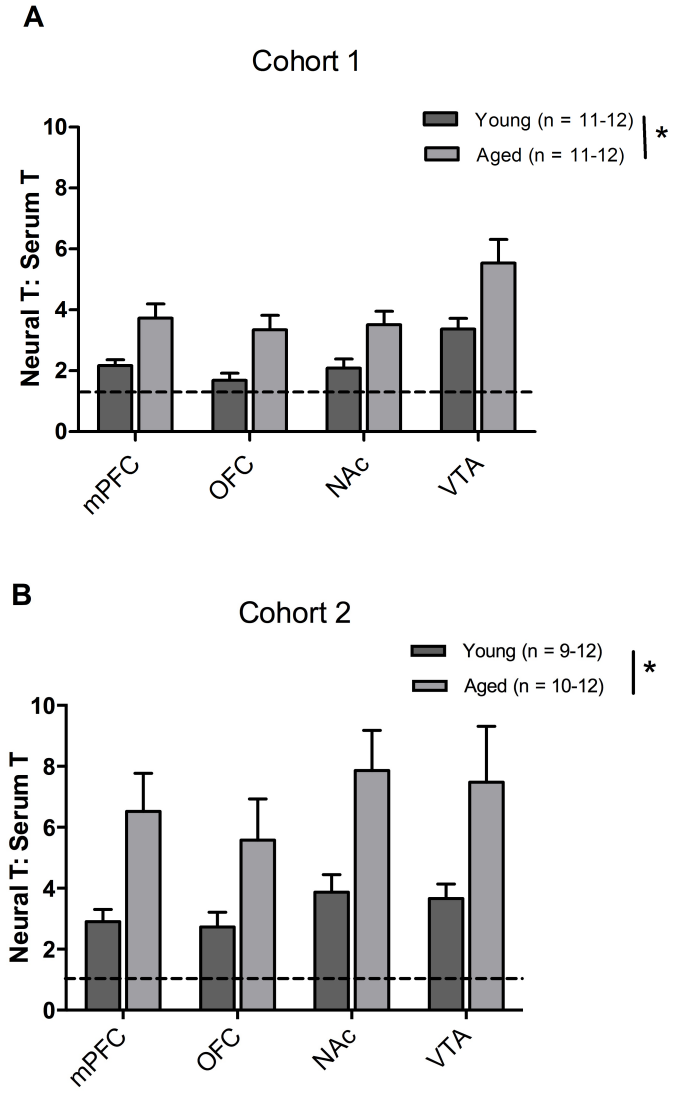

Figure 3.6. Neural T: Serum T ratio in young and aged male rats, separated by cohort. (A) Cohort 1, (B) Cohort 2. Although there were lower absolute T levels in aged rats, the neural T: serum T ratio in mesocorticolimbic regions was higher than in young. Horizontal line represents a ratio of 1. *P<.05

3.4.3 AR Levels in the Brain

3.4.3.1 Overall Pattern of AR Immunoreactivity

In all brain regions examined, AR staining appeared mostly concentrated in cell nuclei. This is consistent with previous reports of predominantly nuclear immunoreactivity in rodent

brain (Lu et al., 1998; Zhou et al., 1994; Fernandez-Guasti et al., 2003), although AR-ir at extranuclear sites has also been described (Tabori et al., 2005; Sarkey et al., 2008; DonCarlos et al., 2003). Nuclear immunostaining ranged from even and dark to punctate and spotty in all regions examined (Figure 3.7), similar to patterns of nuclear staining reported by Wu et al. (2009).

The overall distribution of staining was also in line with previous evaluations of nuclear AR distribution in the rat brain (Simerly et al., 1990; Sar et al., 1990). Importantly, in the regions of interest, including the frontal cortex and VTA, the staining pattern closely matched prior studies (Kritzer, 1997; 2004). In the frontal cortex, AR-ir was mainly observed in pyramidal cell layers II/III and to a lesser extent in V/VI, in a similar bilaminar pattern described by Kritzer (2004), and in the VTA, staining was intense but sparse with the majority of ir in the PBP and PN subregions (Kritzer, 1997).

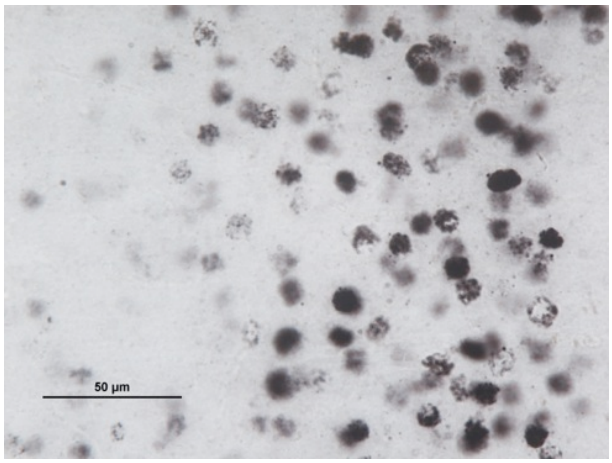

Figure 3.7. Nuclear AR immunostaining. Immunolabelled cells ranged from dark and evenly stained to light and unevenly stained. Representative image from the prelimbic (PrL) region in the medial prefrontal cortex (mPFC).

3.4.3.2 Age Differences in AR Immunoreactivity

Two-factor ANOVA revealed a significant main effect of age on AR-ir, but this effect was observed in cortical regions only, and not in mesolimbic regions. In all cortical forebrain regions examined, including the PrL, IL, LOFC, and MOFC, aged rats had significantly lower AR-ir than young rats (P <.05 in all regions) (Figures 3.8, 3.9, 3.10). In all mesolimbic regions examined, including the NAcS, NAcC, pVTA, and cVTA, there was no age difference in AR-ir (P >.10 for all regions) (Figure 3.11).

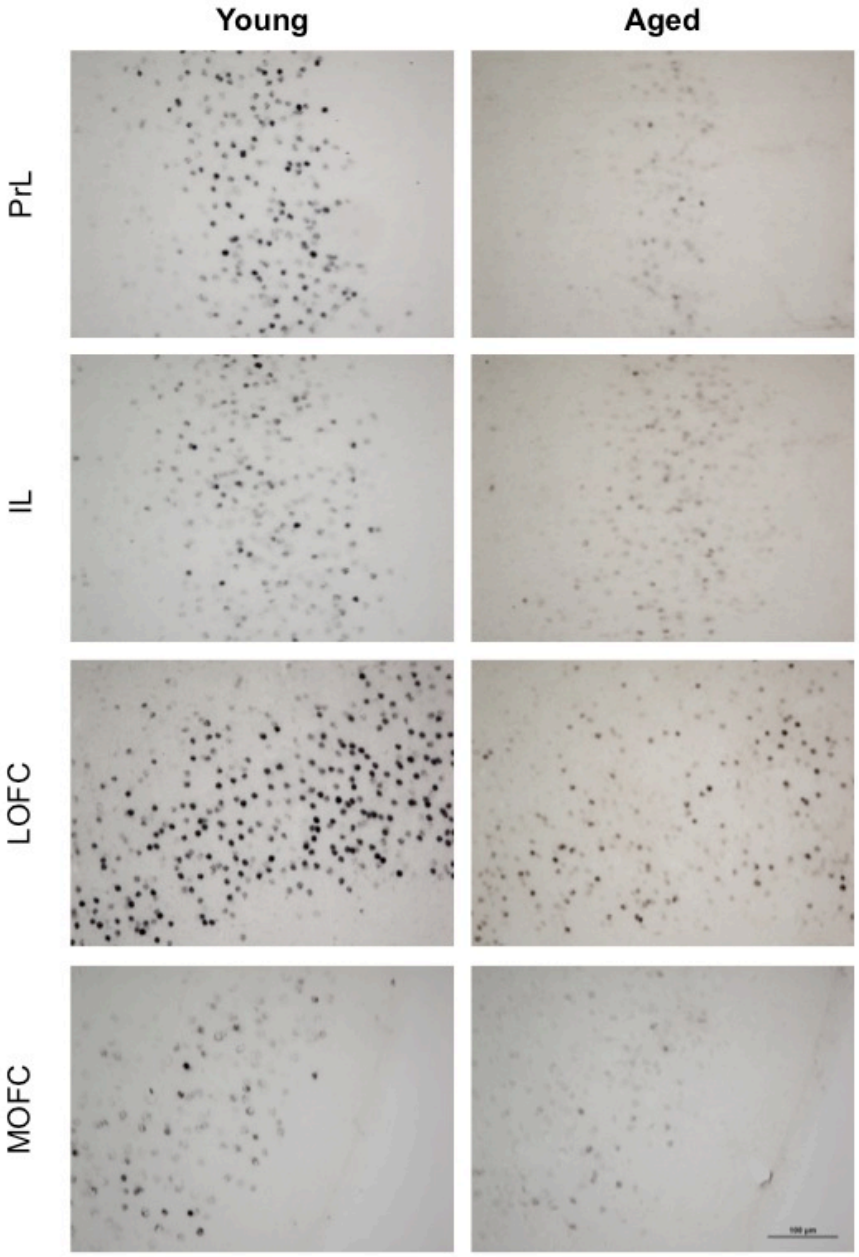

Figure 3.8. Cohort 1 AR-ir decreased with age in cortical regions. Representative images from the prelimbic (PrL), infralimbic (IL), lateral orbitofrontal cortex (LOFC), and medial orbitofrontal cortex (MOFC). Scale bar represents 100 μm.

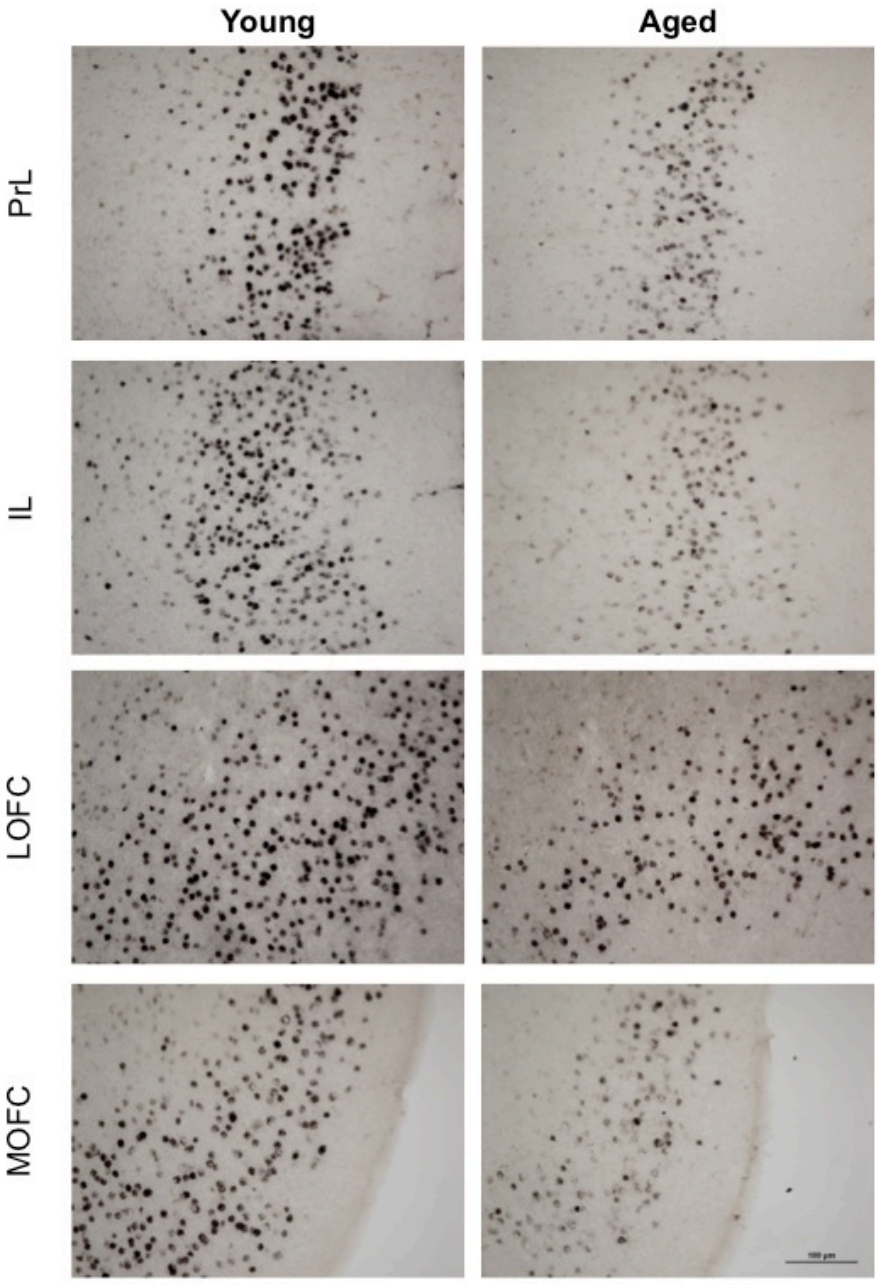

Figure 3.9. Cohort 2 AR-ir decreased with age in cortical regions. Representative images from the prelimbic (PrL), infralimbic (IL), lateral orbitofrontal cortex (LOFC), and medial orbitofrontal cortex (MOFC). Scale bar represents 100 μm.

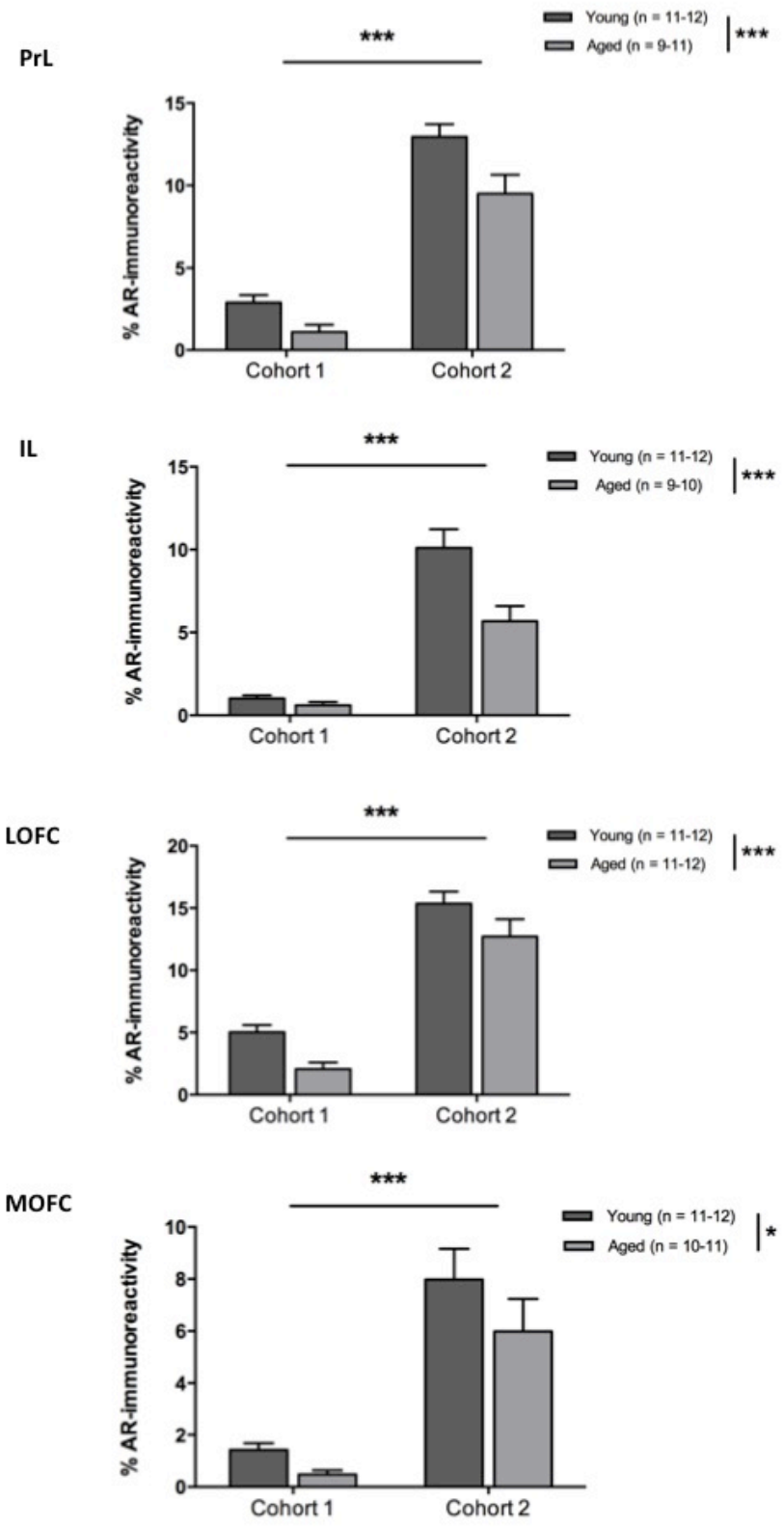

Figure 3.10. AR-ir in cortical regions of young and aged animals. AR-ir decreased with age in Cohorts 1 and 2 in the PrL, IL, LOFC, and MOFC. In each region, Cohort 2 AR-ir was significantly higher than Cohort 1. Data are mean %AR-ir ± SEM. * P < .05, **P < .01, ***P < .001.

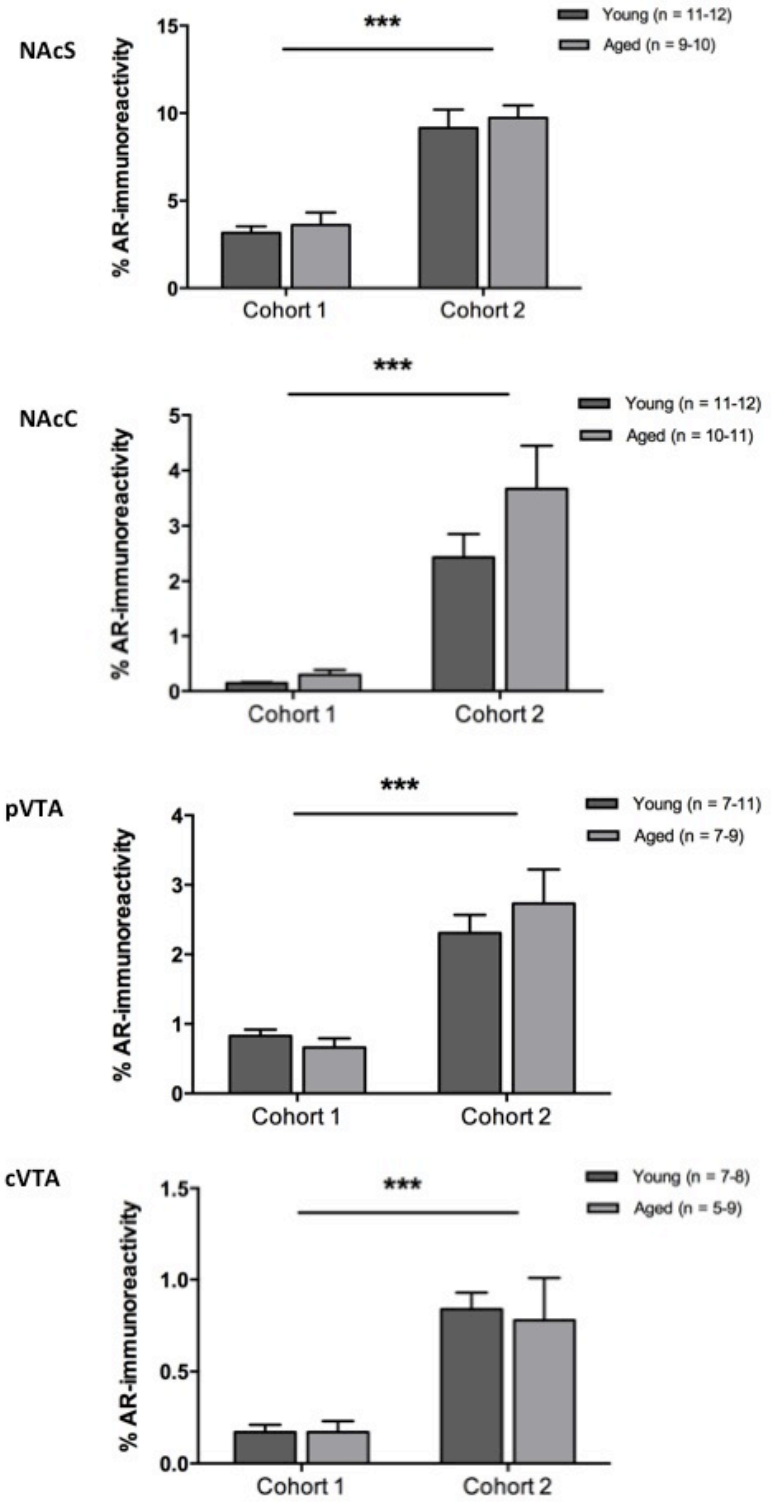

Figure 3.11. AR-ir in mesolimbic regions of young and aged animals. AR-ir did not change with age in Cohorts 1 and 2 in the NAcS, NAcC, pVTA, and cVTA. In each region, Cohort 2 AR-ir was significantly higher than Cohort 1. Data are mean %AR-ir $\pm$ SEM. ***P < .001

In all brain regions, there was a significant main effect of cohort, as expected, showing Cohort 2 AR-ir values to be consistently higher than Cohort 1 values ($P < .001$ in all regions) (Figures 3.10 and 3.11). However, there was no evidence of a significant age x cohort interaction on AR-ir ($P > .10$ in all regions). Consequently, the main effect of age was considered to be similar across both cohorts.

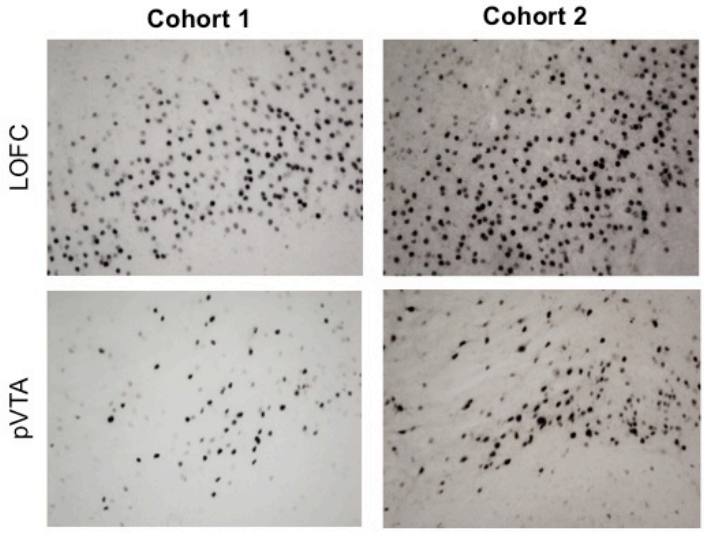

Figure 3.12. Immunostaining intensity differences between cohorts. AR-ir in Cohort 2 was consistently higher than in Cohort 1 across cortical and mesolimbic regions examined. There was a significant effect of Cohort, but no Age x Cohort interaction. Representative images from LOFC (top), and pVTA (bottom).

Region	Young	Aged	
PrL	100 ± 8.36%	57.31 ± 9.43%	*t*(41) = 3.40, ***P* = .002****
IL	100 ± 10.65%	57.25 ± 10.70%	*t*(40) = 2.81, ***P* = .008****
LOFC	100 ± 6.56%	60.96 ± 8.15%	*t*(44) = 3.73, ***P* = .001****
MOFC	100 ± 11.50%	53.58 ± 10.32%	*t*(42) = 2.98, ***P* = .005****
NAcS	100 ± 7.99%	110.47 ± 12.13%	*t*(40) = -0.74, *P* = .46
NAcC	100 ± 11.09%	175.88 ± 33.52%	*t'*(42) = -1.68, *P* = .10
pVTA	100 ± 7.72%	96.33 ± 13.48%	*t*(32) = 0.24 *P* = .81
cVTA	100 ± 12.50%	95.80 ± 23.45%	*t*(27) = 0.16, *P* = .87

Table 3.2. Normalized AR-ir in young and aged rats from both cohorts (pooled). Data are represented as a percentage of the young mean. Data were analyzed by independent samples t-tests, and appear as normalized mean ± SEM. *P < .05, **P < .01.

Region	Cohort 1		Cohort 2	
	Young	**Aged**	**Young**	**Aged**
PrL	100 ± 15.46%	37.64 ± 16.16%	100 ± 5.87%	73.41 ± 8.88%
IL	100 ± 18.21%	58.48 ± 20.97%	100 ± 11.11%	56.14 ± 9.10%
LOFC	100 ± 11.44%	40.92 ± 10.51%	100 ± 6.40%	82.82 ± 9.03%
MOFC	100 ± 18.03%	34.04 ± 11.17%	100 ± 14.77%	75.07 ± 15.69%
NAcS	100 ± 11.61%	114.32 ± 22.53%	100 ± 11.49%	106.19 ± 7.74%
NAcC	100 ± 14.72%	203.06 ± 61.90%	100 ± 17.48%	151.17 ± 32.04%
pVTA	100 ± 10.58%	79.39 ± 16.20%	100 ± 11.10%	118.12 ± 21.15%
cVTA	100 ± 25.32%	97.30 ± 34.26%	100 ± 10.27%	93.08 ± 27.86%

Table 3.3. Normalized AR-ir in young and aged rats, separated by cohort. Data are represented as a percentage of the young mean. Data are normalized mean ± SEM.

3.4.4 Correlations between T and AR-ir Levels

Pearson correlations between T and AR-ir levels are shown in Tables 3.4 and 3.5. In Cohort 1, T and AR were not significantly correlated, except for the LOFC and serum T (Table 3.4). In Cohort 2, AR-ir correlated significantly with serum T levels in cortical regions, but not

mesolimbic regions (Table 3.5). AR-ir was also significantly correlated with neural T in the PrL, IL, MOFC, and cVTA.

AR-ir Region	Correlation with Neural T	Correlation with Serum T
PrL	*r* = .16	*r* =.27
IL	*r* = .01	*r* = .11
LOFC	*r* = .37	*r* = **.44***
MOFC	*r* =.35	*r* = .40
NAcS	*r* = .15	*r* = .18
NAcC	*r* = .00	*r* = .05
pVTA	*r* = -.04	*r* = .22
cVTA	*r* = .16	*r* =.13

Table 3.4. Cohort 1 Pearson correlations between T and AR levels, n = 14-24. Neural T levels corresponded to T in the mPFC (for correlations with AR-ir in the PrL and IL), OFC (for the LOFC and MOFC), NAc (for NAcS and NAcC), and VTA (for pVTA and cVTA). *P < .05.

AR-ir Region	Correlation with Neural T	Correlation with Serum T
PrL	***r* =.57****	***r* = .58***
IL	***r* =.56****	***r* = .55***
LOFC	*r* =.41	***r* = .48***
MOFC	***r* = .53***	***r* = .64***
NAcS	*r* = -.28	*r* = -.14
NAcC	*r* = .04	*r* = .06
pVTA	*r* = .21	*r* = .03
cVTA	***r* = .79***	*r* = .02

Table 3.5. Cohort 2 Pearson correlations between T and AR levels, n = 8-22. Neural T levels corresponded to T in the mPFC (for correlations with AR-ir in the PrL and IL), OFC (for the LOFC and MOFC), NAc (for NAcS and NAcC), and VTA (for pVTA and cVTA). *P < .05; **P < .001.

3.5 Discussion

The overarching goal of this study was to examine how aging affects endogenous levels of T and ARs in male rats. We measured and compared serum T levels, neural T levels, and neural AR levels between groups of young (5 months) and aged (22 months) F344/BN rats. Accumulating evidence suggests that androgens influence executive functions through effects on mesocorticolimbic DA signaling (Kritzer et al., 2007), so we were interested specifically in T and AR levels in mesocorticolimbic brain regions including the mPFC and OFC (cortical

regions), and NAc and VTA (mesolimbic regions). We found that both serum and neural T levels declined with age, although in both ages, neural T levels were higher than in serum, suggesting that local T synthesis may be occurring. The ratio of neural T: serum T was higher in aged animals relative to young, suggesting that neuroandrogen synthesis may be especially important in aged animals. In addition, levels of cortical ARs were found to decrease with age, while mesolimbic ARs did not change significantly. These results were produced similarly in two cohorts of subjects.

3.5.1 Age-related Changes in Serum Testosterone Levels

A progressive age-associated decline in serum T levels has been consistently described in males of several species, including humans (Harman et al., 2001; Morley et al., 1997; Feldman et al., 2002), monkeys (Downs and Urbanski, 2006), mice (Flood et al., 1995; Machida et al., 1981), guinea pigs (Rigaudiere et al., 1976), and multiple strains of rat, such as Sprague-Dawley (Roselli et al., 1986; Wu et al., 2009), Wistar (Bernardi et al., 1998; Ghanadian et al., 1975; Pirke et al., 1978), Brown Norway (Gruenewald et al., 2000; Gruenewald et al., 1994), and Fischer 344 (Chambers et al., 1991; Luine et al., 2007; McGinnis and Yu, 1995). In rats, lowered T is thought to result from both primary and secondary testicular dysfunction (Gruenewald et al., 2000), including diminished T production within individual Leydig cells of the testes (Chen et al., 1994), Leydig cell loss (Bethea and Walker, 1979), and dysregulation of the HPG axis (Gruenewald et al., 2000).

Within a given age, serum T levels can vary widely depending on strain, sexual experience, and time of day, with ranges of roughly 1-10 ng/mL reported in young rats (Wu and Gore, 2010), and 0.22-1.6 ng/mL in aged rats. Our results demonstrating significantly lowered serum T in aged F344/BN rats relative to young are within this range, and are consistent with a modest but marked decrease with age.

3.5.2 Age-related Changes in Neural Testosterone Levels

Similar to the decline in serum T levels, brain T levels also significantly declined with age across cortical and mesolimbic regions. This decline was statistically significant in all regions except for the VTA, which showed a trend close to significance. Since the brain receives gonadally-produced steroids from the bloodstream, the lowered brain T levels in aged rats relative to young could be reflective of the overall age-related drop in circulating T.

However, the brain is not solely reliant on systemic T as an androgen source; recent research has shown the brain capable of producing its own steroids, so-called “neurosteroids” (Schmidt et al., 2008). Neurosteroid production has been previously demonstrated in the human, songbird, and rat brain, along with the presence of the enzymes necessary to synthesize androgens (Zwain and Yen, 1998; Schumacher et al., 2003; Celotti et al., 1997; London et al., 2009). Neural androgen production may be especially important in the context of aging, when systemic levels fall (Munetomo et al., 2015; Wu et al., 2009; Schumacher et al., 2003). Wu et al. (2009) and Schumacher et al. (2003) propose that neural T production may even change during aging to partially compensate for the age-related loss in systemic T, and Munetomo et al. (2015) suggest that neural androgens may be more important than gonadal androgens during aging. They cite findings that the hippocampus can synthesize estrogens and androgens at higher levels than found in serum as evidence (Hojo et al., 2009; Munetomo et al., 2015). A small but growing number of studies have similarly performed comparisons of steroid levels in the brain and serum in rats, and have demonstrated higher neural T levels than serum T levels (Caruso et al., 2010; Meffre et al., 2007).

Given the importance neural T may have over systemic T, we were interested in a comparison of neural T and serum T levels in young and aged animals. We found that in both age groups, brain T levels in the four regions examined were significantly higher than serum T levels, suggesting that neural production of T may indeed occur in young and aged animals.

Interestingly, although neural T levels decreased with age, neural T levels of aged rats were still fairly high, and exceeded the level of serum T in young rats in all regions except the OFC, further emphasizing the importance of T in the brain.

We were also interested in the relative proportions of T in the brain versus in circulation, so we examined the ratio of neural T: serum T. In all both age groups, this ratio was greater than 1 since neural T levels were higher than serum T levels; but interestingly, aged rats had a significantly higher ratio than young rats in all brain regions examined. So, although the levels of absolute neural T were lower in aged animals compared to young, the neural T: serum T ratio was higher. These results could be interpreted as showing that partially compensatory T production is occurring in the brains of aged animals, as theorized previously (Wu et al., 2009; Schumacher et al., 2003; Munetomo et al., 2015). Alternative interpretations are that in aged animals, more T is sequestered in the brain (e.g. bound to proteins such as AR or sex hormone binding globulin) rather than being produced, or that in young animals, more T is metabolized to other steroids. Although the possibility of increased partially compensatory neurosteroidogenesis in the aged brain is quite compelling, further research should be done to distinguish among these options, none of which are mutually exclusive.

3.5.3 Age-related Changes in AR Immunoreactivity

In addition to examining changes in systemic and neural T levels, examining changes in levels of the corresponding receptor is critical to forming a clearer picture of androgen action in the brain. Age-related changes in neural ARs have been assessed by a number of previous studies in rodents (Munetomo et al., 2015; Wu et al., 2009; Haji et al., 1981; Chambers et al., 1991; McGinnis and Yu, 1995; Kerr et al., 1995; Xiao and Jordan, 2002), whose results suggest that aging does not exert uniform effects on ARs in the brain; rather, effects are region, species, and strain-specific (Wu et al., 2009).

3.5.3.1 Cortical AR Immunoreactivity Decreases with Age

Previous immunohistochemical work has largely focused on "classical" AR regions, since those are sites of high AR density and intensity (Wu et al., 2009; Wood, 2008). However, there is increasing appreciation for the considerable number of AR-expressing cells present in non-classical regions, such as the isocortex (DonCarlos et al., 2006). ARs have been shown to be present in the PFC of primates, including the OFC and dorsolateral PFC, widely considered to be the primate analog to the rodent mPFC (Finley and Kritzer, 1999), and in the frontal cortex of rats (Kritzer, 2004). While no studies have looked specifically at age-related changes in AR in the PFC, some have examined AR mRNA in the cerebral cortex, with an age-associated decrease (Munetomo et al., 2015) and no change (Kerr et al., 1995) reported previously. To our knowledge, our results showing a significant decrease in AR-ir in aged rats relative to young are the first to characterize age-related changes in the mPFC and OFC.

AR is an auto-regulated protein (Lu et al., 1998), and it is well established that neural AR-ir is dependent on circulating T levels (Fernandez-Guasti et al., 2003). In general, and in many previous rodent studies, castration reduces neural AR-ir levels to diminished or undetectable amounts, and immunoreactivity is restored with androgen replacement (Lu et al., 1998; Lynch and Story, 2000; Menard and Harlan, 1993; Xiao and Jordan, 2002; Fernandez-Guasti et al., 2003; Kashon et al., 1996). Supraphysiological doses of androgens have been shown to increase the density of AR-ir in the brain (Fernandez-Guasti et al., 2003; Lu et al., 1998; Wood and Newman, 1993). Consequently, decreases in systemic and neural T levels may be expected to down-regulate AR, although this may be region-specific. Our results showing decreased cortical AR-ir in aged animals with lower circulating and neural T are consistent with this model of traditional AR autoregulation.

The lower % AR-ir observed in the mPFC and OFC may suggest a decreased sensitivity of these regions to T. While we did not explicitly measure cell counts or staining intensity per

cell, our measure of % immunoreactivity encompasses those properties. For example, AR staining was exclusively nuclear and included capture of mostly whole nuclei, so there should be high correlation between AR-ir and number of cells. In addition, our quantification method included thresholding based on staining intensity, and preferentially captured higher intensity immunoreactivity over lower intensity. Finally, even if the decrease in AR-ir cannot be definitively broken down into contributions from decreased number of AR-expressing cells (i.e. cell count) versus a decreased number of ARs per cell (i.e. staining intensity), the overall biological effects (decreased sensitivity and responsivity to T) are similar.

Decreased cortical AR-ir in aged rats is probably not due to age-related loss of overall cell numbers. Except in cases of neurological disorders, the brain does not seem to experience a general loss of neurons in the PFC during aging in rodents, primates, or humans (Finch, 2003; Zamzow et al., 2014; Wu et al., 2009). Rather, aging alters specific cell phenotypes that may be responsible for deficits in brain function (Wu et al., 2009). Other studies of neural ARs have shown age-related decrease in AR binding without accompanying loss of neurons (McGinnis and Yu, 1995). Less cortical AR-ir is also probably not due to a decrease in nuclei size with age. Previous studies examining age-associated changes in nuclear diameter in rodent brain have produced mixed results, and appear to be region- and strain-specific (Flood and Coleman, 1988). Although we did not measure and compare nuclei sizes between age groups, in several frontal regions of the rat brain, neuronal nuclei have not been shown to change size, and may even become larger with age (Flood and Coleman, 1988). So, while diminishing nuclei size could be a factor, it is unlikely to be fully responsible for the age-associated reduction of AR-ir in cortical regions.

3.5.3.2 Mesolimbic AR Immunoreactivity Does Not Change with Age

While cortical AR-ir decreased with age, mesolimbic AR-ir did not. We are not aware of any studies that specifically examine age-related changes in ARs in the NAc or VTA. In fact,

previous studies have been inconclusive on the presence of ARs in these regions. Prior reports have claimed that the NAc has sparse or very few AR (Wood, 2008; Sato et al., 2010). On the other hand, it is also claimed that the NAc is one of the brain areas that controls copulatory and sexual behavior, and consequently may be rich in AR (Fernandez-Guasti et al., 2003).

The VTA is the midbrain site of origin of DAergic cell bodies, including those that project to frontal regions and those that project to limbic regions (Kritzer and Creutz, 2008). Although initially thought to be largely devoid of ARs, the presence of AR mRNA and protein has been demonstrated in parts of the VTA (Simerly et al., 1990; Kritzer, 1997), suggesting that subgroups of neurons might be direct targets of androgens (Kritzer, 1997). Consistent with Kritzer's work, we observed sparsely distributed but strongly staining AR-ir in the VTA, with the majority of immunoreactivity concentrated in the PBP and PN. Our analysis of AR-ir in the PBP and PN, and also the cVTA showed no significant age differences between young and aged animals in two separate cohorts, despite the fact that T levels in the VTA decline with age, suggesting that ARs may not be as sensitive to regulation by declining androgen levels. In line with our results, Menard and Harlan (1993) examined the optical density and number of AR-positive cells in the VTA of male rats after castration, and then with anabolic-androgenic steroid replacement, and found that neither type of hormone manipulation changed measures of AR in the VTA, while they did have significant effects in other brain regions. Taken together, this suggests that ARs in the VTA may not be as sensitive to traditional regulation by changes in androgen levels, may be regulated by T in a non-traditional fashion, or may be regulated by non-androgenic factors.

Interestingly, although we did not observe age-related changes in mesolimbic ARs, there were within-region changes. The NAcS had higher AR-ir compared to the NAcC, and the pVTA had higher staining than the cVTA, suggesting differential roles of each subregion with respect to androgen action.

3.5.3.3 Cohort Staining Differences

The pattern of age-related changes in AR-ir showing decreases in cortical regions of aged animals and no change in mesolimbic regions was observed in two cohorts of subjects, although Cohort 2 immunostaining overall was darker than Cohort 1. We found no interaction of region x cohort, confirming that age had similar effects on AR-ir regardless of cohort. The between-cohort differences in staining intensity likely reflect batch-to-batch immunohistochemical variability. Since each cohort consisted of both young and aged animals, was counterbalanced by age in each plate, and was processed altogether in one run, any variability would affect subjects equally and have no biasing effect on any age differences. In fact, the similar effect of age observed in both cohorts could even serve to further strengthen the reliability of our results.

3.5.3.4 Non-Parallel Changes in Receptor and Ligand Amount

Interestingly, our results also show that ligand and receptor concentration do not always change in parallel in aging – cortical regions had both lower T and less AR, but mesolimbic regions had lower T and no change in AR. In addition, in cortical regions of Cohort 2 subjects, T and AR levels were moderately correlated, while there was no significant relationship in most mesolimbic regions. Taken together, these results suggest that there may be different mechanisms of androgen regulation of AR expression operating in the cortical versus mesolimbic regions. However, the fact that AR-ir and cortical T do not correlate significantly in Cohort 1 subjects is not consistent with this idea. Indeed, the discrepancies between correlations in Cohort 1 and 2 are puzzling, so it is difficult to infer any clear relationship between AR-ir, neural T, and serum T from these data.

3.5.5 Connections to Cognitive Aging of Executive Functions

Although the primary endpoints of this study were endocrine and neurochemical, our results could have interesting cognitive and behavioral consequences as well. There is considerable evidence that a relationship between androgens and executive function exists beyond the fact that both decline with age. Firstly, studies in aged men show relationships between endogenous T levels and performance on tasks measuring executive functions (Yaffe et al., 2002; Barrett-Connor et al., 1999), and that exogenous T administration can improve performance (Cherrier et al., 2001; Janowksy et al., 2000). Secondly, the presence of ARs in cortical regions suggests androgen involvement in higher-order cognitive functions, and that the mPFC and OFC are targets of gonadal hormone stimulation (Finley and Kritzer, 1999). Finally, strong evidence has accumulated for androgen modulation of mescorticolimbic DA signaling, and as a consequence, prefrontal DA-dependent executive functions (Aubele and Kritzer, 2011). For example, GDX impairs and in some cases, T administration restores performance on spatial working memory and behavioral flexibility tasks (Aubele et al., 2008; Kritzer et al., 2001; Bimonte-Nelson et al., 2003). In addition, chronic high doses of anabolic-androgenic steroids impair reversal learning and set-shifting performance (Wallin and Wood, 2015). GDX also increases axon density of cells with tyrosine hydroxylase, a DA-synthesizing enzyme (Adler et al., 1999; Kritzer et al., 2000), and extracellular DA levels in the PFC but not other frontal regions (Aubele and Kritzer, 2010).

Although it is well established that androgens can influence DA-dependent prefrontal executive functions, it remains unclear whether this occurs through indirect effects on circuits and regions afferent to the PFC, through direct effects on the PFC itself, or through a combination of both. Evidence supporting indirect effects of androgens on mesolimbic regions includes the presence of AR-expressing cells in the VTA and NAc, and the fact that prefrontally-projecting DAergic afferents in the VTA are enriched in ARs (Kritzer and Creutz, 2008). In

addition, DA and DA receptor manipulations in subcortical mesolimbic regions like the NAc can also impact prefrontal executive functions including extradimensional set-shifting and reversal learning (Haluk and Floresco, 2009). Evidence supporting direct effects of androgens on the PFC has been fewer, and focuses mainly on the fact that AR-expressing cells are present in the PFC (Kritzer, 2004).

Although previous work has tended to favor indirect effects, citing the fact that the PFC has few ARs, making direct effects unlikely (Sato et al., 2008), our results do not definitively side with one mechanism over the other. For example, we find that ARs are present in mesolimbic regions of young and aged rats, and that systemic T and neural T in the NAc and VTA decline with age. Lowered neural T levels in these regions could contribute to impaired regulation of DA transmission. On the other hand, while ARs are present in mesolimbic regions, the fact that receptor amount does not change during aging suggests that AR-mediated effects of T may not be as impaired compared to cortical regions, where AR-ir decreases in addition to neural T levels. The reduction in cortical AR-ir could reflect a decreased sensitivity of aged PFC cells to T, which could manifest as impaired T modulation of prefrontal functions directly. Alternatively, changes in AR amount may not be important behaviorally. AR levels in aged animals could be lower than in young, but still sufficient, and instead maintaining adequate T levels is the driving factor with regard to cognitive impairment. In that case, lowered neural T levels in both cortical and mesolimbic and brain regions could contribute to impaired regulation of DA transmission, and impairment of executive functions in aging. Further research is needed to dissociate the roles and relative importance of T and AR.

3.6 Conclusion

In summary, we demonstrate age and region-related changes in endogenous levels of T, and its receptor, AR, in male F344/BN rats. Compared to young (5 months old) rats, aged (22 months old) rats have significantly lower serum T, neural T, and cortical ARs. Regardless of

age, neural T levels in mesocorticolimbic brain regions were higher than serum levels, suggesting the importance of neural androgens to brain function. In addition, an increased neural: serum T ratio in aged rats suggests that neural T production may be up-regulated with age. Lower levels of serum T, neural T, and cortical AR may contribute to impairments in prefrontal DA activity through direct or indirect mechanisms, and thereby contribute to cognitive aging of executive functions.

Chapter 4: Conclusion

4.1 Main Conclusions

Altogether, the data presented in Chapters 2 and 3 show that ARs are present in mesocorticolimbic regions of male rats, including the PFC, NAc, and VTA. These regions, particularly the PFC, are underappreciated but important sites of androgen action, and not traditionally considered as classical regions of high AR abundance. One barrier to studying androgen action in the PFC may have been difficulty in visualizing ARs; however, we have addressed this issue by showing that TSA technology facilitates stronger immunolabeling and detection of ARs, which are expressed primarily by neurons and not glia. This will ultimately enable improved examination of cortical ARs, and the role they play in cognition.

Our results also show that not only are ARs present in mesocorticolimbic regions, but that they are also affected by age in a region-dependent manner. In a comparison between young and aged male rats, AR levels were shown to decrease with age in cortical areas, but not in mesolimbic areas. Serum and mesocorticolimbic T levels also decreased with age. Since androgens may be involved in regulating executive functions, these age-related declines in AR and T levels may contribute to executive impairments with age. Additionally, across ages, we showed that T levels in the brain were higher than circulating T levels, suggesting the importance of neural androgen action. We also observed a higher neural T: serum T ratio in aged animals compared to young. Neurosteroids and their function are another underappreciated area of study, and our results suggest that neuroandrogen production may be particularly important in the context of aging.

4.2 Study Limitations

It is important to note that our analysis is limited to the traditional nuclear AR. Although the vast majority of work on androgen action in the brain has focused on nuclear ARs, androgens could also be regulating neural activity through their membrane AR, or through a combination of both. The existence of a membrane AR has been long suspected, but only recently characterized (Thomas et al., 2014), and its distribution and density has yet to be determined in the rat brain. Membrane receptors for other steroid receptors including ER have previously been characterized in the nervous system, and rapid effects of androgens have been observed (Heinlein and Chang, 2002), along with effects of androgens in testicular feminized rats with poorly functioning nuclear ARs (Sato et al. 2010), suggesting that there may be a role for membrane ARs in the brain.

Another limitation concerns the possibility that the primary antibody used in our experiments might preferentially or only recognize liganded ARs, and not unliganded ARs. As with most nuclear receptors, it is thought that bound receptors reside in the nucleus, whereas unbound receptors are found in the cytoplasm. Throughout AR-positive regions of the rat brain, we detected exclusively nuclear AR staining, even in areas of high AR density per cell, such as the hypothalamus, where cytoplasmic staining would be most likely. While it is possible that our antibody does detect unliganded AR, but it is simply present at very low amounts not detectable by IHC, the apparent lack of non-nuclear AR staining suggests that recognition of exclusively liganded AR is also a possibility. This was previously a concern with another widely used AR antibody, PG-21 (Zhou et al., 1994; Lu et al., 1998; Greco et al., 1996), although some have also argued that PG-21 does indeed recognize unliganded receptor (Lu et al., 1998). In studies using PG-21 in rats and songbirds, T was injected into subjects prior to sacrifice in order initiate receptor-ligand translocation to the nucleus to increase immunostaining visibility (Smith et al., 1996; Soma et al., 1999; Xiao and Jordan, 2002). Our antibody, ab133273, and PG-21

recognize very similar epitopes on the AR protein, with PG-21 recognizing an epitope located on the first 21 amino acids (Prins et al., 1991; Smith et al., 1996), and ab133273 recognizing a region within the first 30 amino acids (as per manufacturer). If indeed these antibodies do not recognize unliganded AR, one concern is that our AR-ir observations would not necessarily be reflective of actual AR levels. While this is a distinct possibility, we view this as unlikely to be responsible for our observation of lower AR-ir in cortical regions with age for several reasons. Firstly, although there was an age-related decrease in neural T levels in our regions of interest, T levels were not extremely low. In fact, neural T levels of aged animals were higher than or very close to serum T levels of young animals. Secondly, there were similarly lower neural T levels across cortical and mesolimbic regions of aged animals, yet only cortical regions showed decreased AR-ir. If lowered neural T was responsible for the decrease in bound receptors and AR-ir, we would expect decreased AR-ir across all regions examined.

It is also critical to emphasize that our results are limited to detection of changes in the amount of ARs and not necessarily their functioning. There is an important distinction between an age-related decline in the amount of AR (via increased degradation or decreased synthesis of the protein), and an age-related decline of AR function (without change in absolute amount) (Chang and Roth, 1979). For example, it has been proposed that aging impairs AR's ligand binding affinity, or ability to translocate to nucleus (McGinnis and Yu, 1995), but not concentration (Greenstein, 1979). These possibilities are unlikely for similar reasons as stated above, and also the fact that no cytoplasmic AR-ir was observed.

A final limitation is that our study focuses only on ARs, and does not examine ERs. Since T is a major source of brain estradiol in males (Hojo et al., 2009), understanding ER action is an important part of understanding T action, and any changes in ARs must be understood in the context of changes to ERs. For example, age-associated decrease in cortical ARs could suggest a decreased sensitivity to T, but could also suggest that T conversion to

estradiol and interaction with ERs may be more important in aged rats. Future work should look at how ER and estradiol levels change with age.

4.3 Future Directions

There are several promising directions for future research concerning the relationship between neural androgens, ARs, and cognitive aging of executive functions. One interesting direction is examination of membrane ARs. A novel membrane AR has been characterized in the Atlantic croaker (Thomas et al., 2014), but basic research is still needed on the presence and distribution of membrane ARs in the rodent brain.

Understanding the role of ERs, aromatase, and neuroestrogens is another important piece in understanding the contributions of neuroandrogens and ARs to cognitive aging. As ERs and aromatase enzymes are present throughout the rat brain (Shrughrue et al., 1997; Sanghera et al., 1991) with substantial overlap with the distribution of ARs (Wood, 2004), it is possible for them to have a role in mediating effects of neural T. Future research could examine how levels of ERs, circulating and neuroestrogens, and aromatase change with age in mesocorticolimbic regions.

Future work could also investigate age-related changes in ARs in female rats. While we focused exclusively on males in our aging study, sexual dimorphism in AR expression has been documented in the rodent brain (Xiao and Jordan, 2002), and we observed sex differences in AR immunoreactivity in frontal cortical areas in our immunofluorescent double-labeling study. Prospective studies could also look at behavioral endpoints in females, and further investigate sex differences in cognitive flexibility and working memory.

Finally, future research could also expand on our observational findings by using manipulations of T levels in vivo, coupled with examination of the effects on behavior, systemic and neural T levels, and AR levels. Such T manipulations could include both systemic and

targeted local hormone manipulations, for example, by GDX; by administration of different doses of androgens, inhibitors of steroidogenic enzymes, or AR antagonists; by selective genetic knockouts; and by combinations of the aforementioned techniques. Better understanding the role of neuroandrogens is a particularly exciting area of research. A large amount of work on neurosteroids has focused on demonstrating steroidogenesis in the nervous system, while less is known about the unique functions they could have.

4.4 Clinical Relevance

Our findings may have some relevance for clinical applications of sex steroids in aging. Administration of steroids can ameliorate, to some degree, age-related changes in cognitive function, and sex steroids are considered promising therapeutic candidates for disorders of the nervous system (Schumacher et al., 2003). HRT is currently used to treat symptoms of andropause; however, it is accompanied by poorly understood risks, including an increased risk of prostate cancer and cardiovascular side effects (Bhasin et al., 2010). More work is needed to develop specialized HRT treatments, particularly ones aimed at treating psychological deficits. One promising approach may be stimulation of neural ARs. For example, selective AR modulators (SARMs) are drugs that act as tissue-specific AR ligands, and could be selectively targeted to neural tissues.

References

Adams, J. C. (1992). Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *Journal of Histochemistry & Cytochemistry*, *40*(10), 1457-1463.

Adler, A., Vescovo, P., Robinson, J. K., & Kritzer, M. F. (1999). Gonadectomy in adult life increases tyrosine hydroxylase immunoreactivity in the prefrontal cortex and decreases open field activity in male rats. *Neuroscience*, *89*(3), 939-954.

Alexander, G. E., Ryan, L., Bowers, D., Foster, T. C., Bizon, J. L., Geldmacher, D. S., & Glisky, E. L. (2012). Characterizing cognitive aging in humans with links to animal models. *Frontiers in aging neuroscience*, *4*.

Agranoff, B. W., & Davis, R. E. (1974). More on seasonal variations in goldfish learning. *Science (New York, NY)*, *186*(4158), 65.

Anderson, K. M., & Liao, S. (1968). Selective retention of dihydrotestosterone by prostatic nuclei.

Apostolinas, S., Rajendren, G., Dobrjansky, A., & Gibson, M. J. (1999). Androgen receptor immunoreactivity in specific neural regions in normal and hypogonadal male mice: effect of androgens. *Brain research*, *817*(1), 19-24.

Arnold, A. P., & Gorski, R. A. (1984). Gonadal steroid induction of structural sex differences in the central nervous system. *Annual review of neuroscience*, *7*(1), 413-442.

Asarian, L., & Geary, N. (2007). Estradiol enhances cholecystokinin-dependent lipid-induced satiation and activates estrogen receptor-α-expressing cells in the nucleus tractus solitarius of ovariectomized rats. *Endocrinology*, *148*(12), 5656-5666.

Aubele, T., Kaufman, R., Montalmant, F., & Kritzer, M. F. (2008). Effects of gonadectomy and hormone replacement on a spontaneous novel object recognition task in adult male rats. *Hormones and behavior*, *54*(2), 244-252.

Aubele, T., & Kritzer, M. F. (2010). Gonadectomy and hormone replacement affects in vivo basal extracellular dopamine levels in the prefrontal cortex but not motor cortex of adult male rats. *Cerebral Cortex*, bhq083.

Aubele, T., & Kritzer, M. F. (2011). Androgen influence on prefrontal dopamine systems in adult male rats: localization of cognate intracellular receptors in medial prefrontal projections to the ventral tegmental area and effects of gonadectomy and hormone replacement on glutamate-stimulated extracellular dopamine level. *Cerebral Cortex*, bhr258.

Bao, A. M., Fischer, D. F., Wu, Y. H., Hol, E. M., Balesar, R., Unmehopa, U. A., ... & Swaab, D. F. (2006). A direct androgenic involvement in the expression of human corticotropin-releasing hormone. *Molecular psychiatry*, *11*(6), 567-576.

Barrett-Connor, E., Goodman-Gruen, D., & Patay, B. (1999). Endogenous Sex Hormones and Cognitive Function in Older Men 1. *The Journal of Clinical Endocrinology & Metabolism*, *84*(10), 3681-3685.

Baulieu, E. E., & Robel, P. (1998). Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids.*Proceedings of the National Academy of Sciences*, *95*(8), 4089-4091.

Beas, B. S., Setlow, B., & Bizon, J. L. (2013). Distinct manifestations of executive dysfunction in aged rats. *Neurobiology of aging*, *34*(9), 2164-2174.

Beauchet, O. (2006). Testosterone and cognitive function: current clinical evidence of a relationship. *European Journal of Endocrinology*, *155*(6), 773-781.

Bernardi, F., Salvestroni, C., Casarosa, E., Nappi, R. E., Lanzone, A., Luisi, S., ... & Genazzani, A. R. (1998). Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats. *European journal of endocrinology*, *138*(3), 316-321.

Bethea, C. L., & Walker, R. F. (1979). Age-related changes in reproductive hormones and in Leydig cell responsivity in the male Fischer 344 rat. *Journal of gerontology*, *34*(1), 21-27.

Beyenburg, S., Watzka, M., Clusmann, H., Blümcke, I., Bidlingmaier, F., Elger, C. E., & Stoffel-Wagner, B. (2000). Androgen receptor mRNA expression in the human hippocampus. *Neuroscience letters*, *294*(1), 25-28.

Bezdickova, M., Molikova, R., Bebarova, L., & Kolar, Z. (2007). Distribution of nuclear receptors for steroid hormones in the human brain: a preliminary study. *Biomedical Papers*, *151*(1), 69-71.

Bhasin, S., Woodhouse, L., Casaburi, R., Singh, A. B., Bhasin, D., Berman, N., ... & Dzekov, J. (2001). Testosterone dose-response relationships in healthy young men. *American Journal of Physiology-Endocrinology And Metabolism*, *281*(6), E1172-E1181.

Bhasin, S., Cunningham, G. R., Hayes, F. J., Matsumoto, A. M., Snyder, P. J., Swerdloff, R. S., & Montori, V. M. (2010). Testosterone therapy in men with androgen deficiency syndromes: an Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, *95*(6), 2536-2559.

Bimonte-Nelson, H. A., Singleton, R. S., Nelson, M. E., Eckman, C. B., Barber, J., Scott, T. Y., & Granholm, A. C. E. (2003). Testosterone, but not nonaromatizable dihydrotestosterone, improves working memory and alters nerve growth factor levels in aged male rats. *Experimental neurology*, *181*(2), 301-312.

Birrell, J. M., & Brown, V. J. (2000). Medial frontal cortex mediates perceptual attentional set shifting in the rat. *The Journal of Neuroscience*, *20*(11), 4320-4324.
Buckner, R. L. (2004). Memory and executive function in aging and AD: multiple factors that cause decline and reserve factors that compensate.*Neuron*, *44*(1), 195-208.

Bobrow, M. N., Harris, T. D., Shaughnessy, K. J., & Litt, G. J. (1989). Catalyzed reporter deposition, a novel method of signal amplification application to immunoassays. *Journal of immunological methods*, *125*(1), 279-285.

Bobrow, M. N., Litt, G. J., Shaughnessy, K. J., Mayer, P. C., & Conlon, J. (1992). The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. *Journal of immunological methods*, *150*(1), 145-149.

Bonkhoff, H., Fixemer, T., Hunsicker, I., & Remberger, K. (2001). Progesterone receptor expression in human prostate cancer: correlation with tumor progression. *The Prostate*, *48*(4), 285-291.

Borst, S. E., & Mulligan, T. (2007). Testosterone replacement therapy for older men. *Clinical interventions in aging*, *2*(4), 561.

Buckner, R. L. (2004). Memory and executive function in aging and AD: multiple factors that cause decline and reserve factors that compensate. *Neuron*, *44*(1), 195-208.

Butler, J. A., Kallo, I., Sjöberg, M., & Coen, C. W. (1999). Evidence for extensive distribution of oestrogen receptor alpha-immunoreactivity in the cerebral cortex of adult rats. *Journal of neuroendocrinology*, *11*(5), 325-329.

Caruso, D., Pesaresi, M., Maschi, O., Giatti, S., Garcia-Segura, L. M., & Melcangi, R. C. (2010). Effect of Short-and Long-Term Gonadectomy on Neuroactive Steroid Levels in the Central and Peripheral Nervous System of Male and Female Rats. *Journal of neuroendocrinology*, *22*(11), 1137-1147.

Celotti, F., Negri-Cesi, P., & Poletti, A. (1997). Steroid metabolism in the mammalian brain: 5alpha-reduction and aromatization. *Brain research bulletin*, *44*(4), 365-375.

Chambers, K. C., Thornton, J. E., & Roselli, C. E. (1991). Age-related deficits in brain androgen binding and metabolism, testosterone, and sexual behavior of male rats. *Neurobiology of aging*, *12*(2), 123-130.

Chang, C., & Roth, G. S. (1979). Changes in the mechanisms of steroid action during aging. *Journal of steroid biochemistry*, *11*(1), 889-892.

Charlier, T. D., Po, K. W., Newman, A. E., Shah, A. H., Saldanha, C. J., & Soma, K. K. (2010). 17β-Estradiol levels in male zebra finch brain: combining Palkovits punch and an ultrasensitive radioimmunoassay. *General and comparative endocrinology*, *167*(1), 18-26.

Chen, H., Hardy, M. P., Huhtaniemi, I., & Zirkin, B. R. (1994). Age-Related Decreased Leydig Cell Testosterone Production in the Brown Norway Rat. *Journal of andrology*, *15*(6), 551-557.

Cherrier, M. M., Asthana, S., Plymate, S., Baker, L., Matsumoto, A. M., Peskind, E., ... & LaTendresse, S. (2001). Testosterone supplementation improves spatial and verbal memory in healthy older men. *Neurology*, *57*(1), 80-88.

Cherrier, M. M., & Craft, S. (2003). Androgens and cognition. In *Androgens in Health and Disease* (pp. 291-309). Humana Press.

Clancy, A. N., Bonsall, R. W., & Michael, R. P. (1992). Immunohistochemical labeling of androgen receptors in the brain of rat and monkey. *Life sciences*,*50*(6), 409-417.

Corpechot, C., Robel, P., Axelson, M., Sjövall, J., & Baulieu, E. E. (1981). Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proceedings of the National Academy of Sciences*, *78*(8), 4704-4707.

Creutz, L. M., & Kritzer, M. F. (2004). Mesostriatal and mesolimbic projections of midbrain neurons immunoreactive for estrogen receptor beta or androgen receptors in rats. *Journal of Comparative Neurology*, *476*(4), 348-362.

Dalley, J. W., Cardinal, R. N., & Robbins, T. W. (2004). Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates.*Neuroscience & Biobehavioral Reviews*, *28*(7), 771-784.

Daniel, J. M., Winsauer, P. J., & Moerschbaecher, J. M. (2003). Castration in rats impairs performance during acquisition of a working memory task and exacerbates deficits in working memory produced by scopolamine and mecamylamine. *Psychopharmacology*, *170*(3), 294-300.

Do Rego, J. L., Seong, J. Y., Burel, D., Leprince, J., Luu-The, V., Tsutsui, K., ... & Vaudry, H. (2009). Neurosteroid biosynthesis: enzymatic pathways and neuroendocrine regulation by neurotransmitters and neuropeptides. *Frontiers in neuroendocrinology*, *30*(3), 259-301.

DonCarlos, L. L., Garcia-Ovejero, D., Sarkey, S., Garcia-Segura, L. M., & Azcoitia, I. (2003). Androgen receptor immunoreactivity in forebrain axons and dendrites in the rat. *Endocrinology*, *144*(8), 3632-3638.

DonCarlos, L. L., Sarkey, S., Lorenz, B., Azcoitia, I., Garcia-Ovejero, D., Huppenbauer, C., & Garcia-Segura, L. M. (2006). Novel cellular phenotypes and subcellular sites for androgen action in the forebrain. *Neuroscience*,*138*(3), 801-807.

Downs, J. L., & Urbanski, H. F. (2006). Aging-related sex-dependent loss of the circulating leptin 24-h rhythm in the rhesus monkey. *Journal of endocrinology*, *190*(1), 117-127.

Emmelot-Vonk, M. H., Verhaar, H. J., Pour, H. R. N., Aleman, A., Lock, T. M., Bosch, J. R., ... & van der Schouw, Y. T. (2008). Effect of testosterone supplementation on functional mobility, cognition, and other parameters in older men: a randomized controlled trial. *Jama*, *299*(1), 39-52.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science*, *240*(4854), 889-895.

Feldman, H. A., Longcope, C., Derby, C. A., Johannes, C. B., Araujo, A. B., Coviello, A. D., ... & McKinlay, J. B. (2002). Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. *The Journal of Clinical Endocrinology & Metabolism*, *87*(2), 589-598.

Fernández-Guasti, A., Kruijver, F. P., Fodor, M., & Swaab, D. F. (2000). Sex differences in the distribution of androgen receptors in the human hypothalamus. *Journal of comparative neurology*, *425*(3), 422-435.

Fernandez-Guasti, A., Swaab, D., & Rodríguez-Manzo, G. (2003). Sexual behavior reduces hypothalamic androgen receptor immunoreactivity. *Psychoneuroendocrinology*, *28*(4), 501-512.

Ferris, J. K., Tse, M. T., Hamson, D. K., Taves, M. D., Ma, C., McGuire, N., ... & Soma, K. K. (2015). Neuronal Gonadotrophin-Releasing Hormone (GnRH) and Astrocytic Gonadotrophin

Inhibitory Hormone (GnIH) Immunoreactivity in the Adult Rat Hippocampus. *Journal of neuroendocrinology*, *27*(10), 772-786.

Fibiger, H. C., & Phillips, A. G. (1988). Mesocorticolimbic dopamine systems and reward. *Annals of the New York Academy of Sciences*, *537*(1), 206-215.

Finch, C. E. (2003). Neurons, glia, and plasticity in normal brain aging.*Neurobiology of aging*, *24*, S123-S127.

Finley, S. K., & Kritzer, M. F. (1999). Immunoreactivity for intracellular androgen receptors in identified subpopulations of neurons, astrocytes and oligodendrocytes in primate prefrontal cortex. *Journal of neurobiology*, *40*(4), 446-457.

Flood, D. G., & Coleman, P. D. (1988). Neuron numbers and sizes in aging brain: comparisons of human, monkey, and rodent data. *Neurobiology of Aging*, *9*, 453-463.

Flood, J. F., Farr, S. A., Kaiser, F. E., La Regina, M., & Morley, J. E. (1995). Age-related decrease of plasma testosterone in SAMP8 mice: replacement improves age-related impairment of learning and memory. *Physiology & behavior*, *57*(4), 669-673.

Floresco, S. B., & Magyar, O. (2006). Mesocortical dopamine modulation of executive functions: beyond working memory. *Psychopharmacology*, *188*(4), 567-585.

Floresco, S. B., Block, A. E., & Maric, T. L. (2008). Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. *Behavioural brain research*,*190*(1), 85-96.

Fonda, S. J., Bertrand, R., O'Donnell, A., Longcope, C., & McKinlay, J. B. (2005). Age, hormones, and cognitive functioning among middle-aged and elderly men: cross-sectional evidence from the Massachusetts Male Aging Study. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *60*(3), 385-390.

Funahashi, S. (2006). Prefrontal cortex and working memory processes.*Neuroscience*, *139*(1), 251-261.

Fuxe, K., Hökfelt, T., Johansson, O., Jonsson, G., Lidbrink, P., & Ljundahl, Å. (1974). The origin of the dopamine nerve terminals in limbic and frontal cortex. Evidence for meso-cortico dopamine neurons. *Brain research*, *82*(2), 349-355.

Galea, L. A. M., Kavaliers, M., Ossenkopp, K. P., Innes, D., & Hargreaves, E. L. (1994). Sexually dimorphic spatial learning varies seasonally in two populations of deer mice. *Brain research*, *635*(1), 18-26.

Gallagher, M., Stocker, A. M., & Koh, M. T. (2011). Mindspan: lessons from rat models of neurocognitive aging. *ILAR Journal*, *52*(1), 32-40.

García-Ovejero, D., Veiga, S., García-Segura, L. M., & Doncarlos, L. L. (2002). Glial expression of estrogen and androgen receptors after rat brain injury. *Journal of Comparative Neurology*, *450*(3), 256-271.

Gauthier, S., Reisberg, B., Zaudig, M., Petersen, R. C., Ritchie, K., Broich, K., ... & Cummings, J. L. (2006). Mild cognitive impairment. *The Lancet*,*367*(9518), 1262-1270.

Ghanadian, R., Lewis, J. G., & Chisholm, G. D. (1975). Serum testosterone and dihydrotestosterone changes with age in rat. *Steroids*, *25*(6), 753-762.

Ghods-Sharifi, S., Haluk, D. M., & Floresco, S. B. (2008). Differential effects of inactivation of the orbitofrontal cortex on strategy set-shifting and reversal learning. *Neurobiology of learning and memory*, *89*(4), 567-573.

Gibbs, R. B. (2005). Testosterone and estradiol produce different effects on cognitive performance in male rats. *Hormones and behavior*, *48*(3), 268-277.

Goldman-Rakic, P. S. (1995). Cellular basis of working memory. *Neuron*, *14*(3), 477-485.

Gooren, L. (2007). Testosterone and the brain. *The journal of men's health & gender*, *4*(3), 344-351.

Greco, B., Edwards, D. A., Michael, R. P., & Clancy, A. N. (1996). Androgen receptor immunoreactivity and mating-induced Fos expression in forebrain and midbrain structures in the male rat. *Neuroscience*, *75*(1), 161-171.

Greenstein, B. D. (1979). Androgen receptors in the rat brain, anterior pituitary gland and ventral prostate gland: effects of orchidectomy and ageing. *Journal of Endocrinology*, *81*(1), 75-81.

Gruenewald, D. A., Naai, M. A., Hess, D. L., & Matsumoto, A. M. (1994). The Brown Norway rat as a model of male reproductive aging: evidence for both primary and secondary testicular failure. *Journal of gerontology*, *49*(2), B42-B50.

Gruenewald, D. A., Naai, M. A., Marck, B. T., & Matsumoto, A. M. (2000). Age-related decrease in hypothalmic gonadotropin-releasing hormone (GnRH) gene expression, but not pituitary responsiveness to GnRH, in the male brown Norway rat. *Journal of andrology*, *21*(1), 72-84.

Haji, M., Kato, K. I., Nawata, H., & Ibayashi, H. (1981). Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat. *Brain research*, *204*(2), 373-386.

Haluk, D. M., & Floresco, S. B. (2009). Ventral striatal dopamine modulation of different forms of behavioral flexibility. *Neuropsychopharmacology*, *34*(8), 2041-2052.

Hamson, D. K., Wainwright, S. R., Taylor, J. R., Jones, B. A., Watson, N. V., & Galea, L. A. M. (2013). Androgens increase survival of adult-born neurons in the dentate gyrus by an androgen receptor-dependent mechanism in male rats.*Endocrinology*, *154*(9), 3294-3304.

Handa, R. J., Roselli, C. E., Horton, L., & Resko, J. A. (1987). The Quantitative Distribution of Cytosolic Androgen Receptors in Microdissected Areas of the Male Rat Brain: Effects of Estrogen Treatment*. *Endocrinology*,*121*(1), 233-240.

Harman, S. M., Metter, E. J., Tobin, J. D., Pearson, J., & Blackman, M. R. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. *The Journal of Clinical Endocrinology & Metabolism*,*86*(2), 724-731.

Hazra, R., Corcoran, L., Robson, M., McTavish, K. J., Upton, D., Handelsman, D. J., & Allan, C. M. (2012). Temporal role of Sertoli cell androgen receptor expression in spermatogenic development. *Molecular Endocrinology*, *27*(1), 12-24.

Heinlein, C. A., & Chang, C. (2002). Androgen receptor (AR) coregulators: an overview. *Endocrine reviews*, *23*(2), 175-200.

Hill, E. L. (2004). Executive dysfunction in autism. *Trends in cognitive sciences*, *8*(1), 26-32.

Hojo, Y., Hattori, T. A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H. T., ... & Harada, N. (2004). Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017α and P450 aromatase localized in neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(3), 865-870.

Hojo, Y., Higo, S., Ishii, H., Ooishi, Y., Mukai, H., Murakami, G., ... & Kawato, S. (2009). Comparison between hippocampus-synthesized and circulation-derived sex steroids in the hippocampus. *Endocrinology*, *150*(11), 5106-5112.

Holland, J., Bandelow, S., & Hogervorst, E. (2011). Testosterone levels and cognition in elderly men: a review. Maturitas, 69(4), 322-337.

Hösli, E., Jurasin, K., Rühl, W., Lüthy, R., & Hösli, L. (2001). Colocalization of androgen, estrogen and cholinergic receptors on cultured astrocytes of rat central nervous system. *International Journal of Developmental Neuroscience*, *19*(1), 11-19.

Hunyady, B., Krempels, K., Harta, G., & Mezey, E. (1996). Immunohistochemical signal amplification by catalyzed reporter deposition and its application in double immunostaining. *Journal of Histochemistry & Cytochemistry*, *44*(12), 1353-1362.

Janowsky, J. S., Oviatt, S. K., & Orwoll, E. S. (1994). Testosterone influences spatial cognition in older men. *Behavioral neuroscience*, *108*(2), 325.

Janowsky, J. S., Chavez, B., & Orwoll, E. (2000). Sex steroids modify working memory. *Journal of cognitive neuroscience*, *12*(3), 407-414.

Janowsky, J. S. (2006). The role of androgens in cognition and brain aging in men. *Neuroscience*, *138*(3), 1015-1020.

Janowsky, J. S. (2006). Thinking with your gonads: testosterone and cognition. *Trends in cognitive sciences*, *10*(2), 77-82.

Johnson, R. T., Schneider, A., DonCarlos, L. L., Breedlove, S. M., & Jordan, C. L. (2012). Astrocytes in the rat medial amygdala are responsive to adult androgens. *Journal of Comparative Neurology*, *520*(11), 2531-2544.

Jung-Testas, I., & Baulieu, E. E. (1998). Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system.*The Journal of steroid biochemistry and molecular biology*, *65*(1), 243-251.

Kadi, F., Bonnerud, P., Eriksson, A., & Thornell, L. E. (2000). The expression of androgen receptors in human neck and limb muscles: effects of training and self-administration of androgenic-anabolic steroids. *Histochemistry and cell biology*, *113*(1), 25-29.

Kaler, L. W., & Neaves, W. B. (1981). The Androgen Status of Aging Male Rats. *Endocrinology*, *108*(2), 712-719.

Kallo, I., Butler, J. A., Barkovics-Kalló, M., Goubillon, M. L., & Coen, C. W. (2001). Oestrogen Receptor β-Immunoreactivity in Gonadotropin Releasing Hormone-Expressing Neurones: Regulation by Oestrogen. *Journal of neuroendocrinology*, *13*(9), 741-748.

Kashon, M. L., Arbogast, J. A., & Sisk, C. L. (1996). Distribution and hormonal regulation of androgen receptor immunoreactivity in the forebrain of the male European ferret. *Journal of Comparative Neurology*, *376*(4), 567-586.

Kaufman, J. M., & Vermeulen, A. (2005). The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocrine reviews*,*26*(6), 833-876.

Kerr, J. E., Allore, R. J., Beck, S. G., & Handa, R. J. (1995). Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus. *Endocrinology*, *136*(8), 3213-3221.

Kesner, R. P., & Churchwell, J. C. (2011). An analysis of rat prefrontal cortex in mediating executive function. *Neurobiology of learning and memory*, *96*(3), 417-431.

Kim, S. H., Shin, Y. K., Lee, K. M., Lee, J. S., Yun, J. H., & Lee, S. M. (2003). An improved protocol of biotinylated tyramine-based immunohistochemistry minimizing nonspecific background staining. *Journal of Histochemistry & Cytochemistry*, *51*(1), 129-132.

Koibuchi, H., & Hayashi, S. (2007). Development of neurons expressing estrogen receptor α transiently in facial nucleus of prenatal and postnatal rat brains. *Neuroscience research*, *58*(2), 190-198.

Koob, G. F., & Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology*, *35*(1), 217-238.

Krémarik-Bouillaud, P., Schohn, H., & Dauça, M. (2000). Regional distribution of PPARβ in the cerebellum of the rat. *Journal of Chemical Neuroanatomy*, *19*(4), 225-232.

Kritzer, M. F. (1997). 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.*Journal of Comparative Neurology*, *379*(2), 247-260.

Kritzer, M. F. (2000). Effects of acute and chronic gonadectomy on the catecholamine innervation of the cerebral cortex in adult male rats: Insensitivity of axons immunoreactive for dopamine-β-hydroxylase to gonadal steroids, and differential sensitivity of axons immunoreactive for tyrosine hydroxylase to ovarian and testicular hormones. *Journal of Comparative Neurology*, *427*(4), 617-633.

Kritzer, M. F., McLaughlin, P. J., Smirlis, T., & Robinson, J. K. (2001). Gonadectomy impairs T-maze acquisition in adult male rats. *Hormones and behavior*, *39*(2), 167-174.

Kritzer, M. (2004). The distribution of immunoreactivity for intracellular androgen receptors in the cerebral cortex of hormonally intact adult male and female rats: localization in pyramidal neurons making corticocortical connections. *Cerebral cortex*, *14*(3), 268-280.

Kritzer, M. F., Brewer, A., Montalmant, F., Davenport, M., & Robinson, J. K. (2007). Effects of gonadectomy on performance in operant tasks measuring prefrontal cortical function in adult male rats. *Hormones and Behavior*, *51*(2), 183-194.

Kritzer, M. F., & Creutz, L. M. (2008). 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*, *28*(38), 9525-9535.

Lamberts, S. W., Van den Beld, A. W., & van der Lely, A. J. (1997). The endocrinology of aging. *Science*, *278*(5337), 419-424.

Le Moal, M., & Simon, H. (1991). Mesocorticolimbic dopaminergic network: functional and regulatory roles. *Physiological reviews*,*71*(1), 155-234.

Li, M., Nakagomi, Y., Kobayashi, Y., Merry, D. E., Tanaka, F., Doyu, M., ... & Sobue, G. (1998). Nonneural nuclear inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. *The American journal of pathology*, *153*(3), 695-701.

Lindeboom, J., & Weinstein, H. (2004). Neuropsychology of cognitive ageing, minimal cognitive impairment, Alzheimer's disease, and vascular cognitive impairment. *European journal of pharmacology*, *490*(1), 83-86.

Linn, M. C., & Petersen, A. C. (1985). Emergence and characterization of sex differences in spatial ability: A meta-analysis. *Child development*, 1479-1498.

Lisciotto, C. A., & Morrell, J. I. (1994). Sex differences in the distribution and projections of testosterone target neurons in the medial preoptic area and the bed nucleus of the stria terminalis of rats. *Hormones and behavior*, *28*(4), 492-502.

Litvinov, I. V., Vander Griend, D. J., Antony, L., Dalrymple, S., De Marzo, A. M., Drake, C. G., & Isaacs, J. T. (2006). Androgen receptor as a licensing factor for DNA replication in androgen-sensitive prostate cancer cells. *Proceedings of the National Academy of Sciences*, *103*(41), 15085-15090.

London, S. E., Remage-Healey, L., & Schlinger, B. A. (2009). Neurosteroid production in the songbird brain: a re-evaluation of core principles. *Frontiers in neuroendocrinology*, *30*(3), 302-314.

Lorenz, B., Garcia-Segura, L. M., & DonCarlos, L. L. (2005). Cellular phenotype of androgen receptor-immunoreactive nuclei in the developing and adult rat brain. *Journal of Comparative Neurology*, *492*(4), 456-468.

Lu, S. F., McKenna, S. E., Cologer-Clifford, A., Nau, E. A., & Simon, N. G. (1998). Androgen Receptor in Mouse Brain: Sex Differences and Similarities in Autoregulation 1. *Endocrinology*, *139*(4), 1594-1601.

Luine, V. N., Beck, K. D., Bowman, R. E., Frankfurt, M., & Maclusky, N. J. (2007). Chronic stress and neural function: accounting for sex and age.*Journal of neuroendocrinology*, *19*(10), 743-751.

Luine, V. N. (2008). Sex steroids and cognitive function. *Journal of neuroendocrinology*, *20*(6), 866-872.

Luine, V. N. (2014). Estradiol and cognitive function: past, present and future.*Hormones and behavior*, *66*(4), 602-618.

Lund, B. C., Bever-Stille, K. A., & Perry, P. J. (1999). Testosterone and andropause: the feasibility of testosterone replacement therapy in elderly men. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *19*(8), 951-956.

Luu-The, V., Bélanger, A., & Labrie, F. (2008). Androgen biosynthetic pathways in the human prostate. *Best Practice & Research Clinical Endocrinology & Metabolism*, *22*(2), 207-221.

Machida, T., Yonezawa, Y., & Noumura, T. (1981). Age-associated changes in plasma testosterone levels in male mice and their relation to social dominance or subordinance. *Hormones and behavior*, *15*(3), 238-245.

Maki, P. M., Ernst, M., London, E. D., Mordecai, K. L., Perschler, P., Durso, S. C., ... & Resnick, S. M. (2007). Intramuscular testosterone treatment in elderly men: evidence of memory decline and altered brain function. *The Journal of Clinical Endocrinology & Metabolism*, *92*(11), 4107-4114.

Martin, D. M., Wittert, G., Burns, N. R., Haren, M. T., & Sugarman, R. (2007). Testosterone and cognitive function in ageing men: data from the Florey Adelaide Male Ageing Study (FAMAS). *Maturitas*, *57*(2), 182-194.

McAbee, M. D., & DonCarlos, L. L. (1999). Regulation of Androgen Receptor Messenger Ribonucleic Acid Expression in the Developing Rat Forebrain 1.*Endocrinology*, *140*(4), 1807-1814.

McAlonan, K., & Brown, V. J. (2003). Orbital prefrontal cortex mediates reversal learning and not attentional set shifting in the rat. *Behavioural brain research*, *146*(1), 97-103.

McEwen, B. S., Alves, S. E., Bulloch, K., & Weiland, N. G. (1997). Ovarian steroids and the brain implications for cognition and aging. *Neurology*, *48*(5 Suppl 7), 8S-15S.

McGinnis, M. Y., & Yu, W. H. A. (1995). Age-related changes in androgen receptor levels in cranial nerve nuclei of male rats. *Brain research bulletin*,*36*(6), 581-585.
Meffre, D., Pianos, A., Liere, P., Eychenne, B., Cambourg, A., Schumacher, M., ... & Guennoun, R. (2007). Steroid profiling in brain and plasma of male and pseudopregnant female rats after traumatic brain injury: analysis by gas chromatography/mass spectrometry. *Endocrinology*, *148*(5), 2505-2517.

Mellon, S. H., & Griffin, L. D. (2002). Neurosteroids: biochemistry and clinical significance. *Trends in Endocrinology & Metabolism*, *13*(1), 35-43.

Menard, C. S., & Harlan, R. E. (1993). Up-regulation of androgen receptor immunoreactivity in the rat brain by androgenic-anabolic steroids. *Brain research*, *622*(1), 226-236.

Mengel, M., Werner, M., & von Wasielewski, R. (1999). Concentration dependent and adverse effects in immunohistochemistry using the tyramine amplification technique. *The Histochemical journal*, *31*(3), 195-200.

Miller, E. K., & Wallis, J. D. (2009). Executive function and higher-order cognition: definition and neural substrates. *Encyclopedia of neuroscience*,*4*(99-104).

Miller, E. K., & Cohen, J. D. (2001). An integrative theory of prefrontal cortex function. *Annual review of neuroscience*, *24*(1), 167-202.

Mitra, S. W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H. A., Hayashi, S., ... & Alves, S. E. (2003). Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. *Endocrinology*, *144*(5), 2055-2067.

Mizoguchi, K., Shoji, H., Tanaka, Y., Maruyama, W., & Tabira, T. (2009). Age-related spatial working memory impairment is caused by prefrontal cortical dopaminergic dysfunction in rats. *Neuroscience*, *162*(4), 1192-1201.

Mizoguchi, K., Shoji, H., Tanaka, Y., & Tabira, T. (2010). Orbitofrontal dopaminergic dysfunction causes age-related impairment of reversal learning in rats. *Neuroscience*, *170*(4), 1110-1119.

Moffat, S. D., Zonderman, A. B., Metter, E. J., Blackman, M. R., Harman, S. M., & Resnick, S. M. (2002). Longitudinal assessment of serum free testosterone concentration predicts memory performance and cognitive status in elderly men. *The Journal of Clinical Endocrinology & Metabolism*,*87*(11), 5001-5007.

Moffat, S. D., Zonderman, A. B., Metter, E. J., Kawas, C., Blackman, M. R., Harman, S. M., & Resnick, S. M. (2004). Free testosterone and risk for Alzheimer disease in older men. *Neurology*, *62*(2), 188-193.

Morley, J. E., Kaiser, F. E., Perry, H. M., Patrick, P., Morley, P. M., Stauber, P. M., ... & Garry, P. J. (1997). Longitudinal changes in testosterone, luteinizing hormone, and follicle-stimulating hormone in healthy older men. *Metabolism*, *46*(4), 410-413.

Morley, J. E., & Perry, H. M. (1999). Androgen deficiency in aging men. *Medical Clinics of North America*, *83*(5), 1279-1289.

Mukai, H., Tsurugizawa, T., Ogiue-Ikeda, M., Murakami, G., Hojo, Y., Ishii, H., ... & Kawato, S. (2006). Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory.*Neuroendocrinology*, *84*(4), 255-263.

Munetomo, A., Hojo, Y., Higo, S., Kato, A., Yoshida, K., Shirasawa, T., ... & Kawato, S. (2015). Aging-induced changes in sex-steroidogenic enzymes and sex-steroid receptors in the cortex, hypothalamus and cerebellum. *The Journal of Physiological Sciences*, *65*(3), 253-263.

Ostrander, M. M., Richtand, N. M., & Herman, J. P. (2003). Stress and amphetamine induce Fos expression in medial prefrontal cortex neurons containing glucocorticoid receptors. *Brain research*, *990*(1), 209-214.

Overk, C. R., Perez, S. E., Ma, C., Taves, M. D., Soma, K. K., & Mufson, E. J. (2013). Sex Steroid Levels and AD-Like Pathology in 3xTgAD Mice.*Journal of neuroendocrinology*, *25*(2), 131-144.

Pascal, L. E., & Wang, Z. (2014). Unzipping Androgen Action Through ZIP9: A Novel Membrane Androgen Receptor. *Endocrinology*, *155*(11), 4120-4123.

Patchev, V. K., Schroeder, J., Goetz, F., Rohde, W., & Patchev, A. V. (2004). Neurotropic action of androgens: principles, mechanisms and novel targets.*Experimental gerontology*, *39*(11), 1651-1660.

Paxinos, G., & Watson, C. (2009). A stereotaxic atlas of the rat brain. *New York: Academic*.

Perry, R. J., & Hodges, J. R. (1999). Attention and executive deficits in Alzheimer's disease. *Brain*, *122*(3), 383-404.

Pike, C. J., Carroll, J. C., Rosario, E. R., & Barron, A. M. (2009). Protective actions of sex steroid hormones in Alzheimer's disease. *Frontiers in neuroendocrinology*, *30*(2), 239-258.

Prins, G. S., Birch, L., & Greene, G. L. (1991). Androgen Receptor Localization in Different Cell Types of the Adult Rat Prostate. *Endocrinology*,*129*(6), 3187-3199.

Quesada, A., Romeo, H. E., & Micevych, P. (2007). Distribution and localization patterns of estrogen receptor-β and insulin-like growth factor-1 receptors in neurons and glial cells of the female rat substantia nigra: Localization of ERβ and IGF-1R in substantia nigra. *Journal of Comparative Neurology*, *503*(1), 198-208.

Rahman, F., & Christian, H. C. (2007). Non-classical actions of testosterone: an update. *Trends in Endocrinology & Metabolism*, *18*(10), 371-378.

Ramos-Vara, J. A. (2005). Technical aspects of immunohistochemistry. *Veterinary Pathology Online*, *42*(4), 405-426.

Rapp, M. A., & Reischies, F. M. (2005). Attention and executive control predict Alzheimer disease in late life: results from the Berlin Aging Study (BASE). *The American journal of geriatric psychiatry*, *13*(2), 134-141.

Renshaw, S. (Ed.). (2006). *Immunohistochemistry: methods express*. Royal College of General Practitioners.

Reuter-Lorenz, P. A., & Lustig, C. (2005). Brain aging: reorganizing discoveries about the aging mind. *Current opinion in neurobiology*, *15*(2), 245-251.

Rigaudiere, N., Pelardy, G., Robert, A., & Delost, P. (1976). Changes in the concentrations of testosterone and androstenedione in the plasma and testis of the guinea-pig from birth to death. *Journal of reproduction and fertility*,*48*(2), 291-300.

Robbins, T. W., Weinberger, D., Taylor, J. G., & Morris, R. G. (1996). Dissociating executive functions of the prefrontal cortex [and discussion].*Philosophical Transactions of the Royal Society B: Biological Sciences*,*351*(1346), 1463-1471.

Roselli, C. E., Kaler, L. W., & Resko, J. A. (1986). Hypothalamic aromatase activity in young and old male rats. *Neurobiology of aging*, *7*(2), 121-125.

Roselli, C. E. (1991). Sex Differences in Androgen Receptors and Aromatase Activity in Microdissected Regions of the Rat Brain. *Endocrinology*, *128*(3), 1310-1316.

Sader, M. A., McGrath, K. C., Hill, M. D., Bradstock, K. F., Jimenez, M., Handelsman, D. J., ... & Death, A. K. (2005). Androgen receptor gene expression in leucocytes is hormonally regulated: implications for gender differences in disease pathogenesis. *Clinical endocrinology*, *62*(1), 56-63.

Sanghera, M. K., Simpson, E. R., McPhaul, M. J., Kozlowski, G., Conley, A. J., & Lephart, E. D. (1991). Immunocytochemical Distribution of Aromatase Cytochrome P450 in the Rat Brain Using Peptide-Generated Polyclonal Antibodies*. *Endocrinology*, *129*(6), 2834-2844.

Sar, M., Lubahn, D. B., French, F. S., & Wilson, E. M. (1990). Immunohistochemical Localization of the Androgen Receptor in Rat and Human Tissues*. *Endocrinology*, *127*(6), 3180-3186.

Sarkey, S., Azcoitia, I., Garcia-Segura, L. M., Garcia-Ovejero, D., & DonCarlos, L. L. (2008). Classical androgen receptors in non-classical sites in the brain. *Hormones and behavior*, *53*(5), 753-764.

Sato, S. M., Schulz, K. M., Sisk, C. L., & Wood, R. I. (2008). Adolescents and androgens, receptors and rewards. *Hormones and behavior*, *53*(5), 647-658.

Sato, S. M., Johansen, J. A., Jordan, C. L., & Wood, R. I. (2010). Membrane androgen receptors may mediate androgen reinforcement.*Psychoneuroendocrinology*, *35*(7), 1063-1073.

Schmidt, K. L., Pradhan, D. S., Shah, A. H., Charlier, T. D., Chin, E. H., & Soma, K. K. (2008). Neurosteroids, immunosteroids, and the Balkanization of endocrinology. *General and comparative endocrinology*, *157*(3), 266-274.

Schumacher, M., Weill-Engerer, S., Liere, P., Robert, F., Franklin, R. J. M., Garcia-Segura, L. M., ... & Suter, U. (2003). Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. *Progress in neurobiology*, *71*(1), 3-29.

Seidman, S. N., & Walsh, B. T. (2000). Testosterone and depression in aging men. *The American Journal of Geriatric Psychiatry*, *7*(1), 18-33.

Shughrue, P. J., Lane, M. V., & Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-α and-β mRNA in the rat central nervous system. *Journal of Comparative Neurology*, *388*(4), 507-525.

Simerly, R. B., Swanson, L. W., Chang, C., & Muramatsu, M. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *Journal of Comparative Neurology*,*294*(1), 76-95.

Smith, G. T., Brenowitz, E. A., & Prins, G. S. (1996). Use of PG-21 immunocytochemistry to detect androgen receptors in the songbird brain. *Journal of Histochemistry & Cytochemistry*, *44*(9), 1075-1080.

Soma, K. K., Hartman, V. N., Wingfield, J. C., & Brenowitz, E. A. (1999). Seasonal changes in androgen receptor immunoreactivity in the song nucleus HVc of a wild bird. *The Journal of comparative neurology*, *409*(2), 224-236.

Squire, L. R., Stark, C. E., & Clark, R. E. (2004). The medial temporal lobe*.*Annu. Rev. Neurosci.*, *27*, 279-306.

Stanworth, R. D., & Jones, T. H. (2008). Testosterone for the aging male; current evidence and recommended practice. *Clinical interventions in aging*,*3*(1), 25.

Steiner, R. A., Bremner, W. J., Clifton, D. K., & Dorsa, D. M. (1984). Reduced pulsatile luteinizing hormone and testosterone secretion with aging in the male rat. *Biology of reproduction*, *31*(2), 251-258.

Sternbach, H. (1998). Age-associated testosterone decline in men: clinical issues for psychiatry. *American Journal of Psychiatry*, *155*(10), 1310-1318.

Stumpf, W. E., & Sar, M. (1976). Steroid hormone target sites in the brain: the differential distribution of estrogen, progestin, androgen and glucocorticosteroid. *Journal of steroid biochemistry*, *7*(11), 1163-1170.

Suárez-Quian, C. A., Martínez-García, F., Nistal, M., & Regadera, J. (1999). Androgen Receptor Distribution in Adult Human Testis 1. *The Journal of Clinical Endocrinology & Metabolism*, *84*(1), 350-358.

Swerdloff, R. S., & Wang, C. (1993). Androgens and aging in men. *Experimental gerontology*, *28*(4), 435-446.

Tabori, N. E., Stewart, L. S., Znamensky, V., Romeo, R. D., Alves, S. E., McEwen, B. S., & Milner, T. A. (2005). Ultrastructural evidence that androgen receptors are located at extranuclear sites in the rat hippocampal formation. *Neuroscience*, *130*(1), 151-163.

Tapia-Gonzalez, S., Carrero, P., Pernia, O., Garcia-Segura, L. M., & Diz-Chaves, Y. (2008). Selective oestrogen receptor (ER) modulators reduce microglia reactivity in vivo after peripheral inflammation: potential role of microglial ERs. *Journal of Endocrinology*, *198*(1), 219-230.

Taves, M. D., Schmidt, K. L., Ruhr, I. M., Kapusta, K., Prior, N. H., & Soma, K. K. (2010). Steroid concentrations in plasma, whole blood and brain: effects of saline perfusion to remove blood contamination from brain.

Taves, M. D., Ma, C., Heimovics, S. A., Saldanha, C. J., & Soma, K. K. (2011). Measurement of steroid concentrations in brain tissue: methodological considerations. *Frontiers in endocrinology*, *2*.

Thammacharoen, S., Lutz, T. A., Geary, N., & Asarian, L. (2008). Hindbrain administration of estradiol inhibits feeding and activates estrogen receptor-α-expressing cells in the nucleus tractus solitarius of ovariectomized rats. *Endocrinology*, *149*(4), 1609-1617.

Thilers, P. P., MacDonald, S. W., & Herlitz, A. (2006). The association between endogenous free testosterone and cognitive performance: a population-based study in 35 to 90 year-old men and women.*Psychoneuroendocrinology*, *31*(5), 565-576.

Thomas, P., Pang, Y., Dong, J., & Berg, A. H. (2014). Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. *Endocrinology*.

Toda, Y., Kono, K., Abiru, H., Kokuryo, K., Endo, M., Yaegashi, H., & Fukumoto, M. (1999). Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. *Pathology international*, *49*(5), 479-483.

Tomm, R. J., Ma, C. Q., Maric, T. T., Grist, M. M., Low, K. L., Tobiansky, D. J., ... & Soma, K. K. (2015). LBF-088: Effects of Aging on Cognition, Mesocorticolimbic Dopamine System, and Testosterone in Male Rats.

Tuttle, W. W., & Dykshorn, S. (1928). Castration and Ovariectomy on Spontaneous Activity and Ability to Learn. *Experimental Biology and Medicine*, *25*(6), 469-470.

VanderHorst, V. G., Gustafsson, J. Å., & Ulfhake, B. (2005). Estrogen receptor-α and-β immunoreactive neurons in the brainstem and spinal cord of male and female mice: Relationships to monoaminergic, cholinergic, and spinal projection systems. *Journal of Comparative Neurology*, *488*(2), 152-179.

Van Haaren, F., Van Hest, A., & Heinsbroek, R. P. (1990). Behavioral differences between male and female rats: effects of gonadal hormones on learning and memory. *Neuroscience & Biobehavioral Reviews*, *14*(1), 23-33.

Vaughan, C., Goldstein, F. C., & Tenover, J. L. (2007). Exogenous testosterone alone or with finasteride does not improve measurements of cognition in healthy older men with low serum testosterone. *Journal of andrology*, *28*(6), 875-882.

Veiga, S., Melcangi, R. C., DonCarlos, L. L., Garcia-Segura, L. M., & Azcoitia, I. (2004). Sex hormones and brain aging. *Experimental gerontology*,*39*(11), 1623-1631.

Vermeulen, A. (1991). Androgens in the aging male. *The Journal of Clinical Endocrinology & Metabolism*, *73*(2), 221-224.

Vermeulen, A. (2000). Andropause. *Maturitas*, *34*(1), 5-15.

Vida, B., Hrabovszky, E., Kalamatianos, T., Coen, C. W., Liposits, Z., & Kallo, I. (2008). Oestrogen receptor α and β immunoreactive cells in the suprachiasmatic nucleus of mice: distribution, sex differences and regulation by gonadal hormones. *Journal of neuroendocrinology*, *20*(11), 1270-1277.

Wallin, K. G., & Wood, R. I. (2015). Anabolic–androgenic steroids impair set-shifting and reversal learning in male rats. *European Neuropsychopharmacology*, *25*(4), 583-590.

Wise, R. A. (2009). Roles for nigrostriatal—not just mesocorticolimbic—dopamine in reward and addiction. *Trends in neurosciences*, *32*(10), 517-524.

Wolf, O. T., Preut, R., Hellhammer, D. H., Kudielka, B. M., Schürmeyer, T. H., & Kirschbaum, C. (2000). Testosterone and cognition in elderly men: a single testosterone injection blocks the practice effect in verbal fluency, but has no effect on spatial or verbal memory. *Biological Psychiatry*, *47*(7), 650-654.

Wood, R. I., & Newman, S. W. (1999). Androgen receptor immunoreactivity in the male and female Syrian hamster brain.

Wood, R. I. (2004). Reinforcing aspects of androgens. *Physiology & behavior*, *83*(2), 279-289.

Wood, R. I. (2008). Anabolic–androgenic steroid dependence? Insights from animals and humans. *Frontiers in neuroendocrinology*, *29*(4), 490-506.

Wu, D., Lin, G., & Gore, A. C. (2009). Age-related changes in hypothalamic androgen receptor and estrogen receptor α in male rats. *Journal of Comparative Neurology*, *512*(5), 688-701.

Wu, D., & Gore, A. C. (2010). Changes in androgen receptor, estrogen receptor alpha, and sexual behavior with aging and testosterone in male rats. *Hormones and behavior*, *58*(2), 306-316.

Xiao, L., & Jordan, C. L. (2002). Sex differences, laterality, and hormonal regulation of androgen receptor immunoreactivity in rat hippocampus. *Hormones and behavior*, *42*(3), 327-336.

Yaffe, K., Lui, L. Y., Zmuda, J., & Cauley, J. (2002). Sex hormones and cognitive function in older men. *Journal of the American Geriatrics Society*,*50*(4), 707-712.

Zamzow, D. R., Elias, V., Legette, L. L., Choi, J., Stevens, J. F., & Magnusson, K. R. (2014). Xanthohumol improved cognitive flexibility in young mice. *Behavioural brain research*, *275*, 1-10.

Zgaljardic, D. J., Borod, J. C., Foldi, N. S., Mattis, P. J., Gordon, M. F., Feigin, A., & Eidelberg, D. (2006). An examination of executive dysfunction associated with frontostriatal circuitry in Parkinson's disease. *Journal of Clinical and Experimental Neuropsychology*, *28*(7), 1127-1144.

Zhou, L., Blaustein, J. D., & De Vries, G. J. (1994). Distribution of androgen receptor immunoreactivity in vasopressin-and oxytocin-immunoreactive neurons in the male rat brain. *Endocrinology*, *134*(6), 2622-2627.

Zwain, I. H., & Yen, S. S. (1999). Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. *Endocrinology*,*140*(8), 3843-3852.