Implications of Menopause Etiology and Hormonal Alterations in Healthy and

Neurodegenerative Aging: Impacts on Learning, Memory, and Neurobiological

Outcomes

by

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ABSTRACT

Variations in menopause etiologies, from surgical manipulation to a natural transition, can impact cognition in both healthy and neurodegenerative aging. Although abundant research has demonstrated impacts from surgical versus transitional menopause, such as variations in timing of menopause, both variations in initiation of menopause and length of time since menopause, but not all avenues have been systematically evaluated. Further, assessments of variations in hormone therapies have demonstrated marked outcomes on the brain and cognition in different menopause etiologies, and results can differ depending on type of hormone, combination of hormones, dose, route of administration, among other factors, in regard to healthy aging. Further, the impact of the endocrine system on neurodegenerative disease is multifaceted. Research has highlighted that the endocrine system not only impacts neurodegeneration, such as in Alzheimer's disease (AD), but that fluctuations in the endocrine system might be strong mediators in disease prevalence and progression. This dissertation seeks to understand how factors such as menopause etiology, biological sex, and hormone therapy impact normative and neurodegenerative aging. Assessments in a rat model of normal aging of progestogen-based hormone therapy given during the transition to menopause demonstrated attenuation of impairment seen with transitional menopause that was working memory specific. In evaluating a rat model of AD, there were distinct trends in neuropathology and associated cognitive changes in males and females with and without gonadal hormone deprivation. Further, assessment of transitional menopause in this AD model yielded an interaction between follicular depletion and genotype for neuropathology that was not present in cognitive assessments. Together, these dissertation chapters highlight

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that there are a multitude of factors to consider when evaluating effects of menopause and that these variations in experience underscore a need for personalized medicine when selecting therapeutic targets for healthy and neurodegenerative aging that includes consideration of overall hormone milieu and menopause history. Further, these data suggest that the inclusion of males and females in the study of AD-related factors is crucial for understanding disease progression.

DEDICATION

To both of my grandmothers, who passed as I was completing this dissertation. To my grandma Della, who was a treasure to live with and taught me about silent strength and love of family. To my grandma Vi, who had a vivacity of life and taught me to preserve and to value simple joy.

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CHAPTER 1

INTRODUCTION

Clinical and preclinical research has sought to understand impacts of reproductive functioning and senescence on a myriad of factors. Indeed, utilization of rodent models has had goals to identify and clarify neurobiological mechanisms of action that impact cognitive outcomes. Considering that women, half of the human population, undergo unique endogenous biological events such as pregnancy, childbirth, and menopause, investigation into these factors is key to be able to provide optimal health care for women. In the past, this area has been markedly understudied as researchers have focused on male subjects. Newer investigation into female-specific questions has gained traction as the National Institute of Health initiated a movement to require inclusion of both male and female subjects to address discrepancies in sex specific effects. Concerns regarding effects of menopause increased rapidly after the results from the Women's Health Initiative Memory Study (WHIMS) found that the common hormone therapy regimen conjugated equine estrogens and medroxyprogesterone acetate was associated with a small but meaningful increased risk of dementia in women 65 years or older (Shumaker et al., 2003). This finding was surprising, as estrogen-based hormone therapies had been previously thought to be either primarily neutral or beneficial to cognition and overall brain health (Haskell et al., 1997). The inclusion of a progestogen component in the WHIMS highlighted the necessity for understanding the impacts of clinically relevant on neuropathological implications of cognitive decline. Subsequent research demonstrated that oophorectomy, the surgical removal of the ovaries in women, before the age of the natural onset of menopause was also associated with an increased risk in cognitive

decline and dementia. This dissertation will consider preclinical aspects of menopause etiology and progestogen-based hormone therapies, their effects on cognition in healthy aging and in neurodegenerative disease, specifically Alzheimer's disease pathology and symptomatology. To this regard, effects of gonadal hormone deprivation in each sex on AD neurobehaviors are included.

Ovary-Intact Model

In rodents, one model of endocrine aging is the ovary intact model. With this model, rodents do not undergo an intervention (be it surgical or otherwise) and instead are assessed in their natural state. It is important to note that the etiology of reproductive senescence in rodents differs from that of humans. Indeed, rodents undergo estropause, whereby hypothalamic-pituitary-gonadal (HPG) axis dysregulation is the primary driver, while women undergo menopause arising primarily from accelerated atresia of ovarian follicles. For the ovary intact model, there is a substantial amount of research that assesses the influence of hormone fluctuations across the estrous cycle and across aging on the brain and cognition (Quinlan et al., 2010; Spencer et al., 2010; Stackman et al., 1997; Warren et al., 1995; Woolley & McEwen, 1992). Research has demonstrated that rats in proestrus, the phase of the estrous cycle with the highest circulating 17beta-estradiol (E2), had an increased synaptic density of dendritic spines in the CA1 region of the hippocampus compared to rats in estrus, which is the estrous phase represented by lower levels of E2 (Woolley & McEwen, 1992). Further, there was an enhancement of long-term potentiation, the principal molecular model of the formation of memories, in proestrus rats compared to estrus rats (Warren et al., 1995; Woolley & McEwen, 1995).With the ovary intact model, however, it is difficult to differentiate the biological

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mechanisms of action of individual sex steroid hormones and other related impacts on the endocrine system since these are in fluctuation in accordance with the estrous cycle. Further, since rodents do not undergo natural transitional menopause, as women do, and instead undergo estropause, the reproductive senescence of a rodents is biologically distinct from human reproductive senescence. Menopause in women occurs at the average age of 52 and is clinically defined as one year of amenorrhea (NAMS, 2015). Multiple menopause models have been established to assess impacts of various menopause etiologies on the brain and cognitive outcomes (Bimonte-Nelson & Bernaud, 2023).

Bilateral Ovariectomy

A widely used rodent model to test hormone modulation is ovariectomy (Ovx), the term used to describe surgical removal of the ovaries in the rodent. This model causes an abrupt cessation of circulating ovarian-derived hormones (e.g., progestogens, estrogens, and androgens) as well as halting the estrous cycle, and is thus a binary, all-or-nothing model. It is important to note that estrogens can also be produced in other areas of the body, such as in lipids and in the brain, and therefore while there is an abrupt cessation of ovarian-derived estrogens, estrogens are not completely diminished (Kretz et al., 2004; Micevych & Sinchak, 2008; Tuscher et al., 2016). The endocrine profile in these rodents is akin to women who undergo oophorectomy. Surgical menopause, however, is not how most women undergo menopause. Instead, most women undergo a natural transition to menopause in which their ovaries remain intact. Of the 600,000 women per year in the United States that receive a hysterectomy, which is the surgical removal of the uterus, half will receive a concurrent oophorectomy (Lowder et al., 2010;

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Whiteman et al., 2008). Women will opt to have an oophorectomy for a myriad of reasons, such as breast cancer risk reduction, treatment of severe premenstrual syndrome, and treatment for the onset of ovarian cancer (Asante et al., 2010; Cronje et al., 2004; Erekson et al., 2013; Olson et al., 2004). This preclinical model is ideal for understanding effects of surgical menopause as well as determining effects of specific hormone administration on a "blank slate" hormone background.

Unilateral Ovariectomy (ULO)

Another model of interest is the unilateral ovariectomy (ULO) model, whereby only one ovary is surgically removed, while the other remains intact. Since one ovary is present to release endogenous hormones, this model can be used to assess an endocrine system that has a reduced capacity, acknowledging that some studies indicate compensatory phenomena (D. A. Coleman et al., 1984; Fleming et al., 1984; Morales-Ledesma et al., 2011).Utilizing this model, researchers have found that ULO performed during metestrus and diestrus led to a surge of follicle stimulating hormone (FSH) in plasma as well as the pituitary compared to ovary-intact sham counterparts, while amounts of luteinizing hormone (LH) remained the same across both groups (Otani & Sasamoto, 1982). This rise in FSH was posited by the researchers to be a compensatory mechanism of the HPG axis to account for the loss of a singular ovary (Otani & Sasamoto, 1982). In the clinic, ULO has been demonstrated to increase risk of dementia when performed concomitantly with hysterectomy compared to hysterectomy alone and women who did not receive a gynecological surgery (Rocca et al., 2012). This risk increased even further when women received a bilateral oophorectomy with concomitant hysterectomy (Rocca et al., 2012). These data suggest that ULO affects the endocrine

system and can have far-reaching impacts on dementia risk, and can be a translationally-relevant model for a specific population of women. It should be noted, however, that this model is rarely utilized in either clinical or preclinical research and thus effects on brain and cognition are not well understood.

Follicular Depletion (VCD)

Surgical models of menopause do not fully recapitulate the experience of women who undergo menopause. In general, women undergo a natural transition to menopause that can last up to 10 years which is induced by accelerated ovarian follicular depletion (Richardson & Nelson, 1990). In preclinical research, administration of 4-vinylcyclohexene diepoxide (VCD) to induce accelerated atresia of ovarian follicles has been developed to model transition menopause in the rodent. Rodents, as in humans, have four follicle types: primordial, primary, secondary, and antral, as well as corpora lutea, the tissue that is left after an antral follicle has released an egg (Kronenberg et al., 2008). These follicles progress accordingly from primordial to primary to secondary to antral, at which point the ovary is classified as pre-ovulatory and ready to release an egg (Kronenberg et al., 2008). Administration of VCD accelerates the atresia of primordial and primary follicles while leaving the ovaries intact and, thus, halts ovarian cyclicity (Flaws et al., 1994a; Hoyer, Devine, Hu, et al., 2001; S.-W. Kao et al., 1999; L. N. Springer et al., 1996). With this model, the endocrine profile becomes more similar to what women undergo in the clinic; in mice, estrogens and progesterone decrease while FSH and LH increase in response (Mayer et al., 2004). Further, androgens are still able to be produced from the intact ovarian tissue, and while there is a decrease in androgen production overall, since there is a more drastic decrease in estrogens and progesterone,

the hormonal milieu becomes androgen dominant (Mayer et al., 2004). Further, cells

from these VCD-treated ovaries are able to release androgens in culture (Mayer et al.,

2004). Thus, with the more translationally relevant hormone milieu and follicle status,

this model is ideal for understanding nuances of follicular depletion. This model,

however, does have caveats. For example, VCD itself can be somewhat toxic, and is used

to create epoxy (Chhabra, 1989). VCD was first discovered as a byproduct of the

production of rubber and flame retardants, pesticides, and plastics via vulcanization

(Rappaport et al., 1976). Only later was it discovered that it had ovotoxic properties as

well as impacts within multiple body systems, such as increasing oxidative stress in the

liver, ovaries, and uterine horns, and increasing inflammatory factors and apoptosis in the

ovaries and liver (Abolaji, Adedara, et al., 2016; Abolaji, Toloyai, et al., 2016).

Additionally, VCD treatment has demonstrated increases in cholesterol and fatty acids

compared to age-matched controls (Romero-Aleshire et al., 2009). Specifically, when

paired with a high fat diet, VCD treatment yielded decreased glucose tolerance and

increased insulin resistance compared to high fat diet alone (Romero-Aleshire et al.,

2009). Adult VCD-treated rats had increased levels of blood urea nitrogen and creatine

(indicative of decreased renal function), but this may have been attributed to the fact that

four of these rats died of peritonitis, which could naturally increase levels of these

enzymes (Muhammad et al., 2009). Further, while liver weights increased with VCD

treatment, there was no indication of liver injury as assessed via alanine aminotransferase

(Muhammad et al., 2009). When utilizing the VCD model, it is imperative to understand

that VCD affects not only ovarian follicles but can also have impacts on other body

systems that can also affect cognitive outcomes. In experiments from our own laboratory,

VCD treatment induced pain-like behaviors and thus necessitates extensive handling while performing injections which can lead to increases in stress levels (unpublished observations). Further, we have noted that utilizing different vehicles and injection modalities for VCD treatment, such as subcutaneous injections with a sesame oil vehicle can produce skin lesions (upublished data). Newer oral routes of VCD administration utilizing food bait are also available but also have drawbacks such as the inclusion of triptolide, a chinese herb that is a cognitive enhancer (Dyer et al., 2013, Cheng et al., 2014).

It is clear that there are distinct biological differences between surgical menopause and transitional menopause models in the rodent. These differences are also present in effects on cognition and the brain. In studies utilizing VCD, our laboratory has demonstrated that VCD-treated rats are marginally impaired compared to Ovx rats, and are significantly impaired compared to sham counterparts (Acosta et al., 2009). Additionally, there was a positive correlation with errors made on the water radial arm maze (WRAM) and androstenedione levels such that rats that tended to have higher serum androstenedione also tended to make more working memory errors during the memory retention phase, suggesting that androstenedione might be mediating VCD-induced impairment (Acosta et al., 2009). This was further supported in a subsequent study where a similar correlation was found whereby rats that tended to have higher serum androstenedione levels also tended to make more total errors on the WRAM (Acosta et al., 2010). Additional evidence suggests that this androstenedione-induced impairment is due to its conversion to estrone via the aromatase enzyme (Mennenga et al., 2015). Moreover, it is clear that there are differential effects of hormone therapy

based on whether the menopause model is surgical or transitional. One study directly comparing surgical and transitional models of menopause has demonstrated that conjugated equine estrogens are beneficial to cognition when given in an Ovx model, but is detrimental when given in the ovary-intact VCD model (Acosta et al., 2010). These data suggest that effects of hormone supplementation can differ based on menopause etiology and that this might be regulated by the hormonal milieu produced by each model.

Progestogen-Based Hormone Therapy

Although effects of estrogen-based hormone therapies have been frequently assessed, for this dissertation we will focus on progestogen-alone based therapies. Progestogen-based therapies can be given for a multitude of reasons. For example, progestogens can be given as a birth control formulation (Stewart et al., 2021) They can also be administered post menopause in combination with an estrogen to combat the negative effects of estrogens at the uterus to reduce the risk of endometrial hyperplasia and cancer (Baker et al., 2012; Gallos et al., 2010; Schindler, 2009). Further still, progestogens can be administered to attenuate heavy uterine bleeding that can be a symptom during the transition to menopause (Bender, 2022). Progestogens include both endogenous progesterone and synthetic variants, known as progestins. Progesterone has been investigated for impacts on the brain and cognition, and has been administered in an acute (hours to days) schedule to provide protection against, and as a therapeutic treatment for, brain trauma (Stein et al., 2008). Indeed, progesterone and its metabolites can act as a neuroprotectant for traumatic brain injury, as well as hippocampal induced excitotoxicity and ischemic stroke via its proinflammatory properties (Amirkhosravi et

al., 2021; Wali et al., 2014; Shear et al., 2002). Acute progesterone can also be beneficial for non-spatial memory and recognition memory consolidation (Lewis et al., 2008; Harburger et al., 2008). Chronic administration of progesterone (weeks to months) has demonstrated impairments to spatial working memory when assessed on an Ovx background in middle-aged rats (Braden et al., 2015). Further, for spatial reference memory, progesterone attenuated beneficial effects of E2 alone on an Ovx background (Bimonte-Nelson et al., 2006). Also, a ten-day administration of progesterone or allopregnanolone (a metabolite of progesterone) on a long term (16.5 weeks) Ovx background improved novel object recognition performance and increased dendritic spine density in the dorsal hippocampus (Barreto-Cordero et al., 2020).

Another progestin of interest, micronized progesterone, can also be administered to counter unwelcome menopause associated symptoms, such as secondary amenorrhea and endometrial hyperplasia (Stute et al., 2016; Gillet et al., 1994). Micronized progesterone, synthesized from yams (*Dioscorea sp*), is bioidentical to progesterone but smaller in particle size to increase oral bioavailability; this is an improved profile of the low bioavailability and rapid clearance rate of progesterone itself (De Lignières, 1999; Maxson & Hargrove, 1985). Since the two progestogens have the exact same empirical formula ($C_{21}H_{30}O_2$) and molecular weight (314.47) it can be presumed that micronized progesterone acts upon the same targets in the central nervous system, as well as the periphery, as progesterone (De Lignières, 1999). The micronization process for progesterone can be achieved through a variety of measures that involve either particle to particle collision, or particle to solid object collision, to cause enough force to reduce particle size (Joshi, 2011). As a result of the micronization process, overall surface area is

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increased for micronized progesterone while the diameter decreases (diameter of 10μ), ensuring that it comes into contact with more of the mucous membrane of the intestines, increasing the rate at which gastrointestinal absorption occurs (De Lignières, 1999). In clinical research, micronized progesterone, when in combination with conjugated equine estrogens (CEE), demonstrate improvements in spatial working memory compared to age-matched counterparts (Miller et al., 2009; Sherwin & Grigorova, 2011; Gleason et al., 2015; Ryan & Rosner, 2001) or no effects on cognition when combined with either E2 or CEE (Miller et al., 2019; Gleason et al., 2015). To our knowledge, there have been no clinical or preclinical assessments for cognition on micronized progesterone alone and thus, determining the underlying mechanism of action has not been deciphered.

Medroxyprogesterone acetate (MPA) is an acetylated pregnane-derived progestin with androgenic effects (Africander et al., 2014; Gogoi et al., 2008; Stanczyk, 2003; Stanczyk & Bhavnani, 2014). MPA can be prescribed to attenuate secondary amenorrhea and heavy uterine bleeding that can occur during the transition to menopause (Bender 2022). Further, it is available to women in the clinic as Depo-Provera or its generic counterpart (Eugia Product Portfolio, 2023; Depo-Provera Prescribing Information, 2006). MPA administration alone has been demonstrated by our laboratory to impair cognition on an Ovx background compared to Ovx rats alone (Braden et al., 2017; Okojie & Oyekunle, 2014). This impairment is not reversible, as even when MPA treatment was discontinued for four months, MPA impairments persisted compared to age-matched controls (Braden et al., 2011). When combined with E2, MPA treatment on an Ovx background can improve non-spatial learning on a T-maze while MPA impairs spatial reference memory on the Morris Water Maze compared to Ovx controls (Chisholm &

Juraska, 2012; Lowry et al., 2010). MPA also has demonstrated effects on the brain. For instance, MPA increased beta-amyloid through disruption of its degradation in rat glial cells, as well as decreased demyelination in a demyelination mouse model (Mohammadi et al., 2021; Porter et al., 2020). These data suggest that MPA can impair spatial memory yet have beneficial effects on non-spatial memory and the brain.

One potential mechanism of action that the aforementioned progestogens act through is the γ-aminobutyric acid (GABA)-ergic system, the primary inhibitory neurotransmitter system in the central nervous system (Ben-Ari, 2014; Bernard et al., 2000; Croarkin et al., 2011; Mccormick, 1989). MPA has been shown to increase inhibitory tone via the GABAergic system in the dentate gyrus as well as alter glutamic acid decarboxylase (GAD), the synthesizing enzyme for GABA, mRNA and protein levels (Belelli & Herd, 2003; Braden et al., 2010; 2011). Further, MPA serum levels negatively correlated with GAD expression in the dorsal hippocampus (Braden et al., 2011). Impairments in memory seen with progesterone administration were attenuated when combined with bicuculline, a GABA$_A$ receptor antagonist (Braden et al., 2015). MPA is a positive modulator for the alpha 2 and alpha 5 subtypes of the GABA$_A$ receptor (Das et al., 2022). It is plausible that effects of progestogens on cognition is mediated, in part, by the GABAergic system.

Alzheimer's Disease

Alzheimer's disease (AD) pathology is marked, in part, by aggregation of two separate proteins; the beta-amyloid protein into beta-amyloid plaques, and hyperphosphorylated tau into neurofibrillary tangles (Kuchibhotla et al., 2008; Price & Morris, 1999; Serrano-Pozo et al., 2011). This protein aggregation leads to a multitude of

pathological and behavioral changes that are characteristic of the disease. Alzheimer's disease pathology consists of cardiovascular dysregulation, loss of dendritic spines, increases in neuroinflammation, and frank neuronal loss (Price et al., 2001; Scheff & Price, 2003; Zilka et al., 2009). Further, the pathology is distinctly different in AD progression as compared to normal aging (West et al., 1994). Earlier research focused on amyloidogenic pathology in AD, with the amyloid cascade hypothesis rising to prominence as a potential pathway of disease progression (Hardy & Higgins, 1992). This hypothesis suggested that cleavage of the amyloid precursor protein by a beta-secretase (BACE1) and gamma-secretase would result in aggregates of beta-amyloid protein leading to beta-amyloid plaque deposition (Borchelt et al., 1996; De Strooper, 2003; Hardy & Higgins, 1992; Vassar et al., 1999, 2009). Beta-amyloid is an amino acid and exists in many variants, the most common discussed being beta-amyloid 1-40 and 1-42. Beta-amyloid 1-42 (the longer form with 42 amino acids) is the more pathogenic of the two species and more likely to aggregate into oligomers and subsequently into plaques (A. M. Klein et al., 1999; Pesaresi et al., 2006; Roher et al., 1993). Interestingly, research has demonstrated that significant plaque burden does not always correlate with impaired cognition, nor does it necessitate a progression to AD (Davis et al., 1999; Haroutunian et al., 1999). In a classic study colloquially known as the "Nun Study," some subjects at autopsy were found to have amyloid plaque deposition similar to those with AD, but did not show marked cognitive impairment, indicating that amyloid pathology alone is not necessary for onset of AD behavioral clinical symptomatology (Mortimer, 2012; Snowdon et al., 1996). Other research has demonstrated similar findings (Bennett et al., 2006; Santacruz et al., 2011). Further analysis of the Nun Study found that in subjects

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with substantial AD pathology, including beta-amyloid plaques and neurofibrillary tangles, that did not display cognitive impairment (designated as asymptomatic AD patients), there was an increase in the apolipoprotein E (APOE) E2 allele, which has been posited to decrease risk of AD, and increased education in asymptomatic AD patients compared to patients with mild cognitive impairment (MCI) and AD (Iacono et al., 2015). This increased level of education in these individuals supports the idea of cognitive reserve as a protective factor against AD prevalence. Cognitive reserve is defined as an adaptation influenced by lifetime experiences and genetic components that increases resiliency towards brain aging and disease (Iraniparast et al., 2022; Pettigrew & Soldan, 2019; Stern et al., 2018). Cognitive reserve is thought to possibly postpone AD prevalence, although it either does not hinder progression of disease once onset has occurred (Perquin et al., 2015).

It should be noted that there are two subtypes of AD: 1. early-onset, familial AD, which is primarily genetic in which a mutation of the APP, presenilin 1, or presenilin 2 genes, among others, occurs, and 2. the sporadic AD subtype which does not have a clear cause, although there are several risk factors associated with the onset of this subtype (Mendez, 2017, 2019; Reitz et al., 2020). In the early-onset version of AD, age of symptomatology occurs before 65 years of age (Mendez 2017, 2019). In the sporadic or late-onset version of AD, age of symptomatology occurs around 65-70 years of age. Risk factors for the sporadic version of AD include the E4 variant of the APOE gene, hypertension, traumatic brain injury, and female sex (Silva et al., 2019). One limiting factor in curing or providing effective therapeutics for AD is the fact that researchers still do not fully understand disease onset and disease progression. This is further complicated

by the fact that AD clinical symptoms occur 10-20 years after neural pathology has begun which limits therapeutic intervention since pathology is already well-established (Holtzman et al., 2011; W. L. Klein et al., 2001; Mucke et al., 2000). Current clinical interventions include anticholinesterase inhibitors such as donepezil, to increase acetylcholine in the synaptic cleft during early disease progression as well as NMDA receptor inhibitors such as memantine to prevent calcium excitotoxicity during late disease progression; a combination of both has demonstrated greater efficacy than donepezil alone (Chen et al., 2017; Jelic & Darreh-Shori, 2010; Kumar et al., 2021; Kuns et al., 2022). Newer medications have targeted amyloid specific pathology, but these interventions have not proven effective at reversing symptoms in patients (Barage & Sonawane, 2015; Castello et al., 2014). The currently available therapeutics for AD are only capable of halting disease progression for the short term and are not capable of reversing clinical symptoms or disease pathology. This lack of efficacy is likely twofold: pathology is too engrained at time of clinical symptom onset, and there is no definitive understanding of the onset of pathology. It is likely that disease onset is not caused by a singular event but related to a multisystem multi-impact cascade of events that can include environment, genetic, cardiovascular, and endocrine factors, among others (Breijyeh & Karaman, 2020). Thus, further investigation into factors impacting AD prevalence and progression should be thoroughly assessed. Work is already underway to identify biomarkers that can be measured at early time points in disease progression to aid in more timely therapeutic interventions to offset early disease progression (Blennow & Zetterberg, 2018; Caroli & Frisoni, 2010; Jack et al., 2016). Indeed, biomarkers such as CSF beta-amyloid (1-42) and phosphorylated tau as well as imaging of beta-amyloid

and tau via PET scans have been identified (Besson et al., 2015; Buerger et al., 2006; Klunk et al., 2004; Visser et al., 2009).

One risk factor of interest for this dissertation relates to the sex difference in AD disease prevalence and progression. Of the 6.5 million Americans diagnosed with AD, roughly two-thirds are women. Although some of this increased risk is attributed to the fact that women on average live 4.5 years longer than men, other aspects of womanhood, such as biological events unique to women including but not limited to pregnancy and menopause, might be related to this increased risk as well (Dye et al., 2012). Women with five or more pregnancies have a two-fold increased risk of developing AD compared to women with four or fewer pregnancies or women who have never been pregnant (Jang et al., 2018). Amount of parity has also been implicated in increased AD neuropathology (Beeri et al., 2009). There are clear sex differences in the pathology of AD. While men have a 10% lifetime risk of AD, women have double this risk, with a 20% lifetime risk (Chene et al., 2015). In women with AD, there is an increase in neuronal loss and overall increased atrophy compared to men with AD (Elbejjani et al., 2015; Mielke et al., 2014; Sampedro et al., 2015). Additionally, women who have at least one E4 allele have an increased cardiovascular risk associated with tau hyperphosphorylation early in disease progression, indicating an increased vulnerability to AD (Tsiknia et al., 2021). Further, there is an increase in hippocampal atrophy and cognitive decline, as well as an increase in beta-amyloid (1-42) and total tau in cerebral spinal fluid in women with AD compared to men (Koran et al., 2017). Faster age-related cognitive decline has been noted in women with AD compared to men (Proust-Lima et al., 2008; Read et al., 2006; Henderson & Buckwalter, 1994). Preclinically, young female mice are protected against

beta-amyloid-induced toxicity in mitochondria, potentially due to mitochondrial responsivity to estrogens that declines with age (Viña & Lloret, 2010). In older female rodents, there is an increase in reactive oxygen species in mitochondria, another pathological impact found in AD progression, in the presence of beta-amyloid (Viña & Lloret, 2010).

In preclinical research, animal models in which AD-like pathology is induced are utilized since rodents do not naturally develop AD. There are a multitude of animal models of AD, some of which attempt to recapitulate the entirety of disease progression, both in pathology and symptomatology, and some that are specific to certain aspects of the disease. For instance, early models of AD include the senescence accelerated mouse (SAMP8) that shows impairments in learning and memory which are age-associated and related to beta-amyloid deposition (Del Valle et al., 2010; Yagi et al., 1988). Further, chemical interventions have also been utilized, such as scopolamine-induced amnesia which targets the cholinergic system, heavily impacted by AD pathology (Ebert & Kirch, 1998; Sunderland et al., 1986). Additionally, lesions to brain regions impacted by AD and important for learning and memory, such as the hippocampus, have also been utilized as models of AD (Gray et al., 1983). These models, however, do not assess or modulate either the amyloid or tau pathology inherent in AD. Thus, amyloid centric models were created to more accurately model AD in accordance with the amyloid cascade hypothesis (Selkoe, 2000). These models involved intracerebroventricular (ICV) injections into either the lateral ventricles or a particular brain region impacted by AD, such as the hippocampus (Harkany et al., 1998; Yamada et al., 2008). These methods, however, are unsound since this only recapitulates part of the disease progression and involves neural

damage during injection and canula implantation. Transgenic models, whereby genes associated with AD are implanted into rodents to be passed along to offspring, are utilized to account for neural damage associated with ICV and to more accurately model early-onset AD. There are a multitude of AD-associated transgenic models. Some models are able to isolate amyloid only pathology, such as the APP/PS1 mouse model (Garcia-Alloza et al., 2006), while others isolate tau pathology with P301L and PS19 genes (J. Lewis et al., 2000; Yoshiyama et al., 2007). The 3xTG-AD mouse model exhibits both amyloid and tau pathology utilizing the APP_{Swe}, $PS1_{M146V}$, and tau_{P301L} genes and is commonly used in AD research to assess pathology and its effects on cognition (Oddo et al., 2003). These transgenic models, however, like all models, have shortcomings. Some do not recapitulate the correct temporal progression of the disease, while some only target distinct molecular aspects of the disease. Additionally, the fact that transgenic models only truly model the early-onset subtype of AD is problematic, as the sporadic subtype might have different biological underpinnings of disease onset. These models, however, do allow us to ask targeted questions about therapeutic interventions at key timepoints in disease progression. Further, we are able to establish relationships between pathology and cognitive outcomes that are not possible to elucidate in the clinic. For this dissertation, we have selected a transgenic rat model of AD to aid in our understanding of the relationships between the endocrine system, cognition, and AD pathology. We chose to utilize a rat model since rats display a more complex array of behaviors that allow for the assessment of prodromal symptoms of AD that are working memory specific (Draoui et al., 2019; Goodman et al., 2021). Further, rats have six isoforms of tau, as do humans,

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while mice only have four, which allows for more translational interpretations of tau pathology (Hanes et al., 2009).

The TgF344-AD rat model stood out as a premier model of AD since the temporal progression of disease, including pathology and behavioral deficits, mapped more closely to the human disease progression than other models of AD. This model has two genes of interest, the Swedish version of the APP gene, and the PS1dE9 gene, which has an exon at the delta 9 position removed (Cohen et al., 2013). These genes are only associated with amyloid pathology. However, there is a natural progression of endogenous hyperphosphorylated tau leading to neurofibrillary tangle-like structures (Cohen et al., 2013; Joo et al., 2017b). Additionally, there is an accumulation of pathogenic soluble beta-amyloid (1-42) that leads to aggregation of beta-amyloid oligomers to beta-amyloid plaques (Cohen et al., 2013; Joo et al., 2017). Further, this model presents with neuroinflammatory markers via reactive microglia and astrocytes, dendritic spine loss, impairments in spatial working and reference memory as well as recognition memory, cardiovascular dysfunction, and frank neuronal loss at disease appropriate time points (Berkowitz et al., 2018; Cohen et al., 2013; Joo et al., 2017a; Smith & McMahon, 2018; Bernaud et al., 2022). We will utilize this model to assess the effects of menopause etiology and gonadal hormone deprivation in female and male rats.

One plausible reason for sex differences in the pathology in AD is that women undergo menopause while males undergo the less extreme andropause. Andropause includes a slow decline of androgens, including testosterone, which decreases at a rate of roughly 2% per year, over the course of decades (Feldman et al., 2002; Muller et al., 2003). Assessing different menopause etiologies and their impacts on AD pathology is a

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key factor to deciphering sex differences in disease prevalence. Prior research has primarily focused on effects in female rodents after surgical menopause via Ovx and effects of add-back hormone treatment, particularly estrogens and progesterone. Abrupt cessation of ovarian-derived hormones via Ovx can exacerbate AD neuropathology (Carroll et al., 2007; Heikkinen et al., 2004; Hu et al., 2016; Yue et al., 2005). Further, Ovx was found to increase sensitivity of neurons to toxic effects of beta-amyloid (1-42) as well as ischemic stress in the CA3 region of the hippocampus, and to potentiate the switch from non-amyloidogenic to amyloidogenic processing following ischemic stress (Q. G. Zhang et al., 2013). These impairments were reversed if E2 was given at surgery, but not if given 10 weeks post-surgery, supporting the critical window of opportunity hypothesis (Zhang et al., 2013). E2 administration can be beneficial for AD pathology. For example, E2 promotes utilization of the MAPK/ERK pathway, a non-amyloidogenic pathway, and decreases BACE1 (an enzyme necessary to cleave beta-amyloid from the amyloid precursor protein), and increases clearance of beta-amyloid via microglial phagocytosis (Singh et al., 1999). E2 can also affect tau pathology such that E2 administration decreases hyperphosphorylated tau compared to Ovx alone via phosphatases and kinases such as Wnt, GSK-3beta, and PKA (Zhang et al., 2008). Decreased E2 in the brain leads to earlier and more severe AD progression in APP mice compared to Ovx alone (Yue et al., 2005).

Progesterone can also impact AD variables in females when administered in various animal models of AD. Progesterone administered on an Ovx background modulates gamma-secretase and downregulates BACE1 gene expression, but does not affect alpha-secretase (Amtul et al., 2010; Zhao et al., 2013; Jung et al., 2013).

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Progesterone can also increase beta-amyloid clearance compared to Ovx only via the insulin degradation enzyme (Jayaraman & Pike, 2014). In the VCD model of follicular depletion, with APOE-TR mice, genotype and follicular depletion interacted such that APOE4 with follicular depletion could not distinguish familiar from novel objects and had a 30% decline of Ephrin type-B receptor 2 (Ephb2), a protein important for learning and memory, compared to APOE3 controls as well as APOE4 AD mice (Pontifex et al., 2021).

It should be noted that although women have a higher incidence of AD risk and faster AD progression, and the hormone milieu in females has been related to many outcome variables related to AD, the hormone milieu in males might also be related to disease progression. In humans, there is a marked decrease in estrogens in women, and in testosterone in men, with AD as compared to non-AD controls, indicating a potential role for sex hormones to modulate disease progression in women and men (Barron & Pike, 2012; Callahan et al., 2001). Androgens, such as testosterone, can protect against heat shock-induced hyperphosphorylated tau independently of estrogens (S. C. Papasozomenos, 1997). Further, castration impaired spontaneous alternation behavior and increased beta-amyloid levels, which was attenuated by administration of testosterone, in 3xTG-AD rats compared to sham controls (Rosario et al., 2006, 2010, 2012). Increasing endogenous androgens by blocking aromatase decreased ratios of beta-amyloid42/beta-amyloid 40, deposition of beta-amyloid plaques, and improved cognition in APP23 mice (McAllister et al., 2010).

Summary and Dissertation Goals

Fluctuations in the endocrine system across the lifespan, with prominent attention in middle-age, can impact a myriad of other systems to, in turn, affect brain and cognitive outcomes. Clinical research has been able to identify directions for avenues of investigation. However, additional preclinical research is needed to ask targeted questions concerning the mechanisms of action in both healthy and neurodegenerative aging. With a focus on addressing differences in menopause etiology and therapeutic hormone administration, this dissertation seeks to understand effects and interactions of differing menopause models, progestogen-based hormone administration, and AD pathology and symptomatology. Chapter 2 assesses progestogen administration given during the transition to menopause utilizing the VCD model of follicular depletion, testing effects on spatial working and reference memory and on the GABAergic system. Chapter 3 evaluates gonadal hormone deprivation in both females and males of the TgF344-AD rat model for effects on AD-like pathology, spatial working and reference memory, precision based spatial navigation, and anxiety-like behavior. Chapter 4 seeks to build on this understanding of gonadal hormone deprivation in females and provide understanding of a different model of menopause: the VCD model of follicular depletion in the TgF344-AD rat model for effects on spatial working and reference memory, anxiety-like behavior, and AD-like pathology. The outcomes of these chapters highlight the necessity of modeling menopause in the rodent for a translational assessment on neurobiological and cognitive outcomes in aging females. The data herein not only provide an understanding of the mechanisms of action within the brain that are associated with cognition, but also

provide an opportunity to explore avenues of intervention for therapeutic approaches for both healthy and neurodegenerative aging.

CHAPTER 2

PROGESTOGENS IMPACT COGNITION DURING THE TRANSITION TO

MENOPAUSE IN THE RAT: DISSOCIATION OF PROGESTOGEN- AND

MEMORY- TYPE

ABSTRACT

Progestogens, such as progesterone (P4), medroxyprogesterone acetate (MPA), or

micronized progesterone (mP4), can be given to uterus-intact women during the

menopause transition. Both P4 and MPA administration have been shown to be

detrimental to cognition and affect the GABAergic system in surgically menopausal,

ovariectomized (Ovx) rats. mP4, however, has yet to be investigated for cognitive effects

preclinically, despite widespread clinical use. Given that preclinical investigations in

cognitive effects of menopause-related progestogens have thus far been limited to models

of surgical menopause via ovariectomy, we evaluated the cognitive impact of chronic P4,

MPA, or mP4 treatment in an ovary-intact transitional menopause rat model. Hormone

administration began during ovarian follicular depletion, modeling the clinical scenario

when women take progestogens alone. Results indicated that P4 and MPA improved

working memory during learning, while MPA impaired working memory retention.

Additionally, transitional menopause impaired reference memory during learning and

memory retention compared to vehicle controls; progestogens did not prevent this

impairment. Western blot analysis revealed no treatment differences for GAD65+67, the

rate limiting enzyme for GABA production. GAD65+67 expression in progesterone-

treated rats correlated with increased working memory errors. Increases in progesterone

were found in progesterone and micronized progesterone treated rats, while

androstenedione levels were consistently low in MPA-treated rats. Additionally, for VCD-only treated rats, all ovarian follicles and corpora lutea declined, except for primary follicles which increased. In summary, the collective findings show that P4 and MPA elicit divergent effects, whereby in the current study we show that they can improve cognition when given early in follicular depletion in an ovary-intact model, while other studies demonstrate that these progestogens can impair cognition in a surgically menopausal Ovx model. Further investigation into progestogens is warranted to fully understand their impact on cognition and to detail parameters within menopause variations.

INTRODUCTION

Menopause occurs at the average age of 52, and is clinically defined as one year of missed periods (NAMS, 2015). During the natural transition to menopause women experience irregular menstrual cyclicity and fluctuations in ovarian hormones, culminating in a substantial loss of ovarian hormones, including estrogens and progesterone, due to ovarian follicular depletion (Burger et al., 2008; Harlow & Paramsothy, 2011). During this perimenopausal timeframe, progestogens, which include both progesterone and its synthetic analogs, known as progestins, are sometimes prescribed alone to women to combat heavy uterine bleeding as well as other symptoms (Bender 2022). Progesterone (P4) has been methodically assessed in preclinical models for effects on the brain and cognition. Progesterone and some of its metabolites have been shown to be neuroprotective in counteracting damage due to ischemic stroke, traumatic brain injury, and experimentally-induced excitotoxicity in the hippocampus in rodent models (Shear et al., 2002; Wali et al., 2014; Amirkhosravi et al., 2021). Progesterone administration has mixed effects on cognition as evaluated in healthy female rodents. In young, middle-aged, and aged ovariectomized (Ovx, whereby the ovaries are surgically removed) mice, acute (i.e., hours to days) progesterone treatment improved non-spatial memory and memory consolidation on an object recognition task, compared to vehicle controls (Harburger et al., 2008; M. C. Lewis et al., 2008). In contrast, our laboratory has demonstrated that chronic (i.e., weeks to months) progesterone administration in middle-aged Ovx rats impairs spatial working memory (Braden et al., 2015). For example, when chronic progesterone was given in combination with 17β-estradiol (E2; the most potent endogenous circulating estrogen in

mammals) it attenuated the beneficial effects of E2 alone on spatial reference memory (Bimonte-Nelson et al., 2006).

Medroxyprogesterone acetate (MPA), a synthetic acetylated pregnane (Stanczyk, 2003), is administered in women during the menopausal transition to alleviate undesired physiological symptoms, such as heavy uterine bleeding (Bender 2022), and is available in the clinic as a contraceptive under the brand name Depo-Provera as well as generically (Depo-Provera Prescribing Information, 2006; Eugia Product Portfolio, 2023). A handful of preclinical studies have evaluated the impact of MPA on the brain and cognition. MPA has been shown to disrupt degradation of the beta-amyloid protein, a key protein in Alzheimer's disease pathology, in rat glial cells (Porter et al., 2020). Additionally, MPA has anti-inflammatory effects in a demyelination mouse model and decreased demyelination compared to age-matched controls (Mohammadi et al., 2021). MPA alone has been demonstrated by our and other laboratories to have negative effects on spatial memory in Ovx rats compared to vehicle controls (Braden et al., 2017; Okojie & Oyekunle, 2014) and on a novelty memory task in young ovary-intact rats (Okojie & Oyekunle, 2014). The MPA-induced cognitive impairment is likely non-reversible, as Ovx rats tested four months after the cessation of chronic MPA treatment exhibited cognitive impairment compared to age matched vehicle controls (Braden et al., 2011). When combined with E2, MPA treatment improved performance on an alternating T-maze and impaired spatial reference memory on the Morris water maze (MWM) in Ovx rats (Chisholm & Juraska, 2012; Lowry et al., 2010). These findings suggest that MPA alone can impair cognition, with research to date focusing on effects in surgical menopause models.

Women can also be prescribed micronized progesterone (mP4) to combat physiological symptoms associated with perimenopause, including endometrial hyperplasia and secondary amenorrhea (Gillet et al., 1994; Stute et al., 2016). As the more orally bioavailable form of progesterone due to reduced particle size and increased solubility, mP4 is identical in chemical structure to P4; mP4 presumably acts upon similar neurobiological pathways as P4, although the literature is unclear if micronization impacts the pharmacokinetics and/or pharmacodynamics of progesterone (De Lignières, 1999; Maxson & Hargrove, 1985). Administration of micronized progesterone combined with conjugated equine estrogens (CEE) has been shown to improve working memory in menopausal and post-menopausal women (Gleason et al., 2015; Miller et al., 2009; Ryan & Rosner, 2001; Sherwin & Grigorova, 2011), while others demonstrate no cognitive effects when in combination with either CEE or E2 (Gleason et al., 2015; Miller et al., 2009). The role of micronized progesterone in the cognitive outcomes observed in these studies is unclear, as there have been no systematic preclinical or clinical studies evaluating micronized progesterone alone on cognition.

Preclinical assessment of progestogen effects on cognition has been limited to models of surgical menopause via Ovx, which removes the primary source for ovarian hormones, yielding an abrupt, drastic decrease in circulating ovarian hormone levels. Over 80% of women do not undergo surgical menopause and instead undergo a naturally occurring transitional menopause during which there is ovarian follicular depletion (NAMS, 2014). The 4-vinylcyclohexene diepoxide (VCD) model is used to induce ovarian follicular depletion of primary and primordial follicles via accelerated atresia in the female rodent, and produces a circulating ovarian hormone profile which is more similar to a

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woman undergoing transitional menopause than that of the Ovx model (Hoyer, Devine, Xiaoming, et al., 2001; S. W. Kao et al., 1999; L. Springer et al., 1996). Further, the VCD model allows for targeted timing of the menopause transition; the Ovx model lacks a transition stage, while with the VCD model, hormones can be administered during the transition to menopause and scientists can now target perimenopausal timepoints. Assessing exogenous progestogen administration in the VCD model of follicular depletion will help determine these clinically-relevant hormone interactions and their effects on cognition.

Progestogens have been shown to impact the inhibitory γ-aminobutyric acid (GABA)-ergic system and there is increasing evidence that progesterone and MPA can modulate progestogen-induced memory changes. Indeed, studies have shown that in Ovx rats, MPA administration alters glutamic acid decarboxylase (GAD, the GABA synthesizing enzyme) protein and mRNA levels, MPA increases inhibitory tone in the dentate gyrus, memory scores after MPA treatment correlated with GAD levels in the dorsal hippocampus, and the detrimental effects of progesterone on memory were reversed by administration of the $GABA_A$ receptor antagonist bicuculline (Belelli & Herd, 2003; Braden et al., 2010, 2011, 2015). These studies been primarily conducted in the surgical menopause model, and progestogen-based treatments have yet to be assessed for effects during follicular depletion. Understanding the role of a wide variety of clinically used progestogens on the GABAergic system, and extending questions to the transition to menopause, is important since chronic progestogen administration on a surgical menopause background has demonstrated impairing effects on cognition that were reversed by blocking the GABAergic system. Additionally, it cannot be assumed that hormone

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administration outcomes will translate across varied menopause etiologies (Acosta et al., 2010). As such, the goal of this experiment was to systematically investigate the effects of MPA, micronized progesterone, and progesterone on cognition and the GABAergic system during the menopausal transition.

METHODS

Subjects

Fifty six-month-old, female, non-breeder, Fischer-344-CDF rats were obtained from the National Institute on Aging (NIA), Charles River Laboratories (Raleigh, NC). Animals were pair-housed and fed *ad libitum* for two weeks before the study began. Animals were placed on a 12-hour on/off light-dark cycle, and were treated in compliance with the Arizona State University Institutional Animal Care and Use Committee protocol. All procedures adhered to the standards provided by the National Institutes of Health.

VCD Administration

Rats were administered either VCD (160mg/kg/day, in 47%/47% dimethyl sulfoxide (DMSO)/saline, 6% VCD, n=40) (SenesTech Inc., Flagstaff, AZ) or vehicle solution (50%/50% DMSO/saline, n=10) (Sigma-Aldrich, St. Louis, MO) via intraperitoneal (i.p.) injection in accordance with published protocols (Acosta et al., 2009, 2010; Flaws et al., 1994b; Koebele et al., 2017; L. Springer et al., 1996). VCD injections began two weeks after arrival and occurred four times a week (every Monday, Tuesday, Thursday, and Friday) for six weeks. One animal died during VCD injections due to a medical condition unrelated to VCD treatment; thus, 49 rats completed the study. The first day of VCD injections was considered day 1 of the study; the rest of the timeline will be referred to as days from the first injection of VCD (See Fig. 1 for study timeline).

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Progestogen Administration

Daily subcutaneous injections began on day 52, as previous research has indicated that at this timepoint, progesterone levels are similar to vehicle controls for young and middle-aged rats administered VCD (Koebele et al., 2017). Rats received either progesterone (P4) (0.7mg/rat/day in 0.3 ml sesame oil, n=10), micronized progesterone (mP4) (0.7mg/rat/day in 0.3 ml sesame oil, n=9), medroxyprogesterone acetate (MPA) (0.7mg/rat/day in 0.3 ml sesame oil, n=10), or vehicle (0.3 ml sesame oil, n=20). Progestogen treatments were only administered to rats who had received VCD injections yielding three treatment groups that received a progestogen: VCD-P4, VCD-mP4, VCD-MPA. Two groups were given sesame oil vehicle injections as a control for the hormone yielding two different control groups: VCD-Vehicle, Vehicle-Vehicle. Therefore, the VCD-Vehicle was given VCD and then sesame oil (vehicle for progestogen injections) and the Vehicle-Vehicle group was given DMSO (vehicle for VCD treatment) and then sesame oil (vehicle for progestogen injections). For each of the five treatment groups, the wording before the hyphen is what was given relative to the VCD manipulation and the wording after the hyphen is what was given relative to the progestogen administration. The doses of MPA and P4 used herein have been shown by our laboratory to impair spatial working memory in Ovx rats (Braden et al., 2015, 2017). The dose for mP4 was chosen to correspond with the P4 dose so direct comparisons could be made. Daily injections continued until euthanasia at day 132.

Vaginal Smears

Vaginal smears occurred for 10 consecutive days, beginning two weeks before behavioral testing. Each smear was determined to be in either the metestrus, diestrus, proestrus, or estrus phase as indicated in previous protocols (Goldman et al., 2007).

Water Radial Arm Maze (WRAM)

Rats began behavioral testing 111 days after receiving the first injection of VCD, and 59 days after the start of hormone injections. The WRAM is a water-escape task used to assess spatial working and reference memory performance. The maze consisted of eight arms (38.1cm x 12.7cm) radiating from a circular center. Robust visual cues were in the room to assist with spatial navigation. Four of the eight arms contained hidden platforms (10 cm diameter) 2 cm below the surface of the water (18-20°C) which contained non-toxic black paint for opacity. Platforms were kept in consistent locations at the beginning of each testing day for each rat but were semi-randomized between rats.

Rats were tested for 12 consecutive days, with four trials each day. The start arm remained constant for all rats and trials. At the start of the first trial, rats were placed in the start arm and were allowed to swim freely in the maze until they found a platform or until 3 minutes had elapsed, after which rats were led to the closest platform. After rats found a platform, they were kept there for a total platform time of 15 seconds and then were removed and placed in their heated testing cage for an inter-trial interval (ITI) of 30 seconds. During this ITI, the just-found platform was removed and the maze was cleaned to remove debris and distribute olfactory cues. Because of this there were four trials per day (one to represent each platform) and as trials progressed, working memory load increased since rats had to sustain more items in their working memory. Errors were

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quantified as entry into an arm that did not have a platform; arm entries occurred when a rat's snout passed 11 cm into an arm. Performance was assessed as the quantification of total errors, and was further broken into three separate error types: working memory correct (WMC), reference memory (RM), and working memory incorrect (WMI). WMC errors were entries into an arm that previously contained a platform for that day. RM errors were the first entry into an arm for that day that never contained a platform. WMI errors were subsequent entries into an arm that never contained a platform. On the last day of testing, day 13, a six-hour delay was implemented between trial 2 and trial 3 to assess delayed memory retention.

Morris Water Maze (MWM)

The MWM is a water escape task that is used to assess spatial reference memory performance. The maze consisted of a circular pool that was 188 cm in diameter. A platform (10 cm diameter) was hidden 2 cm below the surface of the water (18-20°C), with non-toxic black paint for opacity, in the north-east quadrant of the maze. Robust visual cues were situated on adjacent walls to assist with spatial navigation. A camera was mounted above the maze and the computer program Ethovision 10 (Noldus Instruments, Wageningen, Netherlands) was used to capture video and track the rat's path through the maze.

Rats were tested for five consecutive days, with four trials each day. At the start of the first trial, animals were dropped off at a quadrant (north, south, east or west) semi-randomly assigned to each trial throughout each of the days. Once in the maze, rats were allowed to swim freely until they found the platform or until one minute had elapsed, after which rats were then led to the platform. Rats were allowed to stay on the platform for 15

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seconds, after which time the rat was returned to their testing cage for an 8±2 minute ITI. On Day 5, the last day of testing, after all test trials, a probe trial was introduced to assess whether the animals had spatially localized the position of the platform. Before the probe trial began the platform was removed and rats were allowed to swim freely in the maze for one minute after which they were returned to their testing cages. Distance to platform (cm) from the drop-off point was recorded and used to assess spatial localization of the platform over the testing period of five days.

Visible Platform

The visible platform test was used to assess motor and visual acuity for solving a water escape task. The maze consisted of a rectangular pool (100 x 60 cm) filled with clear water kept at 18-20°C with a platform situated inside the maze (10 cm diameter) protruding 4 cm above the water. The maze was encircled by a curtain so that there were no salient visual cues other than those found inside the maze.

Rats were tested for one day with six trials. At the start of trial 1, animals were placed in the starting position that remained constant throughout trials. The rats were allowed to swim freely until they found the platform or until 90 seconds had elapsed, after which the rats were led to the platform. After the rats found the platform, they were allowed to stay on it for 15 seconds after which the tester returned rats to their testing cage for an ITI of 8±2 minutes. All rats were tested for trial 1 before moving on to trial 2. Platform locations were assigned semi-randomly across trials. Latency (s) to the platform was measured.

Euthanasia

Rats were euthanized over two days, at the end of behavioral testing. Final body weights (g) were obtained earlier in the day directly before daily injections on the first day of sacrifices. Rats were anesthetized via isoflurane, and cardiocentesis was performed for blood collection. Rats were then decapitated and brains removed for dissection. The Rat Brain Atlas was utilized for reference for brain dissections via plate designations (Paxinos & Watson, 1998). Six separate brain regions were raw brain dissected in the right frontal cortex (plates 5-14), basal forebrain (plates 15-17), right dorsal hippocampus (plates 33-35), right perirhinal cortex, right entorhinal cortex, and right CA1/CA2 region of the hippocampus (plates 39-42) directly after removal. Tissue was weighed immediately and stored at -70°C until further analysis.

Uterine Horn and Ovarian Weights

Uterine horns and ovaries were dissected and trimmed of excess fat. Ovaries were cut from the tips of the uterine horns and separately weighed as left and right wet weights (g). Ovaries were placed in 10% formalin for two days, and then switched to 70% ethanol until future analysis. Uterine horn wet weights (g) were also collected. For analysis purposes, uterine horn wet weight (g) was divided by final body weight (g) to account individual weight differences between rats.

Serum Hormone Analyses

P4 and androstenedione serum hormone levels were determined by the FYXX Foundation (Flagstaff, AZ). P4 levels were determined by ELISA (Cat# IB79183 IBL-America, Minneapolis, MN, United States). All samples were run in duplicate. The inter-assay coefficient average was 7.48% with an average value of 16.68 ng/ml. The

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progesterone assay had a functional sensitivity of 0.144 ng/ml. Intra-assay coefficient average was 7.97% for this ELISA kit. Androstenedione levels were also determined by ELISA (Cat # 11-ANRHU-E01, ALPCO, Salem, NH, United States). The inter-assay coefficient average was 4.56% with an average value of 0.50 ng/ml. The intra-assay coefficient average was 7.28% for this ELISA kit. The androstenedione assay had a functional sensitivity of 0.1 ng/ml.

E2 and MPA serum hormone levels were determined by the Wisconsin National Primate Research Center at the University of Wisconsin in Madison, WI. Serum samples (500 µL) for analysis of MPA and E2 were extracted using the following protocol. All subjects were included for E2 analysis (n = 49). All MPA-treated rats were assayed for MPA serum hormone levels. In addition, as a negative control, two randomly selected rats from all non-MPA-treated groups (Vehicle-Vehicle, VCD-Vehicle, VCD-P4, VCD-mP4) were assayed for levels of MPA in serum (n = 18). Unknowns, standards and quality control samples were diluted in 500 µL of ultrapurified bottled water (Fisher Scientific). Internal standard (d6-MPA, d5-E2) was added to all samples. One milliliter of methyl tert butyl ether (Fisher Scientific) was then added, vortexed vigorously, and incubated at room temperature for 5 min. The organic phase was transferred into a new tube, evaporated to dryness, and resuspended in ethanol and water. A second liquid–liquid extraction was performed with dichloromethane (Fisher Scientific) and the organic phase was evaporated to dryness. Samples were then resuspended in $NaHCO_3$ buffer and E2 was derivatized with dansyl chloride and transferred into minivials for LC/MS/MS analysis.

Samples were analyzed on a QTRAP 5500 quadruple linear ion trap mass spectrometer (Sciex) equipped with an electrospray ionization source operated in positive mode. The system included two Shimadzu LC20ADXR pumps and a Shimadzu SIL20ACXR autosampler. A sample of 10 µL was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100×2.1 mm column (Phenomenex) for separation using a mobile phase: water with 0.1% formic acid (Solution A) and acetonitrile with 0.1% formic acid (Solution B), at a flow rate of 200 µl/min with a gradient method from 45% to 98% B over 8 min. Quantitative results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each compound and internal standard. The concentrations for the calibration curve ranged from 0.195 to 25 ng/mL for MPA and 0.0012 to 0.5 ng/mL for E_2. The linearity was $r > 0.9990$ and the curve fit was linear with 1/x weighting. Neither MPA or E_2 were detected in blank or double blank samples. All samples were analyzed in one run and the intra-assay CV ranged from 2.3-4.7%.

Ovarian Follicle Counts

One ovary was randomly selected from each treatment group for histological analysis. The presence of healthy primordial, primary, secondary, and antral follicles, as well as corpora lutea in the ovaries were quantified. Ovaries were paraffin embedded, sectioned at 5µm, mounted (every 10th section), and stained with hematoxylin and eosin Y/phyloxine B. At every 20th section, all follicle types were counted at x20 magnification. Corpora lutea were counted at x10 magnification (3D HisTech DESK Scanner, Budapest, Hungary). In order to count total number of follicles per ovary, we used the following equation: $N_t = (N_0 \times S_t \times t_s)/(S_0 \times d_0)$. N_t is the total approximate number of follicles in the

ovary, N_0 is the number of follicles detected in the ovary, S_t is the number of sections per ovary, t_s is the thickness of each section (μm), S_0 is the number of observed sections per ovary, and d_0 is the mean diameter of the ovary nucleus (Gougeon & Chainy, 1987). Ovary classification was determined by previous protocol (Haas et al., 2007; Koebele et al., 2017).

Western Blot

GAD 65 and GAD 67 expression levels were assessed from the right hemisphere in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex and entorhinal cortex via Western Blot analysis. Samples were homogenized via probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA) in a 1:50 RIPA buffer (1% Triton X-100, 150mM NaCl, 0.5% Na deoxycholate, 0.1% SDS, 50mM Tris, phosphatase inhibitor [Cat#: 524625, Millipore-Sigma], and protease inhibitor [Cat#: 5892791001, Millipore-Sigma]). Samples were then centrifuged for 10 minutes at 10,000 rpm at 4°C. A BCA (bicinchoninic acid) protein assay (ThermoFisher Scientific, Pittsburgh, PA, USA) was utilized to measure protein concentration. Samples were run on a 4-12% Bis-Tris NuPAGE gel using a SureLock Mini-Cell system (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Immobulin-P). Samples were loaded at 10μg of protein per brain region. Treatment groups were counterbalanced across gels and four gels were run per brain region. After transfer, the membrane was blocked in 5% non-fat milk for one hour after which it was incubated overnight at 4°C in primary antibodies anti-GAD 65 (1:5000, Abcam) and anti-GAD 67 (1:10,000, Abcam) as well as primary antibody anti-beta actin (1:20,000, Cell Signaling) as a control measure. The membrane was then incubated in secondary antibodies anti-mouse horseradish peroxidase (1:2000, Cell Signaling) and anti-rabbit horseradish peroxidase (1:2000, Cell Signaling) at

room temperature for one hour. Chemoluminescence (LumiGlo and Peroxide, Cell Signaling) was used to visualize protein expression using a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). ImageJ software was used to perform densitometry. GAD 65 and GAD 67 were analyzed together in accordance with a previous protocol from our laboratory (Braden et al., 2010, 2011). GAD 65+67 levels were normalized to the loading control beta-actin and then to the dual vehicle samples for analysis.

Statistical Analyses

WRAM data were analyzed across Days 2-12 using an omnibus repeated measures analysis of variance (ANOVA) for each of the error types (WMC, WMI, and RM, and Total errors). The repeated measures consisted of Trials nested within Days while the independent variable was Treatment. Data were further separated into three distinct blocks for Days 2-5 (the early acquisition phase), Days 6-9 (the late acquisition phase), and Days 10-12 (the asymptotic phase) and each block was individually assessed via an omnibus repeated measures ANOVA for each of the error types, as previously reported (Prakapenka et al., 2018). Effects of treatment on Day 12 alone were also assessed as previous research in our laboratory has shown impairments after MPA administration in Ovx rats on the last day of WRAM testing (Braden et al., 2010, 2017). Additionally, we compared performance amongst groups for the highest (Trial 4) working memory load as our laboratory has previously shown that effects of several hormone-altering treatments become apparent when working memory load is highly taxed (Bimonte & Denenberg, 1999; Braden et al., 2010; Koebele et al., 2017). For the delay, for each treatment group separately, trials 3 and 4 from baseline (day 12) and postdelay (day 13) were averaged and compared as has been

previously reported (Camp et al., 2012; Engler-Chiurazzi et al., 2011; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018).

MWM total swim distance data were analyzed using an omnibus repeated measures ANOVA for all days of testing. The repeated measures consisted of Trials nested with Days, and the independent variable was Treatment. Additionally, the probe trial was analyzed by assessing percent distance swim distance in the target quadrant (NE quadrant) where the platform had been previously located and in the opposite quadrant (SW quadrant) to assess if the rats had correctly spatially localized the platform location for each treatment group.

Visible platform data were assessed using a repeated measures ANOVA where time to platform was the dependent variable. The repeated measures were Trials and the independent variable was Treatment.

Uterine horn data were assessed using an omnibus ANOVA where wet weight (g) adjusted to final body weight (g) was the dependent variable and Treatment was the independent variable.

Serum hormone data were assessed using an omnibus ANOVA were serum hormone levels for each hormone evaluated (progesterone, androstenedione, and E2) were the dependent variables and Treatment for the independent variable. MPA descriptive statistics were obtained for all samples that had detectable levels. One sample from the VCD-mP4 treatment group was excluded from the androstenedione analysis because the coefficient of variance was above 15% (CV = 29.13%). Samples that were below the quantifiable range for the E2 analysis (0.0024 pg/ml) were set a placeholder of 0.0023 pg/ml for all E2 analyses.

Ovarian follicle data were assessed using an omnibus ANOVA where follicle count for each follicle type was the dependent variable and Treatment was the independent variable.

One-way ANOVAs were utilized to assess GAD 65+67 protein levels in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex, and entorhinal cortex. GAD 65+67 levels were normalized to the dual vehicle treatment data. The independent variable was Treatment. Pearson r correlations were analyzed for GAD 65+67 in each brain region for WMC and WMI errors for Days 2-5 and Days 10-12 of WRAM data where significant main effects of treatment were found. We used a false discovery rate (FDR) limit of 0.1 to protect against multiple correlations. Both FDR-corrected (Q) and uncorrected (p) values are reported (Benjamini & Hochberg, 1995).

RESULTS

WRAM

The omnibus ANOVA for Days 2-12, including all trials, revealed a main effect of Day for WMC, WMI, RM, and Total errors [WMC, $F(10, 880) = 6.283$, $p < 0.001$; WMI, $F(10, 1320) = 24.652$, $p < 0.001$; RM, $F(10, 1320) = 12.430$, $p < 0.001$; Total, $F(10, 1290) = 15.238$, $p < 0.001$], indicating that all animals learned the task across Days 2-12. There was also a main effect of Trial for Days 2-12 for WMC, WMI, RM, and Total errors [WMC, $F(2, 880) = 610.278$, $p < 0.001$; WMI $F(3, 1320) = 154.272$, $p < 0.001$; RM $F(3, 1320) = 25.594$, $p < 0.001$; Total, $F(3, 1290) = 249.703$, $p < 0.001$], with errors escalating as trials and working memory load increased.

For Days 2-5, there was a main effect of Treatment for WMI errors [$F(4, 44) = 2.740$, $p < .05$] as well as RM errors [$F(4, 44) = 3.393$, $p < 0.05$] (See Fig. 2B). Fisher's

post-hoc analyses revealed that for WMI errors, VCD-Vehicle rats made more errors than Vehicle-Vehicle rats [$p < 0.05$], and that VCD-treated rats given MPA and P4 made fewer errors than VCD-treated rats that did not receive hormone administration [VCD-Vehicle *vs.* VCD-MPA, $p < 0.01$, VCD-Vehicle *vs.* VCD-P4, $p < 0.05$], indicating that VCD-induced follicular depletion impaired working memory and that MPA or P4 treatments attenuated this impairment. For RM errors during this testing phase, Fisher's post-hoc analyses revealed that VCD-treated rats given mP4 or P4, and VCD-treated rats that did not receive hormone administration (the VCD-Vehicle group), each made more errors than non-follicle deplete ovary-intact rats receiving dual vehicle (the Vehicle-Vehicle group) [Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$] (see Fig. 2C). Additionally, VCD-treated rats given MPA tended to make more errors than rats given dual vehicle [Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.1$], indicating an impairment with VCD-induced transitional menopause, an effect that was not prevented by the progestogen treatments. There were no effects of Treatment for Days 6-9 for any error type.

For Days 10-12 there was a main effect of Treatment for RM errors [$F(4, 44) = 2.889$, $p < 0.05$] (See Fig. 2). Fisher's post-hoc analyses revealed an impairment of VCD-induced follicular depletion (VCD-Vehicle) compared to dual vehicle treatment (Vehicle-Vehicle); RM was not further impacted by any of the progestogen treatments, as each VCD- plus progestogen- treated group differed from the dual vehicle treatment [Vehicle-Vehicle *vs.* VCD-mP4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, Vehicle-Vehicle *vs.* VCD-P4, $p < 0.05$, Vehicle-Vehicle *vs.* VCD-Vehicle, $p < 0.01$], but not the VCD-Vehicle group (See Fig. 2F).

As we have demonstrated impairments with MPA treatment on the last day of WRAM testing (Braden et al., 2010, 2017), we assessed Day 12 alone. On Day 12, for all trials, there was a Trial x Treatment interaction for WMI errors [$F(4, 44) = 1.882, p < 0.05$] (Fig. 3). Analysis of Trial 4 alone, the highest working memory load trial, revealed a main effect of Treatment [$F(4, 44) = 2.638, p < 0.05$]. Fisher's post-hoc analyses demonstrated an impairment induced by MPA administration relative to all other treatments on Trial 4 during the last day of testing [Vehicle-Vehicle *vs.* VCD-MPA, $p < 0.01$, VCD-Vehicle *vs.* VCD-MPA, $p < 0.05$, VCD-MPA *vs.* VCD-P4, $p < 0.05$, VCD-MPA *vs.* VCD-mP4, $p < 0.05$].

For Day 13, when a six-hour delay was given between trials 2 and 3, there were no main effects of Day for any error type between baseline trials and postdelay trials (data not shown), suggesting that all groups were able to retain baseline working memory performance after a six-hour delay period.

MWM

Swim distance to the platform decreased across days [Main effect of Day: $F(4, 44) = 75.750, p < 0.001$] (See Fig. 3A), indicating that all groups were able to learn the task. There was no main effect of Treatment nor any significant Treatment x Day interactions, indicating that there were no differences between treatment groups across days for reference memory performance. For the probe trial, there was a main effect of Quadrant such that there was a higher percent swim distance in the target quadrant (NE quadrant), which had previously contained the platform, than the opposite quadrant (SW quadrant), indicating spatial localization of the platform [$F(1, 44) = 361.118, p < 0.0001$] (See Fig. 3). There was a main effect of Quadrant for each treatment group, [Vehicle-Vehicle: $F(1,$

10) $= 69.574$, $p < 0.001$; VCD-Vehicle: $F(1, 10) = 48.576$, $p < 0.001$; VCD-MPA: $F(1, 10)$ $= 55.364$, $p < 0.001$, VCD-P4: $F(1, 10) = 57.829$, $p < 0.001$; VCD-mP4 $F(1, 9) = 49.397$, $p < 0.001$] where each treatment group swam a significantly higher percentage of distance in the target quadrant (NE) where the target used to be located versus the opposite quadrant (SW), indicating that treatment groups were able to correctly spatially localize to the platform.

Visible Platform

The visible platform test was used to assess the procedural components of a water escape task. There was a main effect of Trial [$F(5, 44) = 8.479$, $p < 0.0001$] such that as trials progressed latency to the platform decreased, indicating better performance as trials progressed with an average latency of 7.55 seconds +/- 0.417 to reach the platform across all trials (See Fig. 4). There was no main effect of Treatment nor a significant Trial x Treatment interaction, indicating that the groups did not differ in their ability to perform the procedural components of a water-escape task.

Vaginal Smears

Across all days of vaginal smears, vaginal cytology was mixed (primarily metestrus or persistent diestrus) for all VCD-treated rats. These results indicate that ovarian cyclicity was halted for VCD-treated rats. Cytology was not affected by progestogen treatment. Further, non-follicle deplete Vehicle-Vehicle treated rats displayed vaginal cytology that was consistent with a 4-5 day estrous cycle, indicating that this group had normal ovarian cyclicity.

Uterine Horn Weights

For uterine horn weights, there was a main effect of Treatment [$F(4, 44) = 7.811$, $p < 0.001$] (See Fig. 5). Fisher's post-hoc analyses revealed that the VCD-induced follicular depletion decreased uterine horn weight compared to dual vehicle controls [VCD-Vehicle *vs.* Vehicle-Vehicle, $p < 0.05$]. Additionally, all three progestogen-treated groups had uterine horns that weighed less than the dual vehicle group [VCD-MPA *vs.* Vehicle-Vehicle, $p < 0.01$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.01$].

Serum Hormone Levels

For the progesterone assay, there was a significant main effect of Treatment [$F(4,44) = 21.263$, $p < 0.001$] (See Fig. 6). Fisher's post-hoc analyses revealed that VCD-treated rats given P4 and mP4 had higher progesterone levels than all other treatment groups [VCD-P4 *vs.* VCD-MPA, $p < 0.001$, VCD-P4 *vs.* VCD-Vehicle, $p < 0.001$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.01$, VCD-mP4 *vs.* VCD-MPA, $p < 0.001$, VCD-mP4 *vs.* VCD-Vehicle, $p < 0.001$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.01$] (See Fig. 6A). Additionally, MPA treatment decreased progesterone levels compared to dual vehicle controls [VCD-MPA *vs.* Vehicle-Vehicle rats, $p < 0.001$]. For the androstenedione assay, there was a significant main effect of Treatment [$F(4,43) = 22.255$, $p < 0.001$] (See Fig. 6B). Fisher's post-hoc analyses revealed that VCD-treated rats given P4 and mP4 had higher androstenedione levels than all other treatment groups [VCD-P4 *vs.* VCD-MPA, $p < 0.001$, VCD-P4 *vs.* VCD-Vehicle, $p < 0.001$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-mP4 *vs.* VCD-MPA, $p < 0.001$, VCD-mP4 *vs.* VCD-Vehicle, $p < 0.001$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.001$]. Additionally, MPA treatment decreased androstenedione

levels compared to rats with VCD-induced transitional menopause that did not receive hormone administration [VCD-MPA *vs.* VCD-Vehicle rats, $p < 0.05$]. Follicular depletion without hormone administration increased androstenedione levels compared to dual vehicle [VCD-Vehicle *vs.* Vehicle-Vehicle, $p < 0.05$]. For the E2 assay, there were no significant differences among treatment groups [$F(4,44) = 1.785$, $p > 0.05$] (See Fig. 6C). Of note, all MPA-treated rats had undetectable levels of E2. For the MPA assay, all MPA-treated rats demonstrated detectable levels of MPA ($M = 32.05$ ng/ml, $SE = 2.66$), which are similar to those previously reported (Braden et al., 2011), while all other treatment groups had undetectable levels of MPA (as assessed by randomly choosing two samples per non-MPA group).

Ovarian Histology

For ovarian histology, there was a main effect of Treatment for primordial [$F(4,43) = 26.163$, $p < 0.001$], primary [$F(4,43) = 17.889$, $p < 0.001$], secondary [$F(4,43) = 114.415$, $p < 0.001$], and antral [$F(4,43) = 18.990$, $p < 0.001$] follicles, as well as for corpora lutea [$F(4,43) = 50.057$, $p < 0.001$; See Fig. 7]. For primordial follicles, Fisher's post-hoc analyses revealed that dual vehicle rats had more follicles and corpora lutea than all other treatment groups [primordials: $p < 0.001$, antrals: $p < 0.001$, secondaries: $p < 0.001$, corpora lutea: $p < 0.001$]. For primary follicles, Fisher's post-hoc analyses revealed that VCD-Vehicle, VCD-P4, and VCD-mP4 -treated rats had more primary follicles than Vehicle-Vehicle and VCD-MPA -treated rats [VCD-Vehicle *vs.* VCD-MPA, $p < 0.001$, VCD-P4 *vs.* VCD-MPA, $p < 0.001$, VCD-mP4 *vs.* VCD-MPA, $p < 0.001$, VCD-Vehicle *vs.* Vehicle-Vehicle, $p < 0.001$, VCD-P4 *vs.* Vehicle-Vehicle, $p < 0.01$, VCD-mP4 *vs.* Vehicle-Vehicle, $p < 0.01$]. Additionally, VCD-MPA treated rats had more corpora lutea than VCD-Vehicle-

and VCD-P4- treated rats [VCD-MPA *vs.* VCD-Vehicle, $p < 0.05$, VCD-MPA *vs.* VCD-P4, $p < 0.05$].

Western Blot Analysis

Western blots were performed to assess GAD 65 and 67 protein expression in the frontal cortex, dorsal hippocampus, basal forebrain, ventral hippocampus, perirhinal cortex and entorhinal cortex. There were no Treatment effects for GAD 65+67 for any region (data not shown). Using an FDR of 0.1 to account for multiple correlations, a significant relationship was revealed for cognitive performance in the VCD-treated rats given P4, whereby higher levels of GAD 65+67 were associated with worse performance on the WRAM (Benjamini & Hochberg 1989). Specifically, in the ventral hippocampus, there was a significant positive correlation between GAD 65+67 expression and Days 2-5 WMC errors [$r(9) = 0.804$, $p < 0.01$, $Q < 0.1$, Fig. 8], suggesting that the P4-treated rats that had higher GAD 65+67 levels in the ventral hippocampus tended to make more WMC errors during the learning phase of WRAM testing. There were no significant correlations between cognitive performance and GAD 65+67 expression for any other treatment groups for any brain region assessed.

<center>DISCUSSION</center>

The current study is the first to preclinically investigate the effects of progestogens on cognition during the transition to menopause, when follicle depletion is ensuing. Thus far, preclinical research has focused on progestogen-based hormone therapies administered post-menopause, either after surgical menopause via Ovx (Braden et al., 2010, 2011, 2015, 2017; Chisholm & Juraska, 2012; Harburger et al., 2008; Lacasse et al., 2022; Lowry et al., 2010; Russo et al., 2008; Sovijit et al., 2021; Walf et al., 2006) or after substantial (93

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days) experimentally-induced ovarian follicular depletion via VCD (Koebele et al., 2021). The endogenous hormone milieu upon which hormones are exogenously administered, such as with hormone therapy, can impact outcomes (Acosta et al., 2010), and the majority of menopausal women receiving hormone therapy have their uterus and ovaries with some follicular depletion at the time of treatment. In the current experiment we used our established rat model of experimental follicular depletion to study the cognitive effects of three different clinically-used progestogens. Specifically, we confirmed the negative mnemonic impact of experimentally-induced follicular depletion via VCD, and evaluated the effects of each progestogen given during the experimentally-induced follicular depletion timeframe to model the profile of the menopause transition.

In the current study, we found that VCD-induced ovarian follicular depletion impaired performance on the WRAM, a cognitively complex spatial working and reference memory task, as shown in prior work (Koebele et al., 2017). Further, in the context of this follicular deplete background, both progesterone and MPA treatment initiated mid-follicular depletion improved spatial working memory during learning. In addition, ovarian follicular depletion impaired reference memory on this complex WRAM task for both learning and asymptotic phases of testing. An impairing effect persisted even when each of the progestogen treatments were mapped onto this follicle-deplete background; thus, progestogens neither impaired nor enhanced reference memory on the WRAM task in this study using experimentally-induced follicular depletion. In fact, this was found for progesterone, MPA, and micronized progesterone treatments. The MPA-induced impairment of spatial working memory during the lattermost part of WRAM testing in the current study corresponds with MPA-induced impairments on this portion of testing that

we have shown previously in the rat after surgical menopause induction via Ovx (Braden et al., 2010, 2017). The collective cognitive profile demonstrates that the benefit of progestogens started during the transition to menopause is spatial working memory specific. Additionally, none of the progestogen treatments prevented the VCD-induced impairment on the reference memory portion of the WRAM. Spatial reference memory on the MWM was not impacted by any progestogen treatment, nor by transitional menopause status.

Timing of hormone administration could play a role in the outcomes of progesterone and MPA administration. Prior research evaluating hormone therapies for effects on cognition in rat menopausal models has focused primarily on the Ovx model of surgical menopause (Daniel et al., 2006; Gibbs, 2000; Korol & Kolo, 2002; Luine et al., 2003; Markowska & Savonenko, 2002; Prakapenka et al., 2018). Thus, these hormone therapy evaluations have been limited since Ovx does not have a transition stage. In previous VCD studies, hormone administration usually occurred after rats had become fully follicle deplete (Acosta et al., 2010; Koebele et al., 2017, 2021; Koebele, Mennenga, et al., 2020). To our knowledge, the current study is the first to test hormone administration effects during the transition to menopause on the background milieu of induced follicular depletion. The choice of administering progestogen therapy during a timepoint where circulating progesterone has yet to differ in VCD-treated rats compared to vehicle counterparts (Koebele et al., 2017) was especially salient since post-follicle deplete rodents usually display decreased progesterone compared to counterparts who did not receive experimentally-induced follicle depletion (Acosta et al., 2009; Frye et al., 2012; Koebele et al., 2017; Mayer et al., 2004). Timing of administration of natural progesterone and its

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synthetic variants, either during the transition to menopause, or after follicle depletion has occurred, could account for the differential progestogen impacts on cognition mapped onto the VCD and Ovx models. Indeed, progestogen treatment has been shown to impair spatial working and reference memory in Ovx rats (Braden et al., 2010, 2011, 2015, 2017; LaCasse et al., 2022) as well as in VCD-treated rats when treatment is started is after follicle depletion (Koebele et al., 2021). These findings differ from our current results which demonstrate MPA- and progesterone- induced spatial working memory benefits when treatment is started during the transition to menopause in the VCD. Additionally, since micronized progesterone did not have a similar effect, it is possible that the micronization process itself to enhance bioavailability obviated the overall effects of progesterone. For cognition specifically, the translational ramifications of these data yield consideration of differential hormone therapy approaches based upon whether surgical or transitional menopause has ensued and that the timing of administration plays a key role in whether progestogens are beneficial.

Length of progestogen treatment plays a key role in mediating cognitive outcomes. Typically, hormone therapies can be chronic (multiple boluses administered across weeks and months) or acute (a single bolus evaluated after hours or days). Results from evaluations of chronic administration of progestogens alone is somewhat limited. Research from our laboratory suggests that chronic MPA or progesterone treatments impair spatial working memory in surgically menopausal Ovx rats (Braden et al., 2010, 2011, 2015, 2017). In addition to the current report and our other evaluations, on a spontaneous alternation task, in Ovx rats, chronic administration of MPA in combination with E2 benefited memory for novelty compared to E2 alone and to vehicle controls (Chisholm &

Juraska, 2012). In contrast, chronic MPA plus E2 treatment given to Ovx rats impaired spatial MWM performance compared to vehicle-treated Ovx controls (Lowry et al., 2010). These findings demonstrate that continuous and chronic administration of progestogen-based hormone therapies can detrimentally impact spatial memory even in the presence of an estrogen given at a regimen shown to be beneficial (previously or in the respective study). More extensive work has been done to understand the effects of acute progestogen administration, evaluating not only cognitive outcomes but the mechanistic properties associated with those outcomes (Shear et al., 2002; Wali et al., 2014). Acute progestogen treatment can benefit cognition when assessed for non-spatial tasks. Specifically, acute progesterone treatment improved or had neutral effects on non-spatial object recognition in rats and mice, yet it impaired spatial reference memory on the MWM or the object placement task compared to vehicle-treated controls in Ovx and ovary-intact rodents (Harburger et al., 2008; Russo et al., 2008; Sun et al., 2010; Walf et al., 2006). These collective results demonstrate that acute progestogen treatment alone can be beneficial for non-spatial memory, but impair spatial memory, in Ovx and ovary-intact rodents.

The progestogen-induced spatial working memory enhancements found herein are likely due, in part, to the follicle deplete ovary-intact background in rats given these hormone regimens. The VCD-induced follicular depletion rat model results in a markedly different circulating hormonal milieu in comparison to Ovx. For the current study, in VCD-only treated rats there was a decrease in circulating progesterone and an increase in circulating androstenedione, while circulating E2 levels did not differ, compared to the dual vehicle controls. These results are consistent with prior work demonstrating that there is an androgen-rich hormone profile with VCD-induced follicular depletion (Acosta et al.,

2009; Frye et al., 2012; Koebele et al., 2017; Mayer et al., 2004). Conversely, the Ovx model yields an abrupt cessation of all ovarian-derived hormones, including androgens, and our laboratory has demonstrated that follicular depletion-induced cognitive impairment is partially mediated through the conversion of the androgen androstenedione to estrone (Acosta et al., 2009; Camp et al., 2012; Mennenga, Koebele, et al., 2015). Further, in the current study MPA administration given to follicle deplete rats decreased circulating androstenedione levels compared to follicle-deplete rats given vehicle administration. These data parallel the decrease in androstenedione associated with MPA treatment in women (Segall-Gutierrez et al., 2012). The MPA-induced androstenedione decrease could be related to the beneficial cognitive effects during learning in MPA-treated follicular-deplete rats tested in this study. In addition, it is important to note that all MPA-treated rats in the current study had undetectable levels of circulating E2, along with decreases in progesterone and androstenedione. This overall decrease in ovarian-derived hormones indicates that MPA treatment potentially acts through a negative feedback loop that is distinct from either progesterone or micronized progesterone, the progesterone treatments assessed herein. This may be due, in part, to pro-androgenic effects of MPA that are distinct from progesterone (Africander et al., 2014). Indeed, both progesterone and micronized progesterone treatments increased circulating levels of progesterone and androstenedione. These findings indicate that exogenous progesterone treatment, whether micronized or not, leads to elevations in endogenous androstenedione corresponding to previously publications in rats (Koebele et al., 2021) and other species (Matsunaga et al., 2002). Further, when using a rat ovary-intact but follicle-deplete model such as VCD, exogenous progestogen administration is mapped on to endogenously circulating E2 from

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the intact ovaries, the latter of which does not differ between VCD-only and dual vehicle rats not receiving VCD. Thus, it might be useful to entertain interpretations of these findings in comparison to experiments including combination estrogen plus progestogen containing therapies. We previously found that P4 given alone after VCD-induced post-follicle depletion impaired working memory compared to VCD-induced post-follicle deplete vehicle controls, and that a high working memory load impairment was attenuated when E2 administration was given in addition to P4 (Koebele et al., 2021). Taken together, these data suggest that the modulation of the hormonal milieu is a mediating factor in the beneficial effects of MPA and progesterone.

In the current experiment, VCD-P4 rats that had higher expression of GAD 65+67 in the ventral hippocampus tended to have poorer working memory in the early acquisition phase, as shown via correlation. This is consistent with previous research indicating that increased activation in the GABAergic system leads to memory impairments (Curran, 1986; Naderipoor et al., 2021). Effects of GABA are not limited to progesterone alone; indeed allopregnanolone, a progesterone metabolite, positively regulates the effects of GABA on $GABA_A$ receptors (Backstrom et al., 2014). Some studies suggest that allopregnanolone treatment inhibits spatial reference memory on the MWM, and researchers have posited that chronic exposure of allopregnanolone in women due to their menstrual cycle, pregnancy, and hormone therapy after menopause might affect cognitive outcomes in women that could be mediated by the GABAergic system (Johansson et al., 2002; Türkmen et al., 2006). MPA inhibits metabolites of progesterone, such as allopregnanolone, and increases inhibitory tone of neurons in the dentate gyrus (Belelli & Herd, 2003). Thus, this MPA attenuation of impaired spatial working memory could be

mediated by allopregnanolone via the GABAergic system. Further, MPA itself is also positive modulator of the $GABA_A$ receptor (Das et al., 2022). Determining the interplay of progestogens and the GABAergic system, especially focusing on neurobiological circuits that modulate working memory, can inform putative pathways of efficacy to highlight potential clinical treatments, and be used to optimize progestogen-based hormone therapies given to women during this menopause transition.

The ovary data reported here are consistent with previous VCD research, whereby VCD-induced follicular depletion dramatically reduces primordial follicles (Koebele et al., 2017; Koebele, Mennenga, et al., 2020; Mayer et al., 2004; L. Springer et al., 1996). In the current study, we demonstrated characteristic ovarian failure using the VCD model, as VCD-treated rats showed a decrease in secondary and antral follicles, as well as corpora lutea. Referring to primary follicles specifically, some of the initial landmark research in rodents have reported decreased primary follicle counts after VCD administration (Koebele et al., 2017; Koebele, Mennenga, et al., 2020; Mayer et al., 2004; L. Springer et al., 1996); however, in our recent study (Koebele et al., 2021), there was an increase in primary follicles compared to a respective ovary-intact reference group following VCD treatment, consistent with findings from our current study where VCD treatment showed an increase in primary follicles compared to the dual vehicle. In the current study, the lack of non-steroidogenic primary follicles transitioning to later stage follicles, as well as the vaginal smear data, confirm halted ovarian cyclicity. We postulate that this comparative increase in primary follicles is due to changes in availability of specific F344 rat strains. Specifically, the current study used the F344-CDF strain, while most prior work used the F344-NIH strain. Although these two strains show 94.3% genetic similarity across 106

single nucleotide polymorphisms, one of the markers absent in the F344-CDF strain is present in the F344-NIH strain (National Institute on Aging, 2019). This absent marker may account, in part, for the increase of primary follicles in the rats given no hormone administration, possibly via Kit ligand signaling (Fernandez et al., 2008; Kezele et al., 2005). To our knowledge, the current and prior study (Koebele et al., 2021) are the first to use the new F344-CDF strain which might account for this change in primary follicle counts. The increase in number of primary follicles was attenuated by MPA treatment but not by either progesterone treatment, indicating that MPA treatment given during the transition to menopause had an effect on the primary follicle pool. It is possible that attenuation of primary follicles by MPA is due to its androgenic effects. MPA has been noted to be pro-androgenic and is an effective androgen receptor agonist that has similar activity to 5α-dihydrotestosterone (DHT) (Africander et al., 2014; Gogoi et al., 2008). Further investigation of effects of MPA on primary follicles in this newer strain of rat is warranted to assess mechanisms modulating follicle atresia.

In conclusion, progestogen-based hormone therapy has been utilized for decades to combat the unwelcome symptoms that occur during the transition to menopause. This study demonstrates that the synthetic progestin hormone therapy component, MPA, can have differential impacts on spatial working memory dependent upon stage of learning and memory retention. The current work also suggests that MPA and natural progesterone are beneficial for spatial memory when administered during perimenopause; prior to this study, most research identified MPA and progesterone as cognitively-impairing for spatial tasks. It is likely that these differential progestogen cognitive effects depend upon etiology and temporal parameters of menopause, specifically, whether surgical or transitional and how

close to menopause onset progestogen treatment occurs. In fact, timing has been a prevalent discussion in the preclinical (Daniel, 2013; K. Zhang et al., 2019) and clinical (Maki et al., 2013; Wu et al., 2020) hormone therapy literature; this is true for timing relative to menopause onset and length of progestogen treatment. Preclinical investigation into chronic administration of progestogens at various time points throughout the menopause transition will provide a better understanding into the nuanced and complex interactions between factors associated with menopause (e.g. hormone milieu, cognitive and neurobiological changes, hormone therapy formulations and treatment schedules, etc.). More exploration of how endogenous progesterone fluctuates as follicles decrease would provide greater insight into whether there is a critical window for beneficial effects of progestins during this transition to follicle depletion. Further, the interaction between progestogens and the GABAergic system via systematic analysis of metabolites such as allopregnanolone should include a more precise evaluation focusing on the status of brain regions associated with working memory. As we continue exploring progestogen-based therapies for effects on spatial memory, we must further refine parameters involved in the administration of such therapies and the reasons women opt to take such therapies, as these have the potential to have profound effects on efficacy.

CHAPTER 3

COGNITIVE CHARACTERIZATION AND EVALUATION OF GONADAL

HORMONE DEPRIVATION IN A RAT MODEL OF ALZHEIMER'S DISEASE

ABSTRACT

Women have an increased risk of developing Alzheimer's disease (AD), possibly

due to unique biological events (e.g. pregnancy and menopause). To assess

neuroendocrine-related effects of AD we utilized the TgF344-AD transgenic rat model of

AD which exhibits Aβ plaque-like pathology, tau-like pathology leading to

neurofibrillary tangles, and neuronal loss, and less observed behavioral impairment. The

current project utilized the TgF344-AD model to systematically characterize cognition in

male and female rats with or without gonadectomy (the surgical removal of the gonads,

Gdx) for effects on behavioral impairments and AD-like pathology. Eight treatment

groups were included in this study: male wild type (WT) Sham, female WT Sham, male

WT Gdx, female WT Gdx, male TG Sham, female TG Sham, male TG Gdx, and female

TG Gdx (n = 10 for each group). All animals received surgery at 6 months of age. At 9-

10 months of age all animals underwent a behavioral battery consisting of the Water

Radial Arm Maze (WRAM) to test spatial working and reference memory, the Morris

Water Maze (MWM) to test spatial reference memory, the visual platform task to test

motor and visual acuity, and the Open Field Test (OFT) to probe for locomotor activity

and anxiety-like behavior. During learning when working memory was highly taxed,

female TG rats were impaired on the WRAM compared to female WT rats regardless of

surgical status, while for male rats there was an interaction between genotype and

treatment. Specifically, male Sham TG and WT rats did not differ, while male TG

castrated rats made more working memory errors at the highest load compared to male WT castrated rats. These data suggest that during learning males are particularly sensitive to gonadal hormone deprivation at the age tested for learning, as a Genotype-related impairment was induced after gonad removal in males, but not females. For both sexes, during memory retention testing, the loss of gonadal hormones appears to be driving the effects, such that gonadectomy improves working memory performance. As shown on the MWM, the AD genotype impaired reference memory. For pathology, there was a significant interaction for tau pathology in females where WT GDX rats had higher AT8/Tau 5 ratios compared to WT Sham rats while TG Sham rats had higher AT8/Tau 5 ratios compared to TG GDX rats with no effects for beta-amyloid. Taken together, these data demonstrate that hormone loss impacts AD-related behavioral outcomes, and future investigations are warranted to better understand associations between sex, hormones, and AD-driven behavior and pathology.

INTRODUCTION

There are roughly 6.5 million people who have Alzheimer's disease (AD) in the United States, of which roughly two-thirds are women (American Alzheimer's Association, 2022). AD pathology consists of well-defined mechanisms in the brain, consisting of aggregation of beta-amyloid plaques, hyperphosphorylation of tau leading to neurofibrillary tangles, neuroinflammation, cardiovascular dysfunction, synaptic loss, network dysfunction, and eventual neuronal loss (Guo et al., 2020). Interestingly, cognitive deficits associated with AD are not present until after robust beta-amyloid plaque deposition (Mucke et al., 2000; Klein et al., 2001). Further, different hallmarks of the disease alone have been shown to produce any cognitive deficits. Indeed, robust beta-amyloid plaque deposition can be present without cognitive impairment or incidence of mild cognitive impairment (MCI) (Davis et al., 1999; Haroutunian et al., 1999), and neurofibrillary tangles can endure without neuronal impairment. It is clear that AD is a complex disease that requires many distinct aspects of pathology to produce clinical symptoms. This complexity of pathology before symptom onset as well as the lack of understanding on how pathology actually begins in disease progression has led to no viable cure nor treatments that either halt or reverse disease progression. As such, further understanding mechanisms of this disease and the roles that biological sex and sex hormones take in disease progression are key.

In Alzheimer's disease there are clear sex differences not only in incidence of disease, but also in pathology and symptomatology. Some studies have shown higher incidence rates of MCI in men compared to women (Petersen et al., 2010; Koivisto et al., 1995), although other studies have not shown this increased prevalence (Kivipelto et al.,

2001; Luck et al., 2010). For AD, women have an estimated lifetime risk of 20%

developing the disease compared to a 10% risk for men (Chêne et al., 2015). Although

this risk can be somewhat attributed to the fact that women live longer than men by an

estimated 4.5 years, and the fact that the men in the study might have had more death in

middle age, indicating a survivor bias for men, other research has demonstrated

differences in sex in the underlying neurobiology of AD (Chene et al., 2015; Zhu et al.,

2014; Vina & Lloret, 2010). In AD, men have demonstrated slowed or lesser neuronal

structure loss compared to women (Mielke et al., 2014). Additionally, men with MCI and

AD have slowed atrophy compared to women (Sampedro et al., 2015; Elbejjani et al.,

2015). Further, women have been documented to have increased levels of total tau

protein and beta-amyloid (1-42) in their cerebral spinal fluid as well as increased

cognitive decline and hippocampal atrophy (Koran et al., 2017). Another study has

shown that women with at least one E4 APOE allele have an increased vulnerability to

AD risk as they have an increased cardiovascular disease risk that is associated with early

tau deposition (Tsiknia et al., 2021). The prevalence of sex differences in AD has

demonstrated a need to further understand the underlying neural mechanisms associated

with the endocrine system.

In the clinic it is difficult to study the full progression of AD since clinical

symptoms occur 15-20 years after pathological onset. Thus, understanding of pathology

and symptomatology can be informed by utilizing rodent models of AD. For this study,

we sought to use the TgF344-AD model of AD since the temporal progression of

pathology and subsequent cognitive impairment matches more closely to the human

disease than other transgenic models. The TgF344-AD model has the mutated human

amyloid precursor protein (APP$_{SW}$) and the mutant presenilin protein (PSEN1dE9) without exon 9 (Cohen et al., 2013). While this model has genes only associated with amyloid pathology and produces soluble amyloid oligomers that aggregate to beta-amyloid plaques, it also demonstrates tau pathology via hyperphosphorylated tau leading to neurofibrillary tangles, neuroinflammation, impaired cardiovascular function, and frank neuronal loss (Cohen et al., 2013; Joo et al., 2017; Smith et al., 2018). This model also demonstrates impairments in spatial working and reference memory (Bernaud et al., 2022; Berkowitz et al., 2018; Cohen et al., 2013) as well as recognition memory (Cohen et al., 2013).

In this study, we sought to assess whether gonadectomy in males and females affected AD-like pathology and cognition in the TgF344-AD model. Previous research using this model has assessed males and females to assess sex differences (Berkowitz et al., 2018); however, no work has been done to fully assess gonadectomy in both males and females utilizing a model that more closely resembles the human condition such as the TgF344-AD model. Further, we wish to assess working memory spatial navigation since this type of memory is impaired in earlier, prodromal stages of Alzheimer's disease (Kirova et al., 2015). Finally, we aim to evaluate anxiety-like behavior at this timepoint, as anxiety disorders are comorbid with AD clinical symptomology and can promote AD-related cognitive decline (Mendez 2021). We aim to distinguish relationships between gonadal hormone loss, cognitive outcomes, and pathology.

METHODS

Subjects

Eighty six-month-old (40 female and 40 male), non-breeder, TgF344-AD rats were utilized for this study. Animals were pair-housed and fed *ad libitum* on post-natal day 28. Rats were placed on a 12-hour on/off light-dark cycle, and were treated in compliance with the Arizona State University Institutional Animal Care and Use Committee protocol. All procedures adhered to the standards provided by the National Institutes of Health.

Animal Breeding

Since the TgF344-AD model is a hemizygous transgenic rat model, breeding males and females were partnered where one sex was transgenic (TG) and the other sex was wild type (WT). Rats were harem bred with two females to one male per breeding cage to ensure successful breeding. On postnatal day (PND) 10 tail samples were collected and stored at -70°C for polymerase chain reaction (PCR) analyses to determine genotype (either TG or WT), rats were ear punched for differentiation and sexed via anogenital distance. At PND21 rats were weaned and sex housed into either male or female cages. Housing did not exceed six rats per cage and there was always at least two rats per cage. Female cages were removed to a separate colony room and male cages stayed in the breeding colony room. At PND28 rats were pair-housed with their littermates two to a cage or three to a cage if there was an odd number of rats. Rats were then either selected as breeders for colony maintenance, or non-breeders for experimental use. Litters for this study ranged from 3-15 pups with a minimum of 14 breeding pairs to achieve 80 experimental rats.

PCR

PCR was utilized to determine genotype via the APP transgene using tail samples from every rat as previously published (Mifflin et al., 2021). Tail samples were dissolved using Sodium Chloride-Tris-EDTA buffer as well as proteinase K in a 24:1 ratio (500ul per sample). Samples were then placed in a heated water bath (55°C) overnight. Samples were centrifuged at 15,000 rpm for 10 minutes at 4°C after which 500 ul of isopropyl alcohol was added to the supernatant. The solution was forcefully shaken to separate the DNA. Liquid was extracted placed for 3 minutes in a Speedvac to evaporate all remaining liquid. DNA was then resuspended in TE buffer (150 µM) and incubated in the water bath (55°C) overnight. Samples were then added to a solution of 3 primers (APP, PRP internal control, and PRP reverse), distilled water and Taq buffer in a 1:7:12.5 ration. A thermocycler was used to increase DNA output during which a gel of 1.8% agarose in 1 X Tris-borate EDTA with GelRed nucleic acid gel stain (10ul) to fluoresce DNA bands. 1 x green loading dye was added to each sample after thermocycler completion. Samples were pipetted into the agarose gel with the appropriate ladder. All gels ran for 30 minutes at 200mV and then placed under UV light for photo capture using BioRad Gel Doc XR. Bands were either classified as 400bp indicating presence of the APP transgene or TG rat or 750bp indicating a WT rat.

Gonadectomy Surgery

At sixth months of age, rats either received gonadectomy surgery (consisting of ovariectomy for females and castration for males) or sham surgery. For ovariectomy surgery, female rats received skin and muscle incisions along the dorsolateral aspect with use of an inhaled anesthetic, isoflurane. After the incision was made the ovary was exposed

and ligated at the tip of the uterine horn and subsequently removed. For the sham surgery, only the skin and muscle incisions were made without ovary removal. Rats received muscle sutures (Coated VICRYL Suture, Ethicon) and skin staples and were given subcutaneous saline injections (2ml) to prevent dehydration. For castration, male rats received an incision at the scrotum. The testes were exposed and ligated where the testis and testicular sheath met and subsequently removed. For the sham surgery only the skin incision was made without testis removal. Scrotal skin was stapled and rats were given subcutaneous saline injections (2ml). During the castration surgeries, two rats died post-surgery. Two more rats of the same genotypes were selected and received successful surgery to account for this loss.

Vaginal Smears

Vaginal smears occurred for eight consecutive days for all rats, beginning two weeks before behavioral testing. Each smear was determined to be in either the metestrus, diestrus, proestrus, or estrus phase as indicated in previous protocols (Goldman et al., 2007). Male rats were anally swabbed in a manner that was comparable to the handling and orifice swabbing procedures used for females.

Water Radial Arm Maze (WRAM)

Rats began behavioral testing three months after gonadectomy surgery. The WRAM is a water-escape task used to assess spatial working and reference memory performance. The maze consisted of eight arms (38.1cm x 12.7cm) radiating from a circular center. Robust visual cues were in the room to assist with spatial navigation. Four of the eight arms contained hidden platforms (10 cm diameter) 2 cm below the surface of the water (18-20°C) which contained non-toxic black paint for opacity. Platforms were

kept in consistent locations at the beginning of each testing day for each rat but were semi-randomized between rats.

Rats were tested for 12 consecutive days, with four trials each day. The start arm remained constant for all rats and trials. At the start of the first trial, rats were placed in the start arm and were allowed to swim freely in the maze until they found a platform or until 3 minutes had elapsed, after which rats were led to the closest platform. After rats found a platform, they were kept there for a total platform time of 15 seconds and then were removed and placed in their heated testing cage for an inter-trial interval (ITI) of 30 seconds. During this ITI, the just-found platform was removed and the maze was cleaned to remove debris and distribute olfactory cues. Because of this there were four trials per day (one to represent each platform) and as trials progressed, working memory load increased since rats had to sustain more items in their working memory. Errors were quantified as entry into an arm that did not have a platform; arm entries occurred when a rat's snout passed 11 cm into an arm. Performance was assessed as the quantification of total errors, and was further broken into three separate error types: working memory correct (WMC), reference memory (RM), and working memory incorrect (WMI). WMC errors were entries into an arm that previously contained a platform for that day. RM errors were the first entry into an arm for that day that never contained a platform. WMI errors were subsequent entries into an arm that never contained a platform. On the last day of testing, day 13, a four-hour delay was implemented between trial 2 and trial 3 to assess delayed memory retention.

Morris Water Maze (MWM)

The MWM is a water escape task that is used to assess spatial reference memory performance. The maze consisted of a circular pool that was 188 cm in diameter. A platform (10 cm diameter) was hidden 2 cm below the surface of the water (18-20°C), with non-toxic black paint for opacity, in the north-east quadrant of the maze. Robust visual cues were situated on adjacent walls to assist with spatial navigation. A camera was mounted above the maze and the computer program Ethovision 10 (Noldus Instruments, Wageningen, Netherlands) was used to capture video and track the rat's path through the maze.

Rats were tested for five consecutive days, with four trials each day. At the start of the first trial, animals were dropped off at a quadrant (north, south, east or west) semi-randomly assigned to each trial throughout each of the days. Once in the maze, rats were allowed to swim freely until they found the platform or until one minute had elapsed, after which rats were then led to the platform. Rats were allowed to stay on the platform for 15 seconds, after which time the rat was returned to their testing cage for an 8±2 minute ITI. On Day 5, the last day of testing, after all test trials, a probe trial was introduced to assess whether the animals had spatially localized the position of the platform. Before the probe trial began the platform was removed and rats were allowed to swim freely in the maze for one minute after which they were returned to their testing cages. Distance to platform (cm) from the drop-off point was recorded and used to assess spatial localization of the platform over the testing period of five days.

Open Field Task

The open field task was used to assess anxiety-like behavior and locomotor activity (Hiroi et al., 2016; Koebele, Nishimura, et al., 2020). The open field area consisted of a 100 cm x 100cm x 40 cm square apparatus that was placed in a dark testing room illuminated by red light. Rats were allowed to habituate in an adjacent room for 30 minutes prior to testing. After habituation, rats were brought into the room with the open field arena and dropped off in the center of the north wall. Rats were allowed to explore the arena for 10 mins before being placed back in their home testing cage. Between trials the arena was sprayed with water and swept with paper towels to distribute olfactory cues. Ethovision 12.5 tracking system (Noldus Instruments, Wageningen, Netherlands) was used to assess distance and time spent in the maze. The arena was divided into 25 equal squares (20cm x 20cm) for assessment (see Fig. 11H). Locomotor activity was analyzed as total distance covered in the arena. Anxiety-like behavior was analyzed as distance covered in the Center, Small Center, and Corners of the arena as well as time spent in the Center, Small Center, and Corners of the arena. More distance covered and more time spent in the Center and Small Center of the arena as well as less distance covered, and less time spent in the Corners of the arena, were interpreted as decreased anxiety-like behavior.

Visible Platform

The visible platform test was used to assess motor and visual acuity for solving a water escape task. The maze consisted of a rectangular pool (100 x 60 cm) filled with clear water kept at 18-20°C with a platform situated inside the maze (10 cm diameter) protruding 4 cm above the water. The maze was encircled by a curtain so that there were no salient visual cues other than those found inside the maze.

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Rats were tested for one day with six trials. At the start of trial 1, animals were placed in the starting position that remained constant throughout trials. The rats were allowed to swim freely until they found the platform or until 90 seconds had elapsed, after which the rats were led to the platform. After the rats found the platform, they were allowed to stay on it for 15 seconds after which the tester returned rats to their testing cage for an ITI of 8±2 minutes. All rats were tested for trial 1 before moving on to trial 2. Platform locations were assigned semi-randomly across trials. Latency (s) to the platform was measured.

Euthanasia

Rats were euthanized over two days, at the end of behavioral testing. Final body weights (g) were obtained earlier in the day directly before daily injections on the first day of sacrifices. Rats were anesthetized via isoflurane, and cardiocentesis was performed for blood collection. Rats were then decapitated and brains removed for dissection. The Rat Brain Atlas was utilized for reference for brain dissections via plate designations (Paxinos & Watson, 1998). Two separate brain regions were raw brain dissected in the right frontal cortex (plates 5-14) and right dorsal hippocampus (plates 33-35) directly after removal. Tissue was weighed immediately and stored at -70°C until further analysis.

Western Blot

Beta-amyloid (1-42), AT8, and Tau5 expression levels were assessed from the right hemisphere in the frontal cortex and dorsal hippocampus via Western Blot analysis. Samples were homogenized via probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA) in a 1:50 RIPA buffer (1% Triton X-100, 150mM NaCl, 0.5% Na deoxycholate, 0.1% SDS, 50mM Tris, phosphatase inhibitor [Cat#: 524625, Millipore-Sigma], and

protease inhibitor [Cat#: 5892791001, Millipore-Sigma]). Samples were then centrifuged for 10 minutes at 10,000 rpm at 4°C. A BCA (bicinchoninic acid) protein assay (ThermoFisher Scientific, Pittsburgh, PA, USA) was utilized to measure protein concentration. Samples were run on a 4-12% Bis-Tris NuPAGE gel using a SureLock Mini-Cell system (Invitrogen, Carlsbad, CA, USA) and transferred to a polyvinylidene difluoride membrane (Immobulin-P). Samples were loaded at 10μg of protein per brain region. Treatment groups were counterbalanced across gels and four gels were run per brain region for beta-amyloid and seven gels were run per region for AT8 and Tau5. After transfer, the membrane was blocked in 5% non-fat milk for one hour after which it was incubated overnight at 4°C in primary antibodies anti-beta-amyloid (1-42) (1:500, Thermo Fisher), anti-AT8 (1:500, Thermo Fisher, and anti-Tau5 (1:1000, Cell Signaling) as well as primary antibody anti-beta actin (1:20,000, Cell Signaling) as a control measure. The membrane was then incubated in secondary antibodies anti-mouse horseradish peroxidase (1:2000, Cell Signaling) and anti-rabbit horseradish peroxidase (1:2000, Cell Signaling) at room temperature for one hour. Chemoluminescence (LumiGlo and Peroxide, Cell Signaling) was used to visualize protein expression using a film developer (Konica SRX-101A Film Processor, Tokyo, Japan). ImageJ software was used to perform densitometry. GAD 65 and GAD 67 were analyzed together in accordance with a previous protocol from our laboratory (Braden et al., 2010, 2011). Beta-amyloid levels were normalized to the loading control beta-actin for analysis. AT8 and Tau5 proteins were normalized to the loading control beta-actin and then provided as a ratio of AT8/Tau5 for analysis.

Statistical Analyses

All assessments herein were performed in males and females separately to assess effects within each sex; thus, males and females were not directly compared. WRAM data were analyzed across Days 3-12 using an omnibus repeated measures analysis of variance (ANOVA) for each of the error types (WMC, WMI, and RM, and Total errors). Day 1 was not analyzed, as this was considered an information day when animals were gathering information for the first time about the rules of the task; typically rats do not make as many errors on this day since they have not made the association between entering arms and escape. Additionally, during Day 1, there was a testing error made such that not all rats were tested. Thus, for Day 2 some rats were encountering the water maze for the first time. For this reason, Days 2 was also not included in the statistical analyses. The repeated measures consisted of Trials nested within Days while the independent variable was Treatment. Days 1 and 2 were excluded from data analysis due to tester error where some rats were not tested on Day 1. Therefore, Day 2 was considered as Day 1 for these rats and both days were excluded from analyses. Data were further separated into two distinct blocks for Days 3-8 (the acquisition phase) and Days 9-12 (the asymptotic phase), and each block was individually assessed via an omnibus repeated measures ANOVA for each of the error types. Additionally, we compared performance amongst groups for the highest (Trial 4) working memory load as our laboratory has previously shown that effects of several hormone-altering treatments become apparent when working memory load is highly taxed (Bimonte & Denenberg, 1999; Braden et al., 2010; Koebele et al., 2017). For the delay, for each treatment group separately, trials 3 and 4 from baseline (day 12) were averaged, and trials 3 and 4 from postdelay (day 13) were averaged and compared, as done

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previously (Camp et al., 2012; Engler-Chiurazzi et al., 2011; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018).

MWM total swim distance data were analyzed using an omnibus repeated measures ANOVA for all days of testing. The repeated measures consisted of Trials nested with Days, and the independent variables were Genotype and Treatment. Additionally, the probe trial was analyzed by assessing percent swim distance in the target quadrant (NE quadrant) where the platform had been previously located, as compared to the opposite quadrant (SW quadrant), via repeated measures ANOVA for all experimental groups, to assess if rats had correctly spatially localized the platform for each treatment group.

Open field data were assessed using an omnibus ANOVA where distance traveled in the Total, Center, Small Center, and Corners of the arena and total time spent in the Center, Small Center, and Corners of the arena were the dependent variables, and Genotype and Treatment were the independent variables.

Visible platform data were assessed using a repeated measures ANOVA where time to platform was the dependent variable. The repeated measures were Trials, and the independent variables were Genotype and Treatment.

One-way ANOVAs were utilized to assess beta-amyloid (1-42), AT8, and Tau5 protein levels in the frontal cortex and dorsal hippocampus. Beta-amyloid (1-42), AT8, and Tau5 levels were normalized to the beta-actin loading control. The independent variables were Genotype and Treatment. Pearson r correlations were analyzed for beta-amyloid (1-42), AT8, and Tau5 in each brain region for WMC and WMI errors for Days 3-8 and Days 9-12 of WRAM data. We used a false discovery rate (FDR) limit of 0.1 to protect against

multiple correlations. Both FDR-corrected (Q) and uncorrected (p) values are reported (Benjamini & Hochberg, 1995).

<div align="center">RESULTS</div>

WRAM

Females

For females, the omnibus ANOVA for Days 3-12 revealed a main effect of Day for all error types [WMC: $F(9, 324) = 5.845$, $p < 0.001$; WMI: $F(9, 324) = 6.629$, $p < 0.001$; RM: $F(9, 324) = 6.643$, $p < 0.001$; Total: $F(9, 324) = 8.819$, $p < 0.001$], indicating that all animals were able to learn the task across all testing days (data not shown). Additionally, there was a main effect of Trial for Days 3-12 for all error types [WMC: $F(2, 72) = 141.763$, $p < 0.001$; WMI: $F(3, 108) = 74.917$, $p < 0.001$; RM: $F(3, 108) = 36.258$, $p < 0.001$; Total: $F(3, 108) = 174.135$, $p < 0.001$], indicating errors increasing as trials and working memory load progressed for all animals.

For Days 3-8 for WMC errors, there was a marginal Genotype effect [$F(1, 36) = 2.978$, $p < 0.1$], such that TG rats tended to make less errors compared to WT counterparts (See Fig. 9). There was a marginal Trial x Genotype interaction [$F(2, 72) = 2.467$, $p < 0.1$] led by Trial 4, the highest working memory load trial, and on Trial 4 there was a marginal effect of Genotype [$F(1, 36) = 3.496$, $p < 0.1$] such that TG rats tended to be impaired compared to WT controls.

For Days 3-8 for WMI errors, there was a significant Trial x Genotype interaction [$F(3, 108) = 3.086$, $p < 0.05$], led by Trial 4, where there was a marginal effect of Genotype [$F(1, 36) = 3.046$, $p < 0.1$] whereby TG rats tended to make more WMI errors than WT counterparts (See Fig. 9).

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For Days 3-8 for Total errors, there was a marginal effect of Genotype [$F(1, 36) = 3.098, p < 0.1$] such that TG rats tended to make less errors compared to WT controls. Further, there was a significant Trial x Genotype interaction [$F(3, 108) = 4.075, p < 0.01$] led by trial 4, where there was a significant effect of Genotype [$F(1, 36) = 4.544, p < 0.05$] such that TG rats were impaired compared to WT counterparts (data not shown).

For Days 9-12 for WMC errors, there was a marginal effect of Treatment [$F(1, 36) = 3.317, p < 0.1$] such that GDX rats tended to make fewer errors than Sham counterparts (See Fig. 10). Further, there was a significant Trial x Treatment interaction [$F(2, 72) = 3.765, p < 0.05$], led by trial 4, the highest working memory load trial, where there was a main effect of Treatment [$F(1, 36) = 4.211, p < 0.05$] such that GDX rats made fewer errors than Sham controls.

For Days 9-12 for WMI errors, there was a marginal Trial x Treatment interaction [$F(3, 108) = 2.443, p < 0.1$] but no effects for any individual trial (data not shown).

For Days 9-12 for Total errors, there was a significant Trial x Treatment interaction [$F(3, 108) = 4.425, p < 0.01$], led by trial 4 where there was a significant effect of Treatment [$F(1, 36) = 4.877, p < 0.05$] such that GDX rats made fewer errors compared to Sham counterparts (data not shown).

Males

For males, the omnibus ANOVA for Days 3-12 revealed a main effect of Day for all error types [WMC: $F(9, 324) = 4.090, p < 0.001$; WMI: $F(9, 324) = 3.965, p < 0.001$; RM: $F(9, 324) = 2.815, p < 0.01$; Total: $F(9, 324) = 4.920, p < 0.001$] indicating that all animals were able to learn the task across all testing days (data not shown). Additionally, there was a main effect of Trial for Days 3-12 for all error types [WMC: $F(2, 72) =$

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201.587, $p < 0.001$; WMI: $F(3, 108) = 143.239$, $p < 0.001$; RM: $F(3, 108) = 21.617$, $p <$ 0.001; Total: $F(3, 108) = 168.875$, $p < 0.001$], indicating increasing working memory load as trials progressed for all animals (data not shown).

For Days 3-8 for WMC errors, there was a significant Genotype x Treatment interaction [$F(1, 36) = 4.275$, $p < 0.05$] (See Fig. 11). This effect was led by trial 4, the highest working memory load trial, where there was a significant Genotype x Treatment interaction [$F(1, 36) = 4.289$, $p < 0.05$] where there was an effect of Genotype for GDX rats where TG rats had more errors than WT rats [$F(1, 18) = 5.612$, $p < 0.05$] that was not present in Sham counterparts [$F(1, 18) = 1.309$, $p > 0.1$].

For Days 3-8 for WMI errors, there was a marginal Trial x Genotype interaction [$F(3, 108) = 2.174$, $p < 0.1$], led by Trial 4, where there was a marginal effect of Genotype [$F(1, 36) = 2.374$, $p < 0.1$] whereby TG rats tended to make more WMI errors than WT counterparts.

For Days 3-8 for Total errors, there was a significant Genotype x Treatment interaction [$F(1, 36) = 4.491$, $p < 0.05$] led by trial 4, where there was a significant Genotype x Treatment interaction [$F(1, 36) = 4.750$, $p < 0.05$] where there was an effect of Genotype for GDX rats [$F(1, 18) = 7.166$, $p < 0.05$] that was not present in Sham counterparts [$F(1, 18) = 0.164$, $p > 0.1$] (data not shown).

For Days 9-12 for WMI errors, there was a significant effect of Genotype [$F(1, 36) = 6.179$, $p < 0.05$] where TG rats made significant more errors than WT counterparts (See Fig. 12). Additionally, there was a significant Trial x Genotype interaction [$F(3, 108) = 4.954$, $p < 0.01$], led by trial 4, where there was a significant Genotype effect [$F(1, 36) = 6.004$, $p < 0.05$], where TG rats made more errors than WT controls. Further, there

was a significant Trial x Treatment interaction [$F(3, 108) = 3.546$, $p < 0.05$], led by trial 4, where there was a marginal effect of Treatment [$F(1, 36) = 3.377$, $p < 0.1$], where GDX rats tended to make fewer errors than Sham counterparts (See Fig. 12).

MWM

Females

For females, there was a main effect of Day for swim distance to platform [$F(4, 144) = 69.044$, $p < 0.001$], indicating that all animals learned the task. There was an effect of Genotype [$F(1, 36) = 6.126$, $p < 0.05$] such that TG rats had a higher swim distance to platform indicating impaired spatial reference memory (See Fig. 13). For the probe trial, there was a main effect of Quadrant such that there was a higher percent swim distance in the target quadrant (NE quadrant), which had previously contained the platform, than the opposite quadrant (SW quadrant), indicating spatial localization of the platform [$F(1, 36) = 361.118$, $p < 0.0001$]. For three groups there was a main effect of Quadrant [WT Sham: $F(1, 9) = 34.996$, $p < 0.001$; WT GDX: $F(1, 9) = 43.610$, $p < 0.001$; TG Sham: $F(1, 9) = 58.265$, $p < 0.001$] where each treatment group swam a significantly higher percentage of distance in the target quadrant (NE) where the target used to be located versus the opposite quadrant (SW), indicating that these treatment groups were able to correctly spatially localize to the platform (See Fig. 13). For the TG GDX group there was no effect of Quadrant [$F(1, 9) = 2.581$, $p > 0.1$] indicating that these rats were not able to correctly spatially localize to the platform. For all other experimental groups there was a main effect of Quadrant for each experimental group, [WT Sham: $F(1, 10) = 69.574$, $p < 0.001$; WT GDX: $F(1, 10) = 48.576$, $p < 0.001$; TG Sham: $F(1, 10) = 55.364$, $p < 0.001$, where each experimental group swam a significantly higher percentage of distance in the target

quadrant (NE) where the target used to be located versus the opposite quadrant (SW), indicating that treatment groups were able to correctly spatially localize to the platform.

Males

For males, there was a main effect of Day for swim distance to platform [$F(4, 144) = 126.373, p < 0.001$], indicating that all animals learned the task. There was an effect of Genotype [$F(1, 36) = 5.548, p < 0.05$] such that TG rats had a higher swim distance to platform indicating impaired spatial reference memory (See Fig. 14). For the probe trial, there was a main effect of Quadrant such that there was a higher percent swim distance in the target quadrant (NE quadrant), which had previously contained the platform, than the opposite quadrant (SW quadrant), indicating spatial localization of the platform [$F(1, 36) = 286.884, p < 0.0001$]. For each group there was an effect of Quadrant [WT Sham: $F(1, 9) = 99.661, p < 0.001$; WT GDX: $F(1, 9) = 108.269, p < 0.001$; TG Sham: $F(1, 9) = 59.046, p < 0.001$, TG GDX: $F(1, 9) = 140.666, p < 0.001$] where each treatment group swam a significantly higher percentage of distance in the target quadrant (NE) where the target used to be located versus the opposite quadrant (SW), indicating that treatment groups were able to correctly spatially localize to the platform.

OFT

Females

For females, for distance, there was an effect of Genotype for Corner distance [$F(1, 36) = 5.342, p < 0.05$], where TG rats covered more distance in the corners of the arena compared to WT controls, indicating increased anxiety-like behavior (See Fig. 15). There was no difference for other distance-related anxiety-like (Center distance or Small

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Center distance) for either Genotype or Treatment. There was not an effect of Total

distance for either Genotype or Treatment, indicating no difference in locomotion

between these groups.

For duration, there was an effect of Genotype for Center time [$F(1, 36) = 6.279$, p

< 0.05], where TG rats spent significantly less time in the center of the arena compared to

WT controls, indicating increased anxiety-like behavior. There was no difference for

Small Center time or Corner time for Genotype or Treatment.

Males

For males, for distance, there was an effect of Genotype for Total distance [$F(1,$

$36) = 7.067$, $p < 0.05$], where TG rats moved less compared to WT controls, indicating

decreased locomotion in these rats (See Fig. 16). Additionally, there was a marginal

effect of Genotype for Center distance [$F(1, 36) = 3.961$, $p < 0.05$], where TG rats tended

to cover less distance in the center of the arena compared to WT controls, indicating

increased anxiety-like behavior. There was an effect of Treatment for Total distance [$F(1,$

$36) = 6.021$, $p < 0.05$] where GDX rats moved more than Sham counterparts, indicating

increased locomotion in these rats. There was an effect of Treatment for Center distance

[$F(1, 36) = 13.592$, $p < 0.001$] and Small Center distance [$F(1, 36) = 10.522$, $p < 0.01$],

where GDX rats moved more in the center and small center arenas compared to Sham

controls, indicating decreased anxiety-like behavior. There was no difference for Corner

distance for either Genotype or Treatment.

For duration in the center of the OFT, there was an effect of Genotype for Center

time [$F(1, 36) = 7.083$, $p < 0.05$], where TG rats spent significantly less time in the center

of the arena compared to WT controls, indicating increased anxiety-like behavior. There

was an effect of Treatment for Center time [$F(1, 36) = 7.629$, $p < 0.01$] and Small Center time [$F(1, 36) = 4.881$, $p < 0.05$] where GDX rats spent more time in the center and small center of the arena, indicating decreased anxiety-like behavior. There was no difference for Corner time for either Genotype or Treatment.

Visible Platform Task

Females and Males

The visible platform test was used to assess an animal's ability to solve a water escape task. There was a main effect of Trial for females [$F(5, 36) = 6.222$, $p < 0.001$] such that as trials progressed latency to the platform decreased, indicating better performance as trials progressed with an average latency of 8.046 seconds +/- 0.298 seconds to reach the platform across all trials (data not shown). There was a main effect of Trial for males [$F(5, 36) = 5.931$, $p < 0.001$] such that as trials progressed latency to the platform decreased, indicating better performance as trials progressed with an average latency of 7.071 seconds +/- 0.304 seconds to reach the platform across all trials (data not shown). There was no main effect of Genotype or Treatment nor a significant Trial x Treatment interaction for either females or males, indicating that the groups did not differ in their ability to perform the procedural components of a water-escape task.

Western Blot Analysis

Beta-amyloid

Females and Males

For females, there was no effect of Treatment for normalized beta-amyloid in the dorsal hippocampus $F(1, 17) = 0.022$, $p > 0.1$, or in the frontal cortex $F(1, 17) = 0.126$, $p > 0.1$ (See Fig. 17). For males, there was no effect of Treatment for normalized beta-

amyloid in the dorsal hippocampus $F(1, 17) = 0.023$, $p > 0.1$, or in the frontal cortex $F(1, 17) = 0.028$, $p > 0.1$ (See Fig. 17).

AT8/Tau5

Females

For females, there was a significant Genotype x Treatment interaction $F(1, 29) = 6.167$, $p < 0.05$, whereby WT GDX rats had significantly higher AT8/Tau 5 ratios compared to WT Sham rats and TG Sham rats had significantly higher AT8/Tau 5 ratios compared to TG GDX rats, $p < 0.05$. For Sham rats, TG rats had significantly higher AT8/Tau5 ratios, indicating increased hyperphosphorylated tau compared to WT counterparts $F(1, 7) = 3.897$, $p < 0.05$ (See Fig. 18).

Males

For males there was a marginal effect of Treatment $F(1, 32) = 3.100$, $p < 0.1$ where GDX rats tended to have higher AT8/Tau5 ratios compared to Sham counterparts (See Fig. 18).

DISCUSSION

This current study is the first to assess effects of gonadectomy in the TgF344-AD model of Alzheimer's disease. Here we have shown interactions with gonadectomy and genotype in males when assessing behavioral outcomes. Gonadectomy surgery induced a genotype effect for working memory on the WRAM. Specifically, for rats with gonadectomy, transgenic rats made more working memory errors than wildtype counterparts; however, this effect was not found in rats that had sham surgery. Additionally, there was an increase in locomotion and anxiety-like behavior, as assessed on the open field task, in male TG rats, as well as male gonadectomized rats. For

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pathology assessments, gonadectomized males tended to have increased AT8/Tau5 ratios compared to sham counterparts.

Previous research has demonstrated that Ovx can exacerbate AD pathology and symptomatology (Hu et al., 2016; Carroll et al., 2007; Heikkinen et al., 2014). In the current study, in females, transgenic, gonadectomized rats were not able to spatially localize the platform by the end of testing for the MWM, an effect not found in any other female group. These results indicate that the surgical removal of the ovaries exacerbates reference memory deficits in transgenic female rats since this effect was limited to this group, and this group did not differ on their visible platform scores from any other female group. This finding was limited to the probe day of testing; there were no interactions for reference memory between gonadectomy and genotype for the traditional five days of MWM in females, although transgenic rats were impaired compared to wildtypes as has been previously demonstrated (Berkowitz et al., 2018; Bernaud et al., 2022). Interestingly, there were no interactions on the WRAM which also assesses reference memory, although there were marginal effects of genotype where transgenic rats tended to be impaired for working memory compared to wildtype controls. These results are similar to previous results from our laboratory in ovary-intact females whereby there was no genotype effect for rats at 9 months of age (whereas ours were tested at 10 months of age), yet there were clear impairments at 12 months of age (Bernaud et al., 2022). It is plausible that there is a compensatory effect that occurs around the nine-month time point which overshadows any possible interactions between gonadal hormone loss via ovariectomy and genotype.

For pathology, in females, although there was no effect for beta-amyloid, there was an interaction between gonadectomy and genotype such that sham transgenic rats exhibited increased levels of AT8/Tau5 ratios compared to wildtype rats, while wildtype, gonadectomized rats had increased AT8/Tau 5 ratios compared to wildtype sham rats. Furthermore, there was an increase in the AT8/Tau 5 ratio in transgenic sham rats compared to wildtype sham rats thereby indicating increased tau pathology in this AD model; these results are consistent with previously reported findings (Joo et al., 2017, Cohen et al., 2013).

Our results additionally indicate that tau pathology can be impacted by surgical menopause, and that this could be a potential mechanism of action through which later cognitive decline might occur. This finding is counter to others reporting increased tau hyperphosphorylation with Ovx surgery as compared to Sham surgery in another transgenic model of AD (Carroll et al., 2007). Further, another study found that prolonged time after ovariectomy of five months, 10 months, and 15 months demonstrated increased hyperphosphorylated tau in the hippocampus in age matched controls in non-AD Wistar female rats, indicating a role for aging and surgical menopause in the increase of tau pathology independent of AD pathology (Picazo et al., 2016). Hormone therapy, particularly E2 administration, has been shown to impact tau pathology. It has been posited that E2 prevents phosphorylation of the tau protein through the inhibition of glycogen synthase kinase 3, through which hyperactivation leads to neurofibrillary tangle formation, a key hallmark of AD progression (Hernandez et al., 2013; Pinto-Almazán et al., 2012).

For males, our findings were somewhat inconsistent with past research. Previous research has demonstrated impairment with castration in AD models for cognition and pathology. For instance, castration in 3xTG-AD male mice impaired performance in spontaneous alternation behavior compared to sham counterparts (Rosario et al., 2012, 2006). Regarding pathological assessments, our results were inconsistent with previous research, which has noted impacts of castration and testosterone administration on beta-amyloid pathology. As an example, beta-amyloid was increased in 3xTG-AD male gonadectomized mice compared to sham counterparts, and this was attenuated by testosterone administration (Rosario et al., 2012, 2006). Further, in the APP23 mouse model of AD, increasing endogenous testosterone via aromatase knock-out led to decreased beta-amyloid plaque formation, beta-amyloid 42/beta-amyloid 40 ratios, and improved cognitive function compared to wildtype age-matched controls in male mice (McAllister et al., 2010). It is possible that effects of androgens on beta-amyloid pathology are related to increased neprilysin, which catabolizes beta-amyloid (McAllister et al., 2010; Yao et al., 2008). For tau pathology, there is evidence that heat-shock induced hyperphosphorylated tau is prevented by androgen treatment, independent of estrogen administration (S. Papasozomenos & Shanavas, 2002)

It should be noted that the translational relevance of assessing castration in AD pathology is tempered, as most males do not undergo castration across their lifetime. There is, however, some utility is assessing this phenomenon as males may opt to be chemically castrated as a therapeutic intervention for prostate cancer to suppress testosterone levels (Gomella, 2009). Indeed, research assessing this patient population has found that chemical castration via flutamide, and leuprolide-induced testosterone

decreases, led to increased levels of beta-amyloid and increased scores on depression and anxiety assessments (Almeida et al., 2004). Finally, although there were no impacts of beta-amyloid 1-42 in the current study, further assessment of alternate beta amyloid pathology, such as ratios of beta-amyloid 42/beta-amyloid 40, and oligomeric forms of beta-amyloid, should be conducted to systematically address the complexity of beta-amyloid signaling.

In conclusion, the current study found that in males there is an interaction of genotype and gonad removal in spatial working memory, while for females there is no interaction between these two variables. These variables, however, do interact in tau pathology for females, indicating that females might have pathological changes at the 10-month time point that have yet to correlate to behavioral consequences. Investigations into different time points of surgical treatment and potential hormone therapeutic intervention, with specificity to estrogen- and testosterone- containing therapies, should be investigated for profiles and efficacy for each sex in order to elucidate pathological and mnemonic outcomes. This will aid in fully understanding the time course of disease progression for this model of AD, and hopefully add insight into clinical disease etiologies.

CHAPTER 4

AN ASSESSMENT OF THE TRANSITION TO MENOPAUSE IN THE RAT IN THE

TGF344-AD MODEL OF ALZHEIMER'S DISEASE

ABSTRACT

Women are at disproportionately increased risk of Alzheimer's disease. One

possible reason for this increased risk is that women undergo a transition to reproductive

senescence not seen in men. This current project utilized 4-vinylcyclohexene diepoxide

(VCD) to model follicular depletion and transitional menopause in the ovary-intact

TgF344-AD transgenic rat model of AD, which displays comprehensive AD

neuropathology such as neurofibrillary tangle-like pathology, neuronal loss and

behavioral impairments. Four treatment groups were evaluated: wildtype (WT) VCD

(n=9), WT Sham (n=11), transgenic (TG) VCD (n=10), and TG Sham (n=9). Young rats

received either VCD injections or Sham injections, followed by a behavioral battery that

included the Water Radial Arm Maze (WRAM) to assess spatial working and reference

memory, the Morris Water Maze (MWM) to assess spatial reference memory, the Open

Field Task to assess locomotor activity and anxiety-like behavior, as well as the Visible

Platform Task to assess motor and visual acuity to perform a water-escape task. WRAM

results indicated that TG rats were impaired compared to WT rats during learning, and

that TG rats tended to be impaired compared to WT rats during memory retention for

working memory. TG rats were impaired for referemce memory compared to WT rats on

the MWM. Results for pathological assessments indicated that VCD-treated TG rats

tended to have more beta-amyloid (1-42) in the frontal cortex compared to TG Sham rats.

Collectively, TG animals were impaired for working and reference memory and

transitional menopause might influence changes in AD-like pathology in TG rats. Further

assessment of hormone therapy on this follicle deplete background should be considered

so that relationships between transitional menopause status, behavioral outcomes, and

AD-like pathology can be better understood.

INTRODUCTION

In the United States, there are currently 6.5 million people living with

Alzheimer's disease (AD), of which 4.33 million (roughly two-thirds) are women, and it

is the sixth leading cause of death in the nation (Alzheimer's Association 2021). It is

clear that AD pathology and symptomatology is different than natural aging. Indeed,

neural correlates and protein markers have been identified that mark AD progression, the

most common being beta-amyloid and tau pathology that lead to increases in

neuroinflammation, synaptic pruning, and ultimately neuronal loss (Braak et al., 1995;

Perl, 2010; Vickers et al., 2016). There is no cure for AD. Therapeutic approaches have

thus far been limited and unable to halt progression of this disease, in part because there

is a lack of scientific understanding of the pathological underpinnings of disease etiology.

The natural transition to menopause can impact AD pathology and

symptomatology. Menopause is retrospectively diagnosed after one year of amenorrhea

and occurs at the average age of 52 (NAMS 2015). With this transition comes a decline

of ovarian follicles that leads to an overall decline of ovarian-derived hormones such as

estrogens and progestogens (Harlow & Paramsothy, 2011; Burger et al., 2008). The

perimenopausal timepoint maps onto the prodromal stage of AD which usually occurs

15-20 years before AD-associated clinical symptom onset (Sperling et al., 2013). Clinical

research has demonstrated that women who undergo oophorectomy, the surgical removal

of the ovaries, before the natural onset of menopause, have an increased risk for cognitive

impairment and dementia compared to age-matched controls (Rocca et al., 2007). This

risk is further increased the younger that women were at surgical intervention, indicating

an interaction with age (Rocca et al., 2007). Further, preclinical studies have

demonstrated that surgical removal of the ovaries in rodents, known as ovariectomy (Ovx), can exacerbate AD neuropathology (Carroll et al., 2007, 2010).

AD and other dementias can be further impacted by hormone therapy that women might opt to take to combat the unwanted symptoms associated with menopause. In the Women's Health Initiative Memory Study, it was discovered that for women 65 years or older who were taking a combined estrogen (conjugated equine estrogen) and progestogen (medroxyprogesterone acetate), there was a small but meaningful increased risk of dementia compared to age-matched controls (Shumaker et al., 2003). Additionally, this combination hormone therapy did not decrease the incidence of mild cognitive impairment in these women compared to placebo-treated women (Shumaker et al., 2003). While this combination therapy demonstrated an increased risk profile for dementia, estrogen only containing therapies have been shown to be beneficial for cognition (Henderson, 2006; Wharton et al., 2011). Further, a meta-analysis containing 28 studies (Wu et al., 2020) demonstrated that there was a significant association between hormone therapy and AD which was more pronounced if it contained both an estrogen and progestogen component. Further, a study of over 350,000 women has shown that hormone therapy is associated with a decreased risk for many neurodegenerative diseases, including AD, and that increased duration of hormone therapy is related to greater efficacy in decreasing neurodegenerative disease risk (Kim et al., 2020).

Preclinically, the Ovx model is a suitable starting point since this is a binary model in which ovarian-derived hormones are drastically reduced. However, this model is not as translational as over 80% of women do not undergo surgical menopause and instead undergo a natural transitional menopause (NAMS 2014). To combat this,

researchers can utilize the 4-vinylcyclohexene diepoxide (VCD) model of follicular depletion to model transitional menopause in rodents. This model accelerates the atresia of ovarian follicles, primarily primordial and primary follicles, leaving the ovarian tissue intact and provides a hormone profile that is closer to what women undergo in the clinic (Kao et al., 1999; Springer et al., 1996; Hoyer et al., 2001). With this model we are able to assess a translationally relevant menopause etiology during clinically relevant time periods in AD progression.

For this experiment, we sought to utilize the TgF344-AD rat model of Alzheimer's disease created by Cohen et al. 2013. These transgenic rats express both the Swedish mutation of the human amyloid precursor protein (APP) and the mutant of the presenilin 1 that lacks exon 9 (PSEN1dE9). This model has a temporal development of pathology that is complimentary to the human condition of the disease. In this model, soluble beta-amyloid aggregates into amyloid plaques and endogenous hyperphosphorylated tau aggregates into neurofibrillary-like tangles that have a more traditional tear-drop morphology (Cohen et al., 2013). This model has also been shown to produce cerebrovascular dysfunction, neuroinflammation, synaptic dysfunction, and neuronal loss in areas of the brain that are associated with AD pathology (Joo et al., 2017, Smith et al., 2018, Wu et al., 2020).

In this study, we aimed to assess whether ovarian follicular depletion via VCD impacted subsequent cognitive outcomes and exacerbated AD-like pathology in the TgF344-AD rat model. Prior research has shown that working memory deficits in AD are some of the earliest, prodromal symptoms (Bianchini et al., 2014; Kirova et al., 2015; Zokaei & Husain, 2019). Additionally, we hope to assess precision-based analyses for

spatial navigation. Spatial memory analyses in our laboratory have centered on the differentiation between working and reference memory utilizing the water radial arm maze (WRAM) as well as impacts on reference memory utilizing the Morris Water Maze (MWM) (Bimonte & Denenberg, 1999; Braden et al., 2015, 2017; Koebele et al., 2017, 2019, 2021; Prakapenka et al., 2018, 2020). Precision, in the context of spatial navigation, can be classified as a form of high-resolution binding and is a complex process (Ekstrom & Yonelinas, 2020). Research suggests that the hippocampus is involved in high-resolution binding and involved in working and reference memory processes (Yonelinas, 2013). Lesions in the medial temporal area produce deficits in spatial precision (Kolarik et al., 2016). Further, research shows that there is an age-related decline in precision-based spatial strategies (McAvan et al., 2021) as well as precision in visual working memory (Peich et al., 2013). Precision-based strategies have been assessed for the MWM (Garthe et al., 2009; Gehring et al., 2015; Graziano et al., 2003; Janus, 2004; Ruediger et al., 2012; Stone et al., 2011)and it has been suggested that these strategies could be impacted by Alzheimer's disease since pathology involves hippocampal degradation that leads to impairments of allocentric deficits that can impact spatial strategy (Serino et al., 2014). Further, menopause has impacts on hippocampal function (Goto et al., 2011; Hara et al., 2012; Q. G. Zhang et al., 2013)and spatial memory (Acosta et al., 2010; Daniel & Dohanich, 2001; Koebele et al., 2017, 2019). Thus, it is plausible that transitional menopause can affect precision in spatial navigation; this will be tested here via the ovarian follicular depletion induction protocol given to TgF344-AD female rats.

METHODS

Subjects

Forty five-month-old, female, non-breeder, TgF344-AD rats were bred from a breeding pair obtained from the University of Southern California. Animals were pair-housed and fed *ad libitum* on post-natal day (PND) 28. Animals were placed on a 12-hour on/off light-dark cycle and were treated in compliance with the Arizona State University Institutional Animal Care and Use Committee protocol. All procedures adhered to the standards provided by the National Institutes of Health.

Animal Breeding

Since the TgF344-AD model is a hemizygous transgenic rat model, breeding males and females were partnered whereby one sex was transgenic (TG), and the other sex was wildtype (WT). Rats were harem bred with two females to one male per breeding cage to leverage successful breeding. On PND 10 tail samples were collected and stored at -70°C for polymerase chain reaction (PCR) analyses to determine genotype (either TG or WT), rats were ear punched for differentiation and sexed via anogenital distance. At PND 21 rats were weaned and sex housed into either male or female cages. Housing did not exceed six rats per cage and there were always at least two rats per cage. Female cages were removed to a separate colony room and male cages stayed in the breeding colony room. At PND 28 rats were pair-housed with their littermates, two to a cage or three to a cage if there was an odd number of rats. Rats were then either selected as breeders for colony maintenance, or non-breeders for experimental use. Litters for this study ranged from 3-15 pups with a minimum of 14 breeding pairs to achieve 80 experimental rats.

PCR

PCR was utilized to determine genotype via the APP transgene using tail samples from every rat as previously published (Mifflin et al., 2021). Tail samples were dissolved using Sodium Chloride-Tris-EDTA buffer as well as proteinase K in a 24:1 ratio (500ul per sample). Samples were then placed in a heated water bath (55°C) overnight. Samples were centrifuged at 15,000 rpm for 10 minutes at 4°C after which 500 ul of isopropyl alcohol was added to the supernatant. The solution was forcefully shaken to separate the DNA. Liquid was extracted and tubes were placed for 3 minutes in a Speedvac to evaporate all remaining liquid. DNA was then resuspended in TE buffer (150 µM) and incubated in the water bath (55°C) overnight. Samples were then added to a solution of 3 primers (APP, PRP internal control, and PRP reverse), distilled water and Taq buffer in a 1:7:12.5 ratio. A thermocycler was used to increase DNA output during which a gel of 1.8% agarose in 1 X Tris-borate EDTA with GelRed nucleic acid gel stain (10ul) to fluoresce DNA bands was created. 1 x green loading dye was added to each sample after thermocycler completion. Samples were pipetted into the agarose gel with the appropriate ladder. All gels ran for 30 minutes at 200mV and then placed under UV light for photo capture using BioRad Gel Doc XR. Bands were either classified as either 400bp indicating presence of the APP transgene or a TG rat or 750bp indicating a WT rat.

DNA Synthesis from Brain Tissue

To confirm genotype, right cingulate cortex tissue samples from every rat were utilized to synthesize cDNA from RNA for PCR analysis. 600ul of Buffer RLT (containing 10ul β-mercaptoethanol per 1ml of Buffer RLT; Qiagen, Hilden, Germany) was added to each sample after which samples were homogenized via probe sonicator

(Ultrasonic Processor, Cole Parmer, IL, USA). Samples were centrifuged at 14,000 rpm at 25°C for 3 minutes. The resulting supernatant was added to 600ul of 70% ethanol. 700ul of the solution was added to RNeasy spin columns (Qiagen, Hilden, Germany) and centrifuged for 15 seconds at 10,000 rpm at 25°C. Flow-through was discarded. 700 ul of Buffer RW1 (Qiagen, Hilden, Germany) was added to RNeasy spin columns and centrifuged for 15 seconds at 10,000 rpm at 25°C. Flow-through was discarded. 500ul of Buffer RPE (4:1 ratio 100% ethanol and Buffer RPE) was added to RNeasy spin columns and centrifuged for 15 seconds at 10,000 rpm at 25°C. Flow-through was discarded. An additional 500ul of Buffer RPE (4:1 ratio 100% ethanol and Buffer RPE; Qiagen, Hilden, Germany) was added to RNeasy spin columns and centrifuged for 2 minutes at 10,000 rpm at 25°C. Flow-through was discarded. Samples were centrifuged for 1 minute at 14,000 rpm at 25°C to eliminate any possible carryover of Buffer RPE or residual flow-through. After RNeasy spin columns were placed in new tubes, 50ul of RNase-free water (Qiagen, Hilden, Germany) was added and centrifuged for 1 minute at 10,000 rpm at 25°C to elute RNA and was either stored at -70°C or immediately used for cDNA synthesis. For cDNA synthesis the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) was used. A solution of up to 5ul of total RNA sample, 1ul of Primer (2uM), 1ul of dNTP mix (10mM), and DEPC-treated water (added until total amount was 10ul) (Life Technologies, Carlsbad, CA) was created for each sample and incubated at 65°C for 5 minutes and placed on ice for at least 1 minute. 10ul of cDNA synthesis mix (2ul 10X RT buffer, 4ul 25mM MgCl, 2ul M DTT, 1ul RNaseOUT (40U/ul), 1ul SuperScript III (200U/ul); Life Technologies, Carlsbad, CA)

was added to each RNA/primer mixture and centrifuged at 14,000 rpm for 1 minute at 25°C. Samples were incubated for 50 minutes at 50°C and then chilled on ice. Samples were centrifuged at 14,000 rpm for 1 minute at 25°C. 1ul of RNase H (Life Technologies, Carlsbad, CA) was added to each sample tube and incubated for 20 minutes at 37°C. Samples were stored at -20°C until use for PCR analysis. Using cDNA, PCR analysis was conducted as described above. After re-running PCR with cDNA, one sample was not confirmed (differed from previous PCR) and was relabeled as WT instead of TG. Thus, there were 20 WT rats and 19 TG rats.

VCD Administration

Rats were administered either VCD (160mg/kg/day, in 47%/47% dimethyl sulfoxide (DMSO)/saline, 6% VCD, n=20) (SenesTech Inc., Flagstaff, AZ) or vehicle solution (50%/50% DMSO/saline, n=20) (Sigma-Aldrich, St. Louis, MO) via intraperitoneal (i.p.) injection in accordance with published protocols (Acosta et al., 2009, 2010; Flaws et al., 1994b; Koebele et al., 2017; L. Springer et al., 1996). VCD injections began at approximately five months of age and occurred four times a week (Mondays, Tuesdays, Thursdays, and Fridays) for six weeks. One animal died during VCD injections due to a medical condition unrelated to VCD treatment; thus, 39 rats completed the study. The first day of VCD injections was considered day 1 of the study. VCD and Sham treated rats were equally distributed across Genotype such that there were four groups [WT Sham (n = 11), WT VCD (n = 9), TG Sham (n = 9), and TG VCD (n = 10)].

Vaginal Smears

Vaginal smears occurred for 8 consecutive days, beginning two weeks before behavioral testing. Each smear was determined to be in either the metestrus, diestrus, proestrus, or estrus phase as indicated in previous protocols (Goldman et al., 2007).

Water Radial Arm Maze (WRAM)

Rats began behavioral testing four months after receiving the first injection of VCD. The WRAM is a water-escape task used to assess spatial working and reference memory performance. The maze consisted of eight arms (38.1cm x 12.7cm) radiating from a circular center. Robust visual cues were in the room to assist with spatial navigation. Four of the eight arms contained hidden platforms (10 cm diameter) 2 cm below the surface of the water (18-20°C) which contained non-toxic black paint for opacity. Platforms were kept in consistent locations at the beginning of each testing day for each rat but were semi-randomized between rats. All location patterns used in this study were balanced across the maze such that no quadrant of the maze was underrepresented.

Rats were tested for 12 consecutive days, with four trials each day. The start arm remained constant for all rats and trials. At the start of the first trial, rats were placed in the start arm and were allowed to swim freely in the maze until they found a platform or until 3 minutes had elapsed, after which rats were led to the closest platform. After rats found a platform, they were kept there for a total platform time of 15 seconds and then were removed and placed in their heated testing cage for an inter-trial interval (ITI) of 30 seconds. During this ITI, the just-found platform was removed, and the maze was cleaned to remove debris and distribute olfactory cues. Because of this there were four trials per day (one to represent each platform) and as trials progressed, working memory load

increased since rats had to sustain more items in their working memory. Errors were quantified as entry into an arm that did not have a platform; arm entries occurred when a rat's snout passed 11 cm into an arm. Performance was assessed as the quantification of total errors. Performance was further broken into three separate error types: working memory correct (WMC), reference memory (RM), and working memory incorrect (WMI). WMC errors were entries into an arm that previously contained a platform for that day. RM errors were the first entry into an arm for that day that never contained a platform. WMI errors were subsequent entries into an arm that never contained a platform. On the last day of testing, day 13, a four-hour delay was implemented between trial 2 and trial 3 to assess delayed memory retention.

Morris Water Maze (MWM)

The MWM is a water escape task that is used to assess spatial reference memory performance. The maze consisted of a circular pool that was 188 cm in diameter. A platform (10 cm diameter) was hidden 2 cm below the surface of the water (18-20°C), with non-toxic black paint for opacity, in the north-east quadrant of the maze. Robust visual cues were situated on adjacent walls to assist with spatial navigation. A camera was mounted above the maze and the computer program Ethovision 12.5 (Noldus Instruments, Wageningen, Netherlands) was used to capture video and track the rat's path through the maze.

Rats were tested for five consecutive days, with four trials each day. At the start of the first trial, animals were dropped off at a quadrant (north, south, east or west) semi-randomly assigned to each trial throughout each of the days. Once in the maze, rats were allowed to swim freely until they found the platform or until one minute had elapsed, after

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which rats were then led to the platform. Rats were allowed to stay on the platform for 15 seconds, after which time the rat was returned to their testing cage for an 8 minute $\pm$ 2 minutes ITI. On Day 5, the last day of testing, after all test trials, a probe trial was introduced to assess whether the animals had spatially localized the position of the platform. Before the probe trial began the platform was removed and rats were allowed to swim freely in the maze for one minute after which they were returned to their testing cages. Distance to platform (cm) from the drop-off point was recorded and used to assess spatial localization of the platform over the testing period of five days.

Open Field Task

The open field task was used to assess anxiety-like behavior and locomotor activity (Hiroi et al., 2016; Koebele et al., 2020). The open field area consisted of a 100 cm x 100cm x 40 cm square apparatus that was placed in a dark testing room illuminated by red light. Rats were allowed to habituate in an adjacent room for 30 minutes prior to testing. After habituation, rats were brought into the room with the open field arena and dropped off in the center of the north wall. Rats were allowed to explore the arena for 10 mins before being placed back in their home testing cage. Between trials the arena was sprayed with water and swept with paper towels to distribute olfactory cues. Ethovision 12.5 tracking system (Noldus Instruments, Wageningen, Netherlands) was used to assess distance and time spent in the maze. The arena was divided into 25 equal squares (20cm x 20cm) for assessment (see Fig. 21H). Locomotor activity was analyzed as total distance covered in the arena. Anxiety-like behavior was analyzed as distance covered in the Center, Small Center, and Corners of the arena as well as time spent in the Center, Small Center, and Corners of the arena. More distance covered and more time spent in the Center and Small

Center of the arena as well as less distance covered, and less time spent in the Corners of the arena, were interpreted as decreased anxiety-like behavior.

Visible Platform

The visible platform test was used to assess motor and visual acuity for solving a water escape task. The maze consisted of a rectangular pool (100 x 60 cm) filled with clear water kept at 18-20°C with a platform situated inside the maze (10 cm diameter) protruding 4 cm above the water. The maze was encircled by a curtain so that there were no salient visual cues other than those found inside the maze.

Rats were tested for one day with six trials. At the start of trial 1, animals were placed in the starting position that remained constant throughout trials. The rats were allowed to swim freely until they found the platform or until 90 seconds had elapsed, after which the rats were led to the platform. After the rats found the platform, they were allowed to stay on it for 15 seconds after which the tester returned rats to their testing cage for an ITI of 5-8 minutes. All rats were tested for trial 1 before moving on to trial 2. Platform locations were assigned semi-randomly across trials. Latency (s) to the platform was measured.

Euthanasia

Rats were euthanized over two days, at the end of behavioral testing. Final body weights (g) were obtained earlier in the day directly before daily injections on the first day of sacrifices. Rats were anesthetized via isoflurane, and cardiocentesis was performed for blood collection. Rats were then decapitated, and brains removed for dissection. The Rat Brain Atlas was utilized for reference for brain dissections via plate designations (Paxinos & Watson, 1998). Two separate brain regions were raw brain dissected in the right frontal

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cortex (plates 5-14) and right dorsal hippocampus (plates 33-35) directly after removal. Tissue was weighed immediately and stored at -70°C until further analysis.

Uterine Horn and Ovarian Weights

Uterine horns and ovaries were dissected and trimmed of excess fat. Ovaries were cut from the tips of the uterine horns and separately weighed as left and right wet weights (g). Ovaries were placed in 10% formalin for two days, and then switched to 70% ethanol until future analysis. Uterine horn wet weights (g) were also collected.

Ovarian Follicle Counts

One ovary was randomly selected from each treatment group for histological analysis. The presence of healthy primordial, primary, secondary, and antral follicles, as well as corpora lutea in the ovaries were quantified. Ovaries were paraffin embedded, sectioned at 5μm, mounted (every 10th section), and stained with hematoxylin and eosin Y/phyloxine B. At every 20th section, all follicle types were counted at x20 magnification. Corpora lutea were counted at x10 magnification (3D HisTech DESK Scanner, Budapest, Hungary). In order to count total number of follicles per ovary, we used the following equation: $N_t = (N_0 \times S_t \times t_s)/(S_0 \times d_0)$. N_t is the total approximate number of follicles in the ovary, N_0 is the number of follicles detected in the ovary, S_t is the number of sections per ovary, t_s is the thickness of each section (μm), S_0 is the number of observed sections per ovary, and d_0 is the mean diameter of the ovary nucleus (Gougeon & Chainy, 1987). Ovary classification was determined by previous protocol (Haas et al., 2007; Koebele et al., 2017).

Western Blot

Beta-amyloid (1-42) expression levels were assessed from the right hemisphere in the frontal cortex and dorsal hippocampus via Western Blot analysis. Samples were

homogenized via probe sonicator (Ultrasonic Processor, Cole Parmer, IL, USA) in a 1:50

RIPA buffer (1% Triton X-100, 150mM NaCl, 0.5% Na deoxycholate, 0.1% SDS, 50mM

Tris, phosphatase inhibitor [Cat#: 524625, Millipore-Sigma], and protease inhibitor [Cat#:

5892791001, Millipore-Sigma]). Samples were then centrifuged for 10 minutes at 10,000

rpm at 4°C. A BCA (bicinchoninic acid) protein assay (ThermoFisher Scientific,

Pittsburgh, PA, USA) was utilized to measure protein concentration. Samples were run on

a 4-12% Bis-Tris NuPAGE gel using a SureLock Mini-Cell system (Invitrogen, Carlsbad,

CA, USA) and transferred to a polyvinylidene difluoride membrane (Immobulin-P).

Samples were loaded at 10μg of protein per brain region. Groups were counterbalanced

across gels and two gels were run per brain region for beta-amyloid and four gels were run

for tau antibodies. After transfer, the membrane was blocked in 5% non-fat milk for one

hour after which it was incubated overnight at 4°C in primary antibodies anti-beta-amyloid

(1-42) (1:500, Cell Signaling), as well as primary antibody anti-beta actin (1:20,000, Cell

Signaling) as a control measure. The membrane was then incubated in secondary antibodies

anti-mouse horseradish peroxidase (1:2000, Cell Signaling) and anti-rabbit horseradish

peroxidase (1:2000, Cell Signaling) at room temperature for one hour. Chemiluminescence

(LumiGlo and Peroxide, Cell Signaling) was used to visualize protein expression using a

film developer (Konica SRX-101A Film Processor, Tokyo, Japan). ImageJ software was

used to perform densitometry. Beta-amyloid (1-42) was analyzed together in accordance

with a previous protocol from our laboratory (Bernaud et al., 2022). Beta-amyloid (1-42)

expression was normalized to the loading control beta-actin for analysis.

Statistical Analyses

WRAM data were analyzed across Days 2-12 using an omnibus repeated measures analysis of variance (ANOVA) for each of the error types (WMC, WMI, and RM, and Total errors). The repeated measures consisted of Trials nested within Days while the independent variables were Genotype and Treatment. Data were further separated into two distinct blocks for Days 2-8 (the acquisition phase), and Days 9-12 (the asymptotic phase) and each block was individually assessed via an omnibus repeated measures ANOVA for each of the error types, as previously reported (Koebele et al., 2021). Additionally, we compared performance amongst groups for the moderate (Trial 3) and high (Trial 4) working memory load as our laboratory has previously shown that effects of several hormone-altering treatments become apparent when working memory load is highly taxed (Bimonte & Denenberg, 1999; Braden et al., 2010; Koebele et al., 2017). For the delay, for each treatment group separately, trials 3 and 4 from baseline (day 12) and postdelay (day 13) were averaged and compared as has been previously reported (Camp et al., 2012; Engler-Chiurazzi et al., 2011; Mennenga, Gerson, et al., 2015; Mennenga, Koebele, et al., 2015; Prakapenka et al., 2018).

Two other analyses were utilized to assess WRAM performance. The first analysis, the WRAM precision analysis, re-quantified errors as either one-arm away from the nearest platform, two-arms away from the nearest platform, three-arms away from the nearest platform, and four-arms away from the nearest platform. Within each of these error types, WRAM data were analyzed as an omnibus repeated measures ANOVA across Days 2-12, Days 2-8 (acquisition phase) and Days 9-12 (asymptotic phase) on trial 4, where errors made was the dependent variable and Genotype and Treatment were the independent

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variables. For the second analysis, the rates of learning analysis, linear regressions were run for Total errors, WMC errors, and WMI errors across all testing days (Days 2-12), Days 2-8, and Days 9-12. Differences in rates of learning, as assessed by slopes, were assessed via omnibus ANOVA where slope was the dependent variable and Genotype and Treatment were the independent variables

MWM total swim distance data were analyzed using an omnibus repeated measures ANOVA for all days of testing. The repeated measures consisted of Trials nested with Days, and the independent variables were Genotype and Treatment. Additionally, the probe trial was analyzed by assessing percent swim distance in the target quadrant (NE quadrant) where the platform had been previously located, and in the opposite quadrant (SW quadrant) to assess if the rats had correctly spatially localized the platform location for each group.

Open field data were assessed using an omnibus ANOVA where distance traveled in the Total, Center, Small Center, and Corners of the arena and total time spent in the Center, Small Center, and Corners of the arena were the dependent variables with Genotype and Treatment as the independent variables.

Visible platform data were assessed using a repeated measures ANOVA with time to platform was the dependent variable. The repeated measures were Trials and the independent variables were Genotype and Treatment.

Ovarian follicle data were assessed using an omnibus ANOVA where follicle count for each follicle type was the dependent variable and Genotype and Treatment were the independent variables.

One-way ANOVAs were utilized to assess beta-amyloid (1-42) expression in the frontal cortex and dorsal hippocampus. Beta-amyloid (1-42) expression was normalized to the beta-actin loading control. The independent variables were Genotype and Treatment. Pearson r correlations were analyzed for beta-amyloid (1-42) in each brain region for WMC and WMI errors for Days 9-12 of WRAM data where significant main effects of treatment were found. We used a false discovery rate (FDR) limit of 0.1 to protect against multiple correlations. Both FDR-corrected (Q) and uncorrected (p) values are reported (Benjamini & Hochberg, 1995).

RESULTS

WRAM

The omnibus ANOVA for Days 2-12 of testing revealed a main effect of Day for all animals for all error types (WMC, WMI, RM, and Total), [WMC, $F(10, 880) = 6.283$, $p < 0.001$; WMI, $F(10, 1320) = 24.652$, $p < 0.001$; RM, $F(10, 1320) = 12.430$, $p < 0.001$; Total, $F(10, 1290) = 15.238$, $p < 0.001$] which indicated that all animals were able to learn the task across Days 2-12. Additionally, there was a main effect of Trial for Days 2-12 for all animals for all error types (WMC, WMI, RM, and Total) [WMC, $F(2, 880) = 610.278$, $p < 0.001$; WMI $F(3, 1320) = 154.272$, $p < 0.001$; RM $F(3, 1320) = 25.594$, $p < 0.001$; Total, $F(3, 1290) = 249.703$, $p < 0.001$], in which errors increased as trials increased indicating that working memory load was impaired as trials progressed (data not shown).

For the learning phase (Days 2-8) for WMI errors, there was a marginal effect of Trial x Genotype interaction $F(3, 105) = 2.213$, $p < 0.1$ where TG rats tended to make more errors than WT rats. This trend was led by performance on trial 3, the moderate

working memory load trial, where there was a significant effect of Genotype, $F(1, 35) = 4.954$, $p < 0.05$, where TG rats made more errors than WT counterparts (See Fig. 19).

For the asymptotic phase (Days 9-12) for WMC errors, there was a marginal effect of Treatment $F(1, 35) = 3.886$, $p < 0.1$, such that VCD-treated rats tended to make fewer errors than Sham counterparts. This trend was led by performance on trial 3, the moderate working memory load trial, where there was a significant effect of Treatment $F(1, 35) = 5.922$, $p < 0.05$, where VCD rats made fewer errors than Sham counterparts (See Fig. 19).

For Day 13 there was a four-hour delay between trials 2 and 3. There was no main effect of Delay for any error type for any group between baseline trials and post-delay trials, suggesting that all animals were able to maintain baseline working memory performance (data not shown).

MWM

Swim distance to platform decreased across days of testing [Main effect of Day: $F(4,35) = 62.693$, $p < 0.001$] for all animals, indicating that rats were able to learn the task. There was a significant main effect of Genotype $F(1, 35) = 5.922$, $p < 0.01$ where TG rats had an increased swim distance to platform compared to WT counterparts (See Fig. 20). For the probe trial, there was a significant effect of Quadrant for each group [WT Sham: $F(1, 11) = 69.574$, $p < 0.001$; WT VCD: $F(1, 9) = 48.576$, $p < 0.001$; TG Sham: $F(1, 9) = 55.364$, $p < 0.001$, TG VCD: $F(1, 10) = 57.829$, $p < 0.001$] where each treatment group swam a significantly higher percentage of distance in the target quadrant (NE) where the target used to be located versus the opposite quadrant (SW), indicating

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that treatment groups were able to correctly spatially localize to the platform (See Fig. 20).

OFT

For Total Distance covered, there was a significant effect of Genotype $F(1, 35) = 6.505, p < 0.05$, such that TG rats move significantly more distance than WT counterparts. Additionally, there was a marginal effect of Treatment $F(1, 35) = 3.739, p < 0.1$, such that VCD-treated rats tended to move less total distance than Sham counterparts (See Fig. 21). For Center Distance there was a significant effect of Genotype $F(1, 35) = 10.970, p < 0.01$, such that TG rats move significantly less distance than WT counterparts. This effect was also found for Small Center Distance where there was a significant effect of Genotype $F(1, 35) = 14.589, p < 0.001$, such that TG rats move significantly less distance than WT rats. For Corner distance, there was a marginal Genotype x Treatment interactions $F(1, 35) = 3.837, p < 0.1$, where TG Sham rats moved significantly more distance than TG VCD rats, $p < 0.05$, while this trend was not found in WT rats (WT Sham vs. WT VCD, p > 0.05). Additionally, there was a significant effect of Treatment $F(1, 35) = 5.978, p < 0.05$, such that VCD rats move significantly less distance than Sham rats for Corner distance.

When assessing duration, for Center Time there was a significant effect of Genotype $F(1, 35) = 12.586, p < 0.01$, such that TG rats spent significantly less time in the center of the arena than WT rats (See Fig. 21). This effect was also found for Small Center Time $F(1, 35) = 19.267, p < 0.001$, such that TG rats spent significantly less time in the small center of the arena compared to WT rats. For Corner Time, there was a marginal Genotype x Treatment interaction $F(1, 35) = 3.196, p < 0.05$; for Sham rats,

there was a Genotype effect whereby TG rats spent significantly more time in corners compared to WT rats, $p < 0.05$, while for VCD rats this effect was not present (WT VCD vs. TG VCD, $p > 0.05$).

Visible Platform

The visible platform test was used to assess the ability of a rat to solve a water escape task. There was a main effect of Trial [$F_{(5, 34)} = 6.721$, $p < 0.001$] such that as trials progressed latency to the platform decreased, indicating better performance as trials progressed with an average latency of 8.32 seconds ± 0.303 seconds to reach the platform across all trials (data not shown). There was no effect of Genotype or Treatment, nor a significant Genotype x Treatment interaction, indicating that the groups did not differ in their ability to perform the procedural components of a water-escape task.

Precision Based WRAM Analysis

All analyses assessed are for trial 4 alone for the WRAM. For Days 2-8, there were no main effects of Genotype or Treatment, nor a Genotype x Treatment interaction, for any of the arm away types. For Days 9-12, for the 2-arm away analyses, there was a marginal effect of Genotype $F_{(1, 35)} = 3.639$, $p < 0.1$, whereby TG rats tended to make more errors than WT counterparts. There were no other effects for any other arm type (See Fig. 22).

Rates of Learning Analysis

There were no effects of Genotype or Treatment as well as no Genotype x Treatment interactions for Days 2-12, Days 2-8, or Days 9-12 for Total, WMC, and WMI errors on the WRAM. These data indicate that there was no difference in the rates of learning on the WRAM for any group (data not shown).

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Vaginal Cytology

Across all days of vaginal smears, vaginal cytology was mixed (primarily metestrus or persistent diestrus for all VCD-treated rats). These results indicate that ovarian cyclicity was halted for VCD-treated rats. Cytology was not affected by genotype. Further, non-follicle deplete Sham rats displayed vaginal cytology that was consistent with a 4-5 day estrous cycle, indicating that this group had normal ovarian cyclicity.

Ovarian Histology

For ovarian histology, there was a main effect of Treatment for primordial [$F(1, 35) = 245.441, p < 0.001$], primary [$F(1, 35) = 155.471, p < 0.001$], secondary [$F(1, 35) = 107.128, p < 0.001$], and antral [$F(1, 35) = 138.225, p < 0.001$] follicles, as well as for corpora lutea [$F(1, 35) = 64.150, p < 0.001$] whereby VCD-treated rats had fewer follicles and corpora lutea than Sham counterparts. There were no effects of Genotype on follicle or corpora lutea count (See Fig. 23).

Western Blot Analysis

There was a marginal effect of Treatment for beta-amyloid (1-42) for the frontal cortex $F(1, 9) = 3.720, p < 0.1$, whereby VCD rats tended to have more beta-amyloid than Sham counterparts (See Fig. 24). There was no effect Treatment for beta-amyloid (1-42) for the dorsal hippocampus. After running Pearson r correlations, using an FDR of 0.1 to account for multiple correlations (Benjamini & Hochberg 1989), there was a positive correlation in the dorsal hippocampus such that rats that tended to have more beta-amyloid expression also tended to make more WMC errors during Days 9-12 on the

WRAM on the moderate working memory trial (trial 3) [$r(9) = .707$, $p < 0.05$, $Q < 0.1$] (See Fig. 24).

DISCUSSION

The current study is one of the first to systematically assess effects of follicular depletion on AD-like pathology and symptomatology. Although deficits have been demonstrated where surgical menopause via Ovx can exacerbate AD outcomes (Carroll et al., 2007, 2010; Heikinnen et al., 2014; Hu et al., 2016), a transitional model of menopause is less straightforward. For this study, TG rats were impaired for working memory as assessed by WMI errors on WRAM band for reference memory on the MWM. Follicular depletion improved working memory as assessed by WMC errors when working memory load was moderate. For locomotor and anxiety-like assessments, TG rats had overall heightened locomotion throughout the OFT but within the center, they travelled less distance and spent less time. These results indicate that TG rats exhibit a heightened anxiety-like profile as has been demonstrated before in this model (Cohen et al., 2013; Pentkowski et al., 2018; Tournier et al., 2021). Further there was an interaction between genotype and follicular depletion such that rats with no follicular depletion had a genotype effect where TG Sham rats spent more time in the corners of the open field arena compared to WT Shams. This effect was not found in rats with follicular depletion. For pathological measures in these rats, we found marginally increased expression of beta-amyloid in the frontal cortex in follicle-deplete rats compared to sham counterparts with no difference between treatment groups in the dorsal hippocampus. However, we did see a positive correlation in the dorsal hippocampus, whereby Sham rats that had tended to have increased expression of beta-amyloid also tended to have poorer working

memory performance in the WRAM as assessed by WMC errors during the asymptotic phase of testing.

Previous research in our laboratory has demonstrated marginal impairments with genotype with this model at 6 months of age and robust impairments at 12 months of age (Bernaud et al., 2022). Interestingly, when rats were tested at the 9-month time point, there was no difference overall in errors made on the WRAM (Bernaud et al., 2022). The author's posited that this might be due to a compensatory factor that the animals undergo whereby they choose a different non-spatial strategy (Berkowitz et al., 2018; Bernaud et al., 2022; Korol & Wang, 2018). Typically, rats will adopt an allocentric, hippocampal-based strategy, but rats can also adopt egocentric, striatal-based strategies whereby they simply make consecutive right or left turns to find the platform (Korol et al., 2018). We attempted to address this concern with our secondary precision-based analyses that would focus less on the differentiation between working and reference memory and more on precision. For this assessment, TG rats were marginally impaired for the two-arms away error type during the asymptotic phase of the task compared to WT controls. Since this finding was only for this error type and not closer or farther error types, it might be that TG rats were persisting in arms where they had previously been rewarded compared to WT counterparts. If this was the case, more detailed assessment of perseverance would need to considered. Additionally, it is plausible that we did not see any genotype by treatment interactions because of this compensatory mechanism that overshadowed these possible effects.

Additionally, on the moderate working load trial during the asymptotic phase of testing on the WRAM there was an improvement with VCD which was unexpected.

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Previous research in our laboratory has demonstrated spatial memory impairments with follicular depletion induced via VCD (Acosta et al., 2009; Koebele et al., 2017). Previous research has shown that in an APOE-TR mouse model of AD, VCD treatment interacts with genotype such that the VCD+APOE4 group was unable to distinguish novel from familiar objects (Pontifex et al., 2020). Additionally, growth factors such as BDNF and mTOR were downregulated for both VCD treatment and transgenic rats (Pontifex et al., 2020). Further, there was an interaction between follicular depletion and genotype such that the VCD+APOE4 group had roughly a 30% decrease of Ephrin type-B receptor 2 (Ephb2), an important protein in learning and memory that can rescue cognitive function in models of AD compared to APOE4 transgenic rats and APOE3 controls (Cissé et al., 2011; Pontifex et al., 2021).

Hormone therapy has been implicated as a potential therapeutic in clinical and preclinical AD populations. Studies have demonstrated attenuation of Ovx induced impairments in transgenic rats with 17beta-estradiol treatment for beta-amyloid pathology (Amtul et al., 2010; Carroll et al., 2007; Zheng et al., 2002), tau pathology (Carroll et al., 2010), and cognitive outcomes (Heikkinen et al., 2014). Additionally, progesterone has be implicated to attenuate tau pathology via increased neurofibrillary expression in APP/PS1 Ovx mice (Carroll et al., 2010). Further, in non-transgenic models, such as an intracerebral ventricular injection of beta-amyloid, 17beta-estradiol alone, progesterone alone, and 17beta-estradiol in combination with progesterone has been shown to improve spatial working memory performance in the MWM compared to controls (Hu et al., 2016). It is theorized that some of the neurobiological mechanisms behind these potential neuroprotective effects of hormone therapy are via 17beta-

estradiols interactions with beta-amyloid in the brain (Jayaraman & Pike, 2014; Uddin et al., 2020; Zheng et al., 2002).

In conclusion, our assessments herein utilizing a model of ovarian follicular depletion in the TgF344-AD rat model of AD indicate a somewhat divergent path for interpretation of the effects of follicular depletion on AD-like pathology and symptomatology. These data provide key insight into a critical temporal timepoint in this model and how AD pathology, spatial working and reference memory, and precision-based spatial navigation can potentially interact with ovarian follicular depletion sensitive windows. Future research systematically assessing estrogen containing hormone therapies will provide a clearer understanding of their therapeutic benefits as they map onto transitional menopause in an AD model.

CHAPTER 5

DISCUSSION

General Conclusions and Overarching Goals

This dissertation has investigated, broadly speaking, effects of gonadal hormone deprivation, menopause etiologies, hormone administration, spatial working and reference memory, precision-based spatial navigation, and AD pathology with behavioral symptomatology. The overarching goal of the studies presented herein was to assess whether alterations in the hormonal milieu could have negative impacts on healthy and neurodegenerative aging, and whether these impairments could be attenuated with exogenous hormone intervention. Interactions between the aforementioned factors and key findings from each study are discussed below.

Chapter 2 Key Findings – MPA and progesterone can attenuate follicle depletion-induced impairment

In Chapter 2 of this dissertation, we found that MPA and progesterone administration attenuated impairments seen with follicular depletion alone for working memory on the WRAM during the early learning phase. MPA impaired performance on the last day of testing, as has been previously demonstrated when given in an Ovx model (Braden et al., 2010; 2011). The attenuation of impairment during the early learning phase of the WRAM seems to be working memory specific since follicular depletion also impaired reference memory on the WRAM, and this impairment was not impacted by any of the progestogen treatments. We also found that there were no differences in spatial reference memory between any of the groups for the spatial reference memory MWM, and that all groups were able to spatially localize to the platform on the last day of

testing. Indeed, there were no differences in the visible platform task across groups, indicating that all animals were able to perform the procedural components of a water escape task at similar capacities.

For physiological variables such as serum hormone levels, ovarian histology and uterine horn weights we found effects of treatment. Analysis of serum hormone levels demonstrated that there were no changes in estradiol levels across groups, but with follicular depletion alone there was a decrease in progesterone and an increase in androstenedione, indicating an androgen dominant hormonal profile as has been previously reported (Frye et al., 2012; Acosta et al., 2009; Koebele et al., 2017; Mayer et al., 2004). For progesterone and micronized progesterone treatments, there were marked increases of progesterone and androstenedione, likely to due to synthesis via 17-alpha-hydroxylase/17,20 lyase to 17-OH progesterone to androstenedione (Diotel et al., 2018). For MPA treatment, however, there was a decrease in progesterone and androstenedione serum hormone levels, indicating that MPA initiated a negative feedback loop for both of these endogenous hormones. There was a decrease in uterine horn weights in all VCD-treated rats, and progesterone treatment decreased uterine horn weights compared to MPA treatment, indicating a protective role of the uterus for progesterone above and beyond that of MPA, at least at this dose. For our ovary findings, all VCD-treated rats had significantly fewer primordial, secondary, and antral follicles, as well as corpora lutea, compared to vehicle controls. Interestingly, there was an increase in primary follicles with VCD treatment that was attenuated by MPA treatment. Since all other follicle types were decreased with VCD treatment, ovarian cyclicity was halted, and primary follicles are not known to produce endogenous estrogens, we concluded that this

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increase in primary follicles did not have biological action through sex steroid hormones and that VCD treatment still initiated a form of transitional menopause through follicular depletion.

For our brain assays, there was no difference in GABAergic expression as assessed via changes in GAD 65+67 expression for any of the brain regions assessed. Progesterone treatment, however, did demonstrate a positive correlation GAD expression. Specifically, rats that had higher GAD 65+67 expression in the ventral hippocampus also tended to make more working memory errors on the WRAM, indicating that the GABAergic system could mediate cognitive outcomes with progesterone treatment.

Chapter 3 Key Findings – Gonadectomy impacts behavior for males and AD-like pathology for females

In Chapter 3 of this dissertation, we found female rats with AD-like pathology were impaired for spatial working memory, regardless of surgical treatment during learning on the WRAM, and that this was led by the highest working memory load trial. Further, during the asymptotic phase of the WRAM, gonadectomy improved working memory as assessed by WMI errors and this effect was led by the highest working memory load triala. For males, during learning on the WRAM, there was genotype by surgical treatment interaction whereby gonadectomy induced a genotype effect. Specifically, in gonadectomized males only, transgenic rats were impaired for working memory compared to wildtype counterparts. On the other hand, there was no difference between transgenic and wildtype rats in the sham male group. For males during the asymptotic phase of the WRAM, transgenic rats were impaired for working memory as assessed via WMI errors compared to wildtype controls when assessed for the highest

working memory load trial. Moreover, both males and females were impaired for spatial reference memory as assessed by the MWM. Interestingly, on the probe trial of the MWM task, female transgenic gonadectomized rats were unable to spatially localize to the platform thereby indicating an impairment that was unique to this group. These findings suggest that in female transgenic animals, gonadectomy further exacerbates spatial reference memory impairments.

We also evaluated anxiety-like behaviors in chapter 3, For females, transgenic rats had higher anxiety-like behavior as demonstrated by increased corner distance and decreased center time in the OFT, with no differences in locomotion compared to wildtype controls. For males, both gonadectomized and transgenic rats had increased locomotion in the OFT compared to sham and wildtype counterparts. Further, transgenic rats displayed more anxiety-like behavior, via decreased center distance, small center distance, and center time compared to wildtype controls. Additionally, gonadectomized rats displayed less anxiety-like behavior via increased center distance, small center distance, center time, and small center time compared to sham counterparts.

For pathological assessments, there was no difference in beta-amyloid (1-42) expression in either the frontal cortex or dorsal hippocampus between gonadectomized and sham rats for either males or females. For tau pathology, however, there was an interaction between genotype and surgical treatment in female rats whereby wildtype gonadectomized rats had higher AT8/Tau 5 ratios compared to wildtype sham controls, while transgenic gonadectomized rats had lower AT8/Tau 5 ratios compared to transgenic sham rats. Additionally, there was an overall increase in AT8/Tau 5 ratio indicating more pathogenic tau burden as has been demonstrated previously in this model

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(Joo et al., 2017; Cohen et al., 2013). For males, there was a marginal increase in AT8/Tau 5 ratios in gonadectomized rats versus sham counterparts, but this was not found when assessing transgenic vs wildtype rats. These data suggest that tau pathology can be impacted by gonadal hormone deprivation in males, and that this increase in tau pathology could be independent of AD.

Chapter 4 Key Findings– Follicular depletion can exacerbate AD-like pathology

In Chapter 4 of this dissertation, we found that transgenic rats had impaired working memory compared to wildtype controls during the learning phase of the WRAM for the moderate working memory load trial. These results are consistent with those from chapter 3 which indicate that female transgenic rats have working memory impairments regardless of menopause status, and that this impairment is especially prevalent when working memory load is taxed. Further, follicular depletion via VCD treatment improved working memory during the asymptotic phase of the WRAM compared to vehicle controls during the moderate working memory load trial. This finding is aberrant from others in the literature which suggests an impairment with follicular depletion (Acosta et al., 2009; Koebele et al., 2017). However, these findings are somewhat consistent, with data from chapter 3 whereby gonadectomized female rats had improved working memory when working memory load was highly taxed. It is plausible that assessment at this timepoint induces subtle memory enhancements for working memory in this particular model.

When assessing precision for spatial navigation, transgenic rats were impaired during the asymptotic phase of the WRAM compared to wildtype counterparts for 2-arm away errors. Since there were no differences between groups in 1-arm away, 3-arm away

or 4-arm away errors we inferred that this difference in 2-arm away errors was not simply due to rats making more of another error type and thus, rats were making more errors closer to the target. These data suggest that transgenic rats made more errors that were closer to the target. There were no differences in rates of learning on the WRAM, another analysis of precision in spatial navigation.

For physiological outcomes, there were overall decreases in primordial, primary, secondary, and antral follicles, as well as corpora lutea. This is in distinct contrast to data from chapter 2 whereby primary follicles were increased with VCD treatment. This is likely due to the fact that the rat strain utilized in chapter 2 of this dissertation was the F344-CDF strain, while the wildtype background strain utilized for chapters 3 and 4 of this dissertation was the F344-NHSD strain. Since the increase in primary follicles in VCD-treated F344-CDF rats has been attributed to KIT ligand signaling, which might not be aberrant in the F344-NHSD strain, it is plausible that primary follicles would be decreased in the F344-NHSD strain as they have been for the F344-NIH strain (Kezele et al., 2005; Fernandez et al., 2008).

For pathological assessments in animals from chapter 4, there was a marginal increase in beta-amyloid (1-42) in follicle deplete rats compared to vehicle counterparts, indicating a role for transitional menopause for effects on amyloidogenic pathology. Although no effects were found between sham and VCD-treated animals, indicating no effects on follicular depletion in the dorsal hippocampus, there was a positive correlation in this brain region for sham rats whereby rats that tended to have higher beta-amyloid (1-42) expression also tended to make more working memory correct errors. These data

demonstrate that beta-amyloid (1-42) might be a potential mechanism through which memory impairments are induced in this AD model in sham rodents.

Interpretation of WRAM and MWM effects

It should be noted that while both the WRAM and MWM assess reference memory, the addition of a working memory component to the WRAM potentially does not allow a true delineation between these two memory systems. As previously discussed, the WRAM and the land based version (RAM) utlilize a win-shift paradigm whereby once a reinforcer has been located (at the end of an arm) the rat needs to shift to the next target location where a reinforcer resides (Olton et al., 1979; Jarrad et al., 1984; Bimonte-Nelson et al., 2015) The WRAM can assess only working memory when all arms are reinforced or both working and reference memory when some arms are reinforced and others are not. Traditionally, the working and reference memory components of the RAM and WRAM have been thought to assess two orthogonal memory systems (Jarrad et al., 1985; Olton et al., 1979). However, more recent research has suggested that these two components are linked for this task. Indeed, a previous study has demonstrated that aged rats are impaired on working memory when it is the only component of the WRAM (e.g., when all arms are reinforced and only WMC errors can be made) (Bernaud et al., 2021). When more arms were added without reinforcers and thus a reference memory component was included, there were differences for WMC errors for aged rats (Bernaud et al., 2021). Aged rats, did however, make more WMI errors than young rats; indeed, aged rats were committing errors when returing to RM arms and not arms where they had been previously rewarded (Bernaud et al., 2021). Further, for aged rats, there was a positive correlation whereby RM errors were positively correlated with WMI errors at the

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highest working memory load, indicating that RM and WMI errors are closely linked

(Bernaud et al., 2021). This finding that RM and WMI measures are not truly orthogonal

could account for some of the differing effects in the reference memory-based MWM and

the reference memory component of the WRAM such that group differences can be seen

in what is operationally defined as "reference memory" for one task but not the other

(Camp et al., 2012, Acosta et al., 2009, Braden et al., 2017; Holter et al., 2019). For this

circumstance, since RM errors are linked to WMI errors, there is the possibility that for

reference memory rats are utilizing both memory systems (reference and working

memory) for the WRAM rather than one system (reference memory) as such is the case

for the MWM; therefore, these two measures of reference memory may in fact be tapping

reference memory as operationally defined, but they are also necessitating additional

requirements simultaneously, depending on the task. It is thus plausible that interpretation

of WRAM data, if any type of working memory measurement is present, should focus on

the differentiation between WMC and WMI errors, and acknowledge that WMI errors are

intrinsically linked to the reference memory component of the WRAM.

Alternate Avenues of Pathological Assessments

In the herein research, all pathological assessments were conducted utilizing

western blot protein analysis to determine amyloid and tau relative protein levels in brain

regions impacted by AD, with a specific interest in regions associated with spatial

working and reference memory as our primary behavioral measures assessed these

particular memory types. Although this approach is valid, alternate approaches might

yield further insight into nuances in pathology that are not available using the western

blot approach. Microscopy and immunohistochemistry have been utilized to assess beta-

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amyloid and tau deposition in brain regions impacted by AD, such as the dorsal hippocampus and frontal cortex, in AD models (Cohen et al., 2013; Joo et al., 2017b; Lee et al., 2022; Tournier et al., 2021). Utilizing immunohistochemistry not only provides quantification of beta-amyloid and tau, but it provides neuroanatomical localization in each coronal slice. These data provide a more comprehensive understanding of protein deposition and aggregation, and when assessed at different timepoints during disease progression, a more thorough understanding of where pathological impacts first occur and how they spread as the disease advances.

Another alternate avenue of beta-amyloid and tau assessment would be to utilize enzyme-linked immunosorbent assays (ELISAs) to achieve accurate concentrations of either protein rather than relative expression (Lachno et al., 2015; Song et al., 2016). With this technique, amounts of protein can be compared across different brain regions of interest to ascertain where pathogenic burden is highest. Commercial kits for beta-amyloid (1-42) (Cat# KMB3441, Thermo Fisher Scientific, Waltham, MA) and phosphorylated tau (Cat# KMB7041, Thermo Fisher Scientific, Waltham, MA) are commercially available and have been utilized in the past to assess these proteins with great accuracy. One caveat to our western blot analyses herein was our use of beta-actin as a loading control. Previous research has indicated that for beta-actin, when incubated for long periods of time and when protein loading is high, western blot assays can fail to discern differences in actin levels (Dittmer & Dittmer, 2006). The authors posit that loading controls such as alpha-GAPDH might be more stable as a loading control when accounting for increase protein concentration and longer incubation periods (Dittmer & Dittmer, 2006). Further, utilizing real-time PCR, researchers have found that in AD post-

mortem tissue, beta-actin was differentially expressed while GAPDH remained relatively stable (Gutala & Reddy, 2004). This might be due to the fact that beta-actin targets actin, a protein important for maintaining cytoskeletal structure and muscle contracture (Dominguez & Holmes, 2011). Since AD pathology involves synaptic and neuronal loss, it is plausible that beta-actin expression can decrease as disease progression becomes more severe (Coleman & Yao, 2003; West et al., 1994; Scheff et al., 2003)(Coleman & Yao, 2003; West et al., 1994; Scheff et al., 2003)(Coleman & Yao, 2003; West et al., 1994; Scheff et al., 2003)(Coleman & Yao, 2003; West et al., 1994; Scheff et al., 2003). Even with these flaws, beta-actin is still used as a loading control for western blot procedures in AD (Chaudhary et al., 2021; Hernández et al., 2022; Lerner et al., 2012; Więckowska-Gacek et al., 2021). Future evaluations of AD pathology utilizing western blots should focus on using GAPDH as a loading control rather than beta-actin. Additionally, investiture into alternate techniques for pathological assessments might provide a broader understanding of the underpinnings of neurobiological alterations in AD.

Gonadal Hormone Deprivation Negatively Impacts AD Progression

Previous research has demonstrated that decrements in endogenous circulating gonadally-derived hormones can impact AD pathology, and indeed, might be a mediating variable in AD risk, prevalence, and progression. For example, men and women with AD have lower levels of testosterone and estrogens, respectively, compared to age-matched non-AD individuals (Callahan et al., 2001; Barron & Pike, 2012). Removal of gonadal hormones in either males or females via castration or ovariectomy, respectively, can detrimentally impact AD progression. For example, Ovx can exacerbate AD

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neuropathology and symptomatology. One study found that Ovx increased levels of beta-amyloid while decreasing spontaneous alternation behavior (Carroll et al., 2007). Further, estrogen deficiency via aromatase knock-out, increased beta-amyloid in the APP23 model of AD (Yue et al., 2005). In a model of follicular depletion utilizing VCD, transgenic APOE-TR mice showed an interaction between follicular depletion status and genotype such that mice with AD-like pathology plus follicular depletion could not discriminate between familiar and novel objects compared to all other groups (Pontifex et al., 2021). This inability to discriminate is potentially mediated through expression of Ephrin type-B receptor 2 (Ephb2), which was decreased in APOE4 mice with follicular depletion compared to all treatment groups (Pontifex et al., 2021). With known detriments with gonadal hormone loss, there is potential for a therapeutic effect of estrogen and progestogen containing therapies to attenuate some facets of AD pathology and symptomatology. However, even with this biological feasibility and the many studies supporting the plausibility of such effects, the details and the nuances of efficacy have yet to be understood so that a true clinical impact can be made.

Estrogens and Progesterone as Therapeutics in Healthy and AD Aging

In healthy and neurodegenerative aging, there is a theory known as the "window of opportunity," in which there is an optimal time point were exogenous sex steroid hormone administration will be the most efficacious (Bean et al., 2015; Daniel et al., 2015; Davey, 2013; Maki & Manuscript, 2013; Rocca et al., 2011; Singh et al., 2013; Wu et al., 2020; K. Zhang et al., 2019). This is especially prudent with estrogen administration whereby the beneficial effects of estrogens in humans (Rocca et al., 2010, 2011) and rodents (Daniel et al., 2006; Gibbs et al., 2000) are obviated if estrogens are

administered well after the onset of menopause or for preclinical models, surgical ovarian removal. One study found that E2 administration directly following Ovx, or three months after Ovx, increased spatial working memory in middle-aged rats; this improvement was not seen when E2 was given 10 months post Ovx (Gibbs et al., 2000). This finding was corroborated by another study which found that E2 administration given immediately after Ovx improved spatial working memory, while E2 given five months post Ovx did not modify working memory compared to vehicle controls (Daniel et al. 2006). Further, acute E2 exposure after 10 weeks of Ovx produced decreases in spine density in the hippocampal CA1 region compared to rats that received E2 one week after Ovx, although it should be noted that spine density was increased in both groups compared to vehicle controls (McLaughlin et al., 2008). Additionally, E2 given immediately after Ovx prevented amyloidogenesis, as assessed via rising beta-amyloid (1-42) levels, seen with Ovx alone as well as decreased CA3 hypersensitivity found with Ovx alone (Zhang et al., 2013). When E2 was given at 10 weeks post Ovx, however, there were no differences between groups, indicating that while AD-like pathology can be mediated by estrogenic activity, estrogen administration must be given at appropriate timepoints to rescue impairments seen with gonadal hormone deprivation alone (Zhang et al., 2013).

It is clear that exogenous hormone administration can positively impact AD. In healthy aging, estrogens have been shown to increase neurogenesis in the dentate gyrus and this increase is related to improvements in learning and memory (Galea et al., 2013). In an intracerebroventricular model of AD in rats, chronic estradiol alone, chronic progesterone alone, and estradiol plus progesterone improved performance in the MWM compared to rats that received sham surgery or rats that received OVX only (Hu et al.,

2016). Further, E2 administration attenuated the increase of beta-amyloid seen with Ovx alone in the 3xTG-AD mouse model, while progesterone administration decreased levels of hyperphosphorylated tau compared to Ovx alone (Carroll et al., 2007). Other research has demonstrated that estradiol treatment given to APP/PS1 OVX mice improved performance on a spontaneous alternation T test, as well as on reference memory as tested in the land-based radial-arm maze but did not impact amyloid plaque formation or amyloid accumulation (Heikkinen et al 2004). E2 also increased beta-amyloid clearance via the insulin degradation enzyme and decreased hyperphosphorylated tau via interactions with phosphatases and kinases such as PKA, Wnt, and GSK-3beta (Jayaraman et al., 2012; Zhang et al., 2008). E2 favors non-amyloidogenic pathways such as the MAPK/ERK pathway, and can decrease BACE1, while also clearing beta-amyloid via microglial phagocytosis (Singh et al., 1999). Some researchers have posited that effects of estrogens are relegated to brain-derived estrogens rather than peripheral estrogens (Yue et al., 2005). Indeed, estrogen deficiency in the APP23 model of AD occurred via aromatase knock out and primarily affected levels of estrogen within the brain (Yue et al., 2005). Brain estrogen deficiency increased beta-amyloid deposits and created an early onset of pathology while peripheral deficiency of estrogens using Ovx in the non-aromatase knock-out APP23 model showed similar plaque deposition compared age-matched APP23 sham controls (Yue et al., 2005). Further exploration intro progesterone as a neuroprotectant has found that progesterone can lower levels of BACE1 gene expression as well as modulate gamma-secretase, while alpha-secretase remains unchanged (Zhao et al., 2012; Dang et al., 2013; Amtul et al., 2010; Jung et al., 2013).

Androgens Can Positively Modulate AD Progression

Males with AD have lower testosterone levels than non-AD males (Callahan et al., 2001; Barron & Pike, 2012). Increases in endogenous androgens via aromatase enzyme blockage decreases AD pathology (McAllister et al., 2020). Tau pathology is also affected by androgens as heat-shock induced hyperphosphorylated tau is prevented by androgens a process which is independent of estrogens (Papasozomenos et al., 1997). Further, androgen depletion therapy (ADT) for treatment of prostate cancer, which roughly 500,000 men are prescribed, has been shown to negatively impact cognition (McGinty et al., 2014; Nelson et al., 2008; Shahani et al., 2008). Additionally, ADT is also associated with an increased risk of AD (Nead et al., 2016). This is especially relevant considering that as many as 20-30% of men who are administered ADT can have prolonged suppression of androgens (Pickles et al., 2002; Yoon et al., 2008).

How Endogenous Hormone Loss and Exogenous Hormones Impact Healthy Aging and AD: Implications for Optimal Therapeutic Impact

This dissertation adds to the growing body of research which demonstrates that gonadal hormone deprivation is a mediating factor that increases risk of cognitive impairment in healthy aging, as well as risk of AD. Our data herein, as well as past literature, suggest that gonadal hormone deprivation in males and females can detrimentally impact the brain and cognition. This is especially true in females if gonadal hormone deprivation occurs before the natural onset of menopause (Imtiaz et al., 2014; Phung et al., 2010; Rocca et al., 2007, 2012). This tenet is supported by work in both healthy aging as well as neurodegenerative disease with AD. It is possible that gonadal hormone deprivation at different timepoints drives an individual to increase the

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likelihood of AD development. In clinical literature, women who receive oophorectomy

before the natural onset of menopause have an increased risk of dementia (Rocca et al.,

2007; Phung et al., 2010; Bove et al., 2014). Additionally, both estrogens and AD impact

the cholinergic system. The cholinergic system originates in the basal forebrain, projects

to the hippocampus and prefrontal cortex, and is integral to learning and memory (Gritte

et al., 1997; Baxter et al., 1999; Everitt et al., 1997). With estrogen administration, there

are alterations in basal forebrain cholinergic functioning such that there is an increase in

choline acetyltransferase (ChAT) mRNA, the rate-limiting enzyme for production of

acetylcholine, in the basal forebrain and dorsal hippocampus as well as increases in

ChAT mRNA expression and protein levels in cholinergic neurons (Luine et al., 1985;

Gibbs, 2000; 1997; Gibbs et al., 1994; Bohacek et al., 2008).Studies have demonstrated

that beneficial impacts of estrogens on cognition are mediated in part by the cholinergic

system (Tinkler et al., 2008; Gibbs et al., 2000). Additionally, early progression of AD

pathology impacts the cholinergic system such that basal forebrain cholinergic volume is

decreased as AD clinical symptoms begin to manifest (Grothe et al., 2012). Moreover,

cholinergic markers in patients with AD decrease as the disease progresses and are linked

to severity of cognitive impairment (Perry et al., 1978). Thus, estrogens can impact the

functionality of the cholinergic in a protective manner in AD (Gibbs et al., 2010).

Lending support that hormone modulation can impact AD development, administration

of exogenous estrogens and progesterone in females, as well as androgens in males, has

been shown to attenuate some of these detriments found with gonadal hormone

deprivation alone. For example, 17beta-estradiol treatment in various AD models

decreased beta-amyloid and improved performance on spatial reference memory while

progesterone decreased hyperphosphorylated tau (Carroll et al., 2007; Hu et al., 2016; Heikkinen et al., 2004).

It is important to note that the time of administration of exogenous sex steroid hormones fall within a window of opportunity for beneficial effects on brain and cognition. If administration occurs within an unsuitable or detrimental timeframe, then these hormones will prove less efficacious. Further, appropriate dosages and routes of administration must be considered to provide the most benefits of each type of hormone. I propose that estrogens and testosterone interact with mechanisms involving clearance of beta-amyloid through the neuroinflammatory system, as well as with phosphatases and kinases to prevent hyperphosphorylation of tau to promote healthy aging. 17beta-estradiol has been shown to attenuate the increase in beta-amyloid seen with Ovx alone and this might be mediated by the insulin degrading enzyme which is a beta-amyloid associated clearance factor that has upregulated mRNA expression with 17beta-estradiol and progesterone treatment (Jayaraman et al., 2012). Moreover, 17beta-estradiol attenuates the increase in hyperphosphorylated in cell culture compared to cells without estrogen treatment and this is potentially mediated through upregulation of protein kinase A (Liu et al., 2008). When these hormones of interest are decreased in some capacity, primarily through normal processes associated with aging, such as menopause and andropause, this alteration of the hormonal milieu can lead to impaired cognition and negative impacts on brain functions. Although at times these effects can be at least partially ameliorated with exogenous hormone administration to improve brain and cognitive functioning, this improvement is reliant on a myriad of factors such as timing, dosage, hormone type, and route of administration, for optimal effects.

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Further, I propose that assessment of AD-like pathology and cognitive profiles in the TgF344-AD model is inherently different in males and females, and that this could yield crucial insights into understanding the disparity in AD prevalence and progression attributed to sex in the clinic. For females, at the timepoint assessed, there were pathological changes for both beta-amyloid and tau assessments, such that follicular depletion increased beta-amyloid expression in the frontal cortex while gonadectomy decreased AT8/Tau 5 ratios in transgenic rats, indicating a potential attenuation in pathology. These changes in pathology were not reflected in assessments of spatial navigation or anxiety-like behavior since there were not interactions between genotype and either surgical treatment or follicular depletion. This was counter to the findings in males whereby there was a significant interaction for spatial working memory such that gonadectomy induced a genotype effect that was not seen in sham counterparts. Moreover, impacts on pathology were not interactive, indicating that assessments of beta-amyloid (1-42) and AT8 were not driving these cognitive changes. Therefore, alternate pathological targets for therapeutic intervention for males must be investigated. Additionally, although interactive impacts on cognition were not demonstrated for females, it is possible that the timepoint assessed was not advanced enough for changes in pathology to effect downstream symptomatology. With this understanding, I propose that one mechanism for why AD is more prevalent in women is due to variations in unique endocrine-related events (with a focus on menopause) that women undergo that can disrupt the endocrine milieu leading to an unbalanced and non-homeostatic environment which can increase risk of AD pathology development.

Future research should focus on distinguishing characteristics in exogenous hormone administration while also acknowledging that healthy aging and AD are impacted by many body systems, not just the endocrine and neural systems, to affect behavior. Investigation into how the endocrine and neural systems interact with the gastrointestinal, cardiovascular, and immune systems to impact disease progression will also likely shed light on critical variables impacting outcomes that are not yet understood. This multi-systems perspective is necessary, as increasing evidence has identified that the commitment to personalized medicine and the inclusion of a whole-body approach to scientific investigation to aging and menopause becomes more prominent. Results from this dissertation underscore the importance of utilization of personalized medicine to target Alzheimer's disease-related pathology and symptomatology, particularly regarding sex, current hormone milieu, and menopause history for females.

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APPENDIX A

DISSERTATION FIGURES
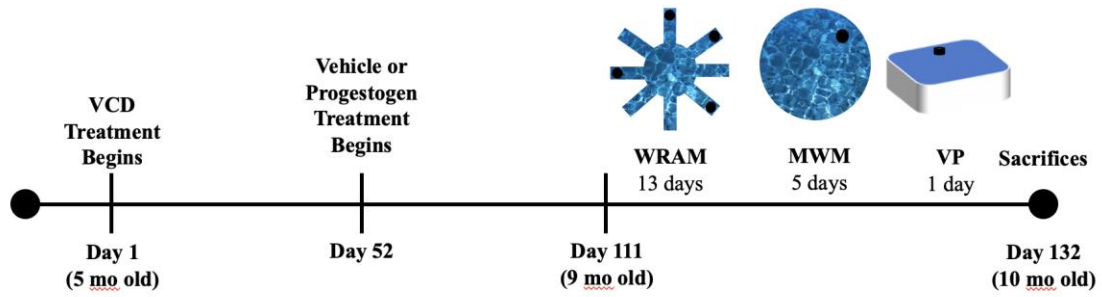
Figure 1. Study timeline. Timeline depicting initiation of VCD treatment to induce follicle depletion, initiation of hormone injections, and the order of tasks for the behavioral battery.
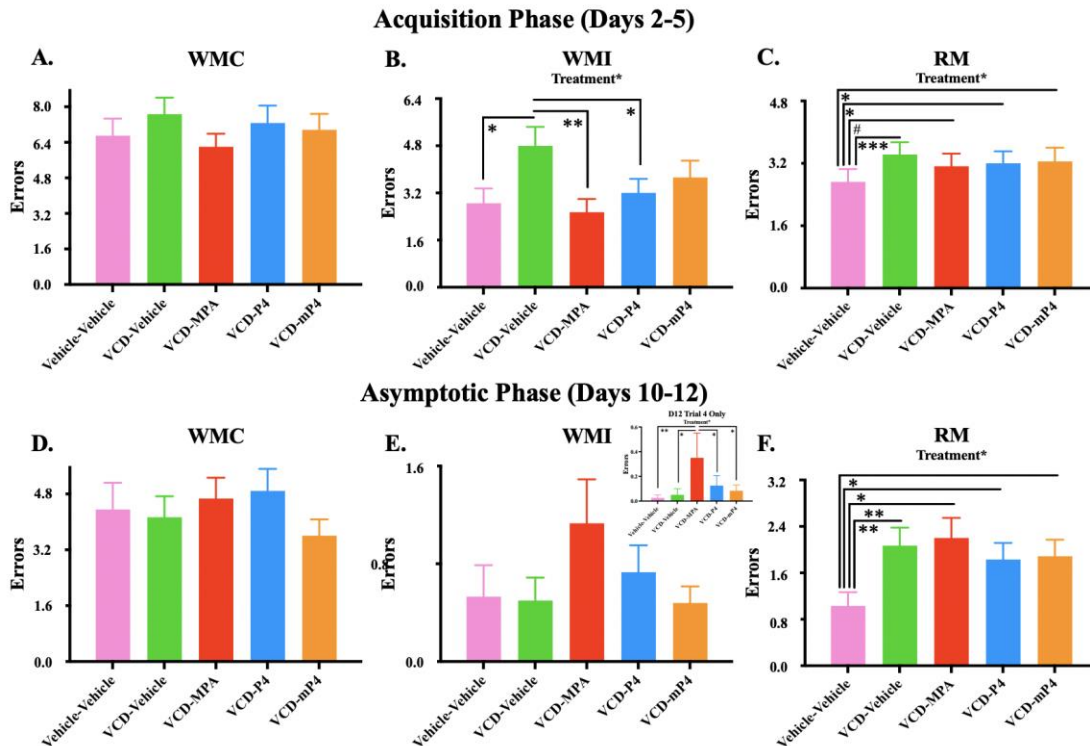
Figure 2. WMC, WMI, and RM errors during the early acquisition phase (Days 2-5) and the asymptotic phase (days 10-12) for the water radial arm maze. There were no effects of treatment for WMC errors during the early acquisition phase (A). (B) WMI errors during the early acquisition phase of testing; VCD-only treated rats made significantly more WMI errors than vehicle controls. This impairment was attenuated by MPA and P4 treatment. (C) RM errors during the early acquisition phase of testing; All VCD-treated groups made significantly more RM errors than vehicle controls. There were no effects of (D) WMC errors during the asymptotic, and (E) WMI errors during the asymptotic phase. (F) RM errors during the asymptotic phase of testing; All VCD-treated rats made significantly more errors than vehicle controls, $\#p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Abbreviations: WMC – working memory incorrect, WMI – working memory incorrect, RM – reference memory, VCD – 4vinylcyclohexene diepoxide, MPA – medroxyprogesterone acetate, P4 – progesterone, mP4 – micronized progesterone.
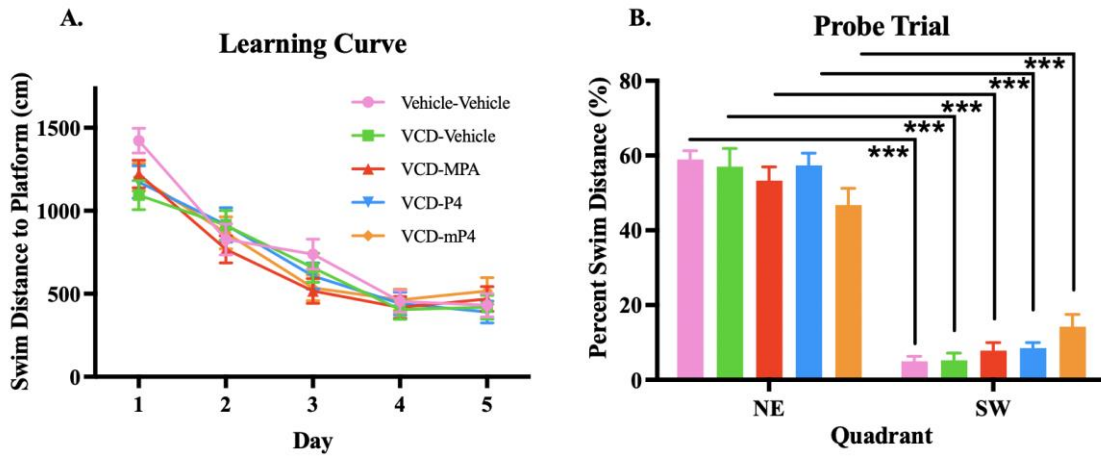
Figure 3. Morris water maze performance. (A) Swim distance to platform across all days of testing; all rats demonstrated decreased swim distance across days, indicating that all rats were able to learn the task. (B) Performance on probe trial, percent swim distance spent in NE vs SW quadrants; all rats demonstrated increased swim distance in the NE quadrant that used to contain the platform compared to the SW quadrant indicating all rats had intact spatial localization, ***$p < 0.001$. Abbreviations: VCD – 4vinylcyclohexene diepoxide, MPA – medroxyprogesterone acetate, P4 – progesterone, mP4 – micronized progesterone, NE – northeast, SW – southwest.
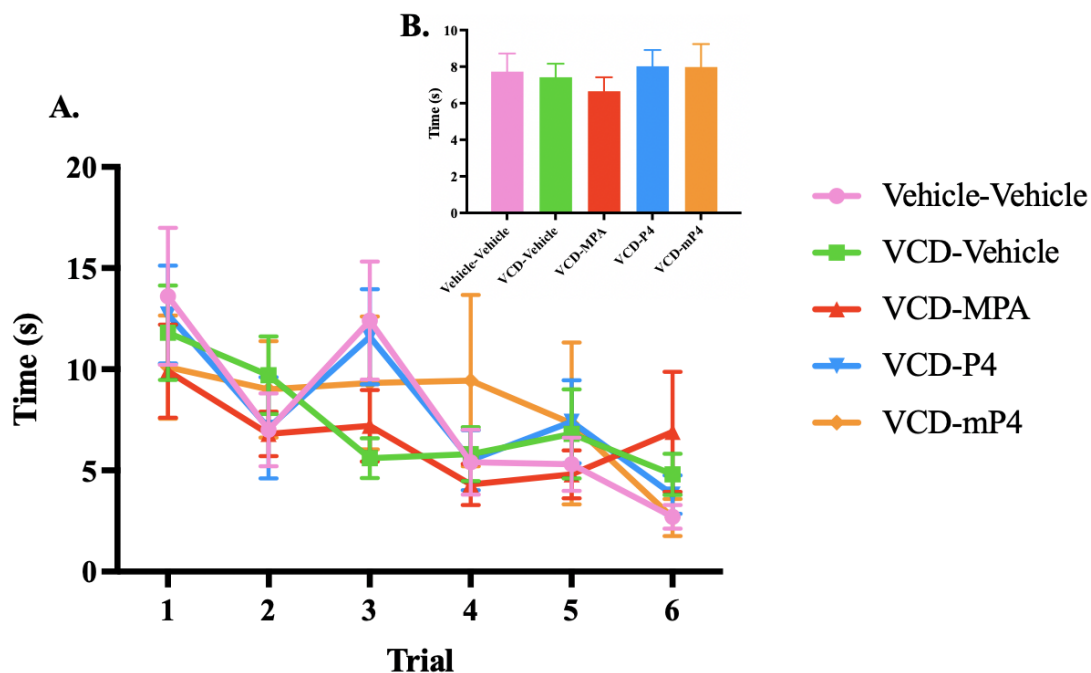
Figure 4. Performance on the visible platform task. (A) Latency to platform during each trial. (B) Average latency to platform collapsed across all six trials. There were no significant differences for any treatment group for the visible platform task indicating that all animals had the visual and motor acuity required to solve a water escape task. Abbreviations: VCD – 4vinylcyclohexene diepoxide, MPA – medroxyprogesterone acetate, P4 – progesterone, mP4 – micronized progesterone.
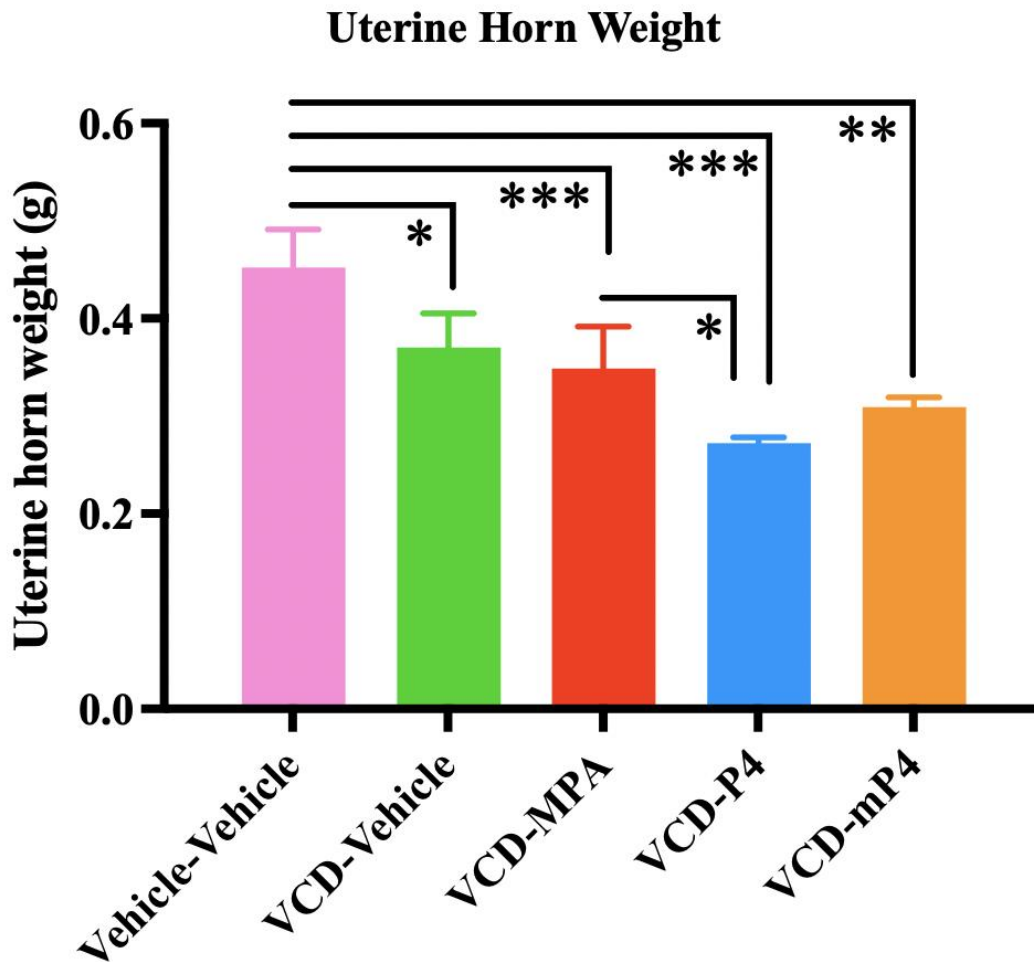
Figure 5. Uterine horn wet weights. All VCD-treated rats had uterine horns that weighed significantly less than vehicle controls. Additionally, progesterone-treated rats had uterine horns that weighed significantly less than MPA-treated rats, *$p < 0.05$, **$p < 0.01$, ***$p <$ 0.001. Abbreviations: VCD – 4vinylcyclohexene diepoxide, MPA – medroxyprogesterone acetate, P4 – progesterone, mP4 – micronized progesterone.
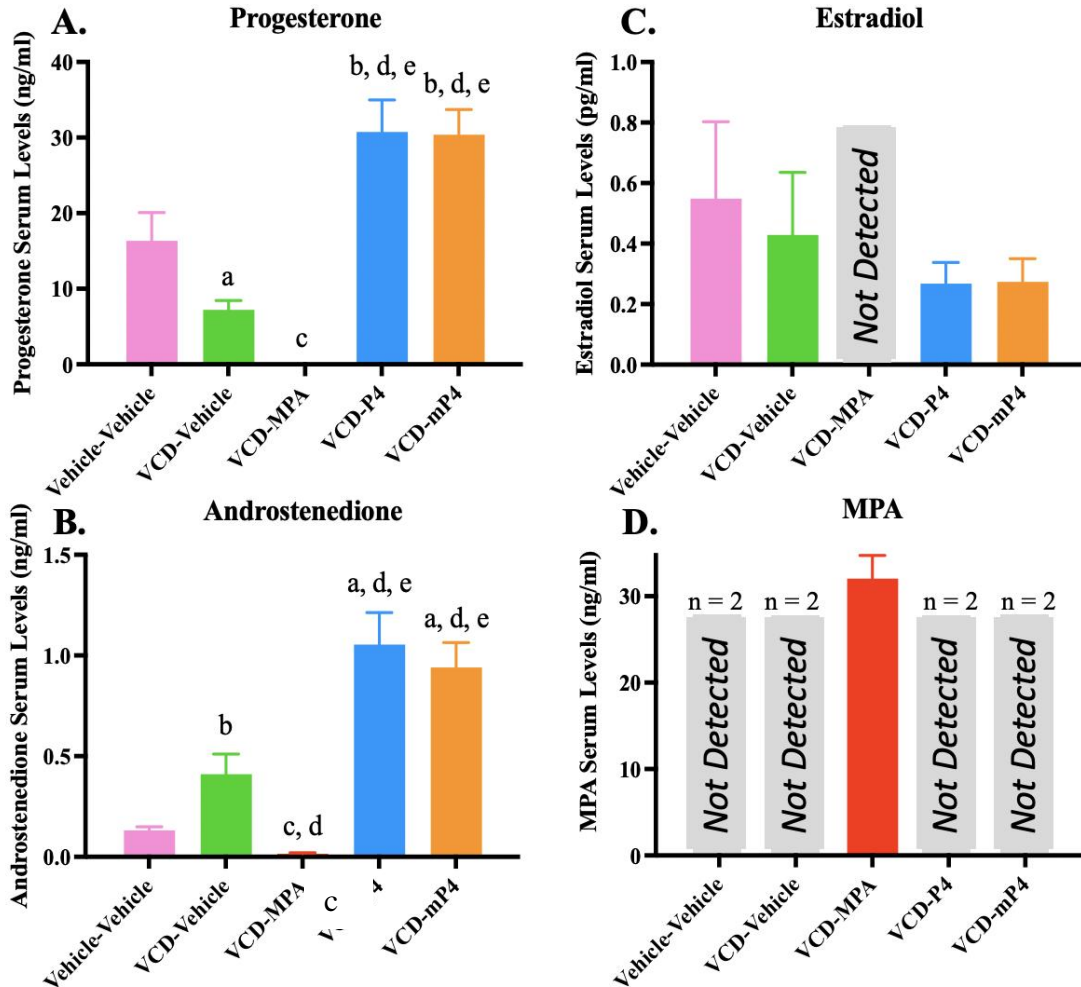
Figure 6. Circulating serum hormone levels. (A) Progesterone levels; P4- and mP4-treated rats had significantly higher progesterone levels than all other treatment groups, MPA-treated rats and VCD-only treated rats had significantly lower progesterone than all treatment groups, a: $p < 0.05$ vs. Vehicle-Vehicle, b: $p < 0.01$ vs. Vehicle-Vehicle, c: $p < 0.001$ vs. Vehicle-Vehicle, d: $p < 0.001$ vs. VCD-Vehicle, e: $p < 0.001$ vs. VCD-MPA. (B) Androstenedione levels; P4- and mP4-treated rats had significantly higher androstenedione levels than all other treatment groups, VCD-only treated rats had significantly higher androstenedione levels than MPA-treated rats and marginally higher androstenedione levels than vehicle controls, a: $p < 0.001$ vs. Vehicle-Vehicle, b: $p < 0.1$ vs. Vehicle-Vehicle, c: $p < 0.05$ vs. VCD-Vehicle, d: $p < 0.001$ vs. VCD-Vehicle, e: $p < 0.001$ vs. VCD_MPA. (C) Estradiol levels; there were no differences in estradiol levels between treatment groups. All MPA-treated rats had undetectable levels of estradiol. (D) MPA levels; the MPA-treated rats had detectable levels of MPA. A random subset of two rats per group were also included in the assay and demonstrated undetectable levels of MPA,

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Ovarian Outcomes
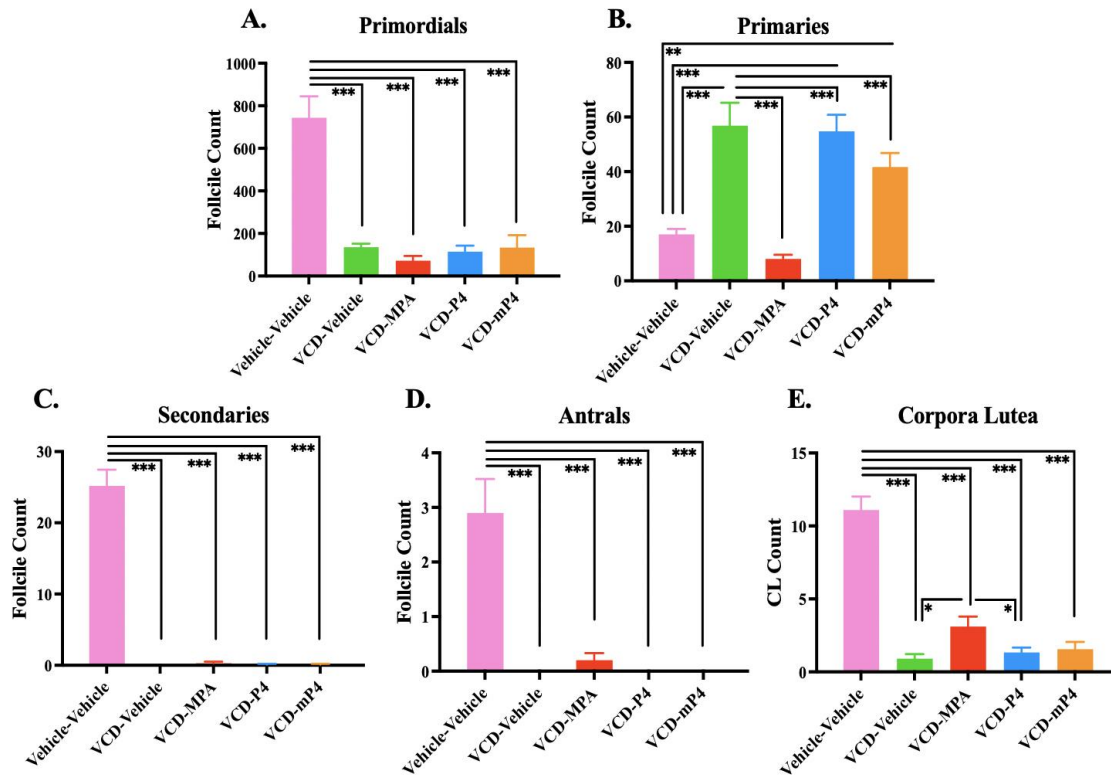
Figure 7. Ovarian follicle status. (A) Primordial follicles; All VCD-treated rats had significantly fewer primordial follicles than vehicle controls. (B) Primary follicles; VCD-only treated rats has significantly more primary follicle than vehicle controls, as did P4 and mP4 treated rats, MPA treatment attenuated the increase in primary follicles with VCD-only treatment. (C) Secondary follicles; All VCD-treated rats had significantly fewer secondary follicles than vehicle controls. (D) Antral follicles; All VCD-treated rats had significantly fewer antral follicles than vehicle controls. (E) Corpora lutea; All VCD-treated rats had significantly fewer corpora lutea than vehicle controls, MPA-treated rats had significantly more corpora lutea than VCD-P4 or VCD-only –treated rats, $**p < 0.01$, $***p < 0.001$. VCD – 4vinylcyclohexene diepoxide, MPA – medroxyprogesterone acetate, P4 – progesterone, mP4 – micronized progesterone.

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VCD-Progesterone Only

r = 0.804
$p < 0.01$, *$Q < 0.1$

GAD 65
GAD 67

Beta-actin
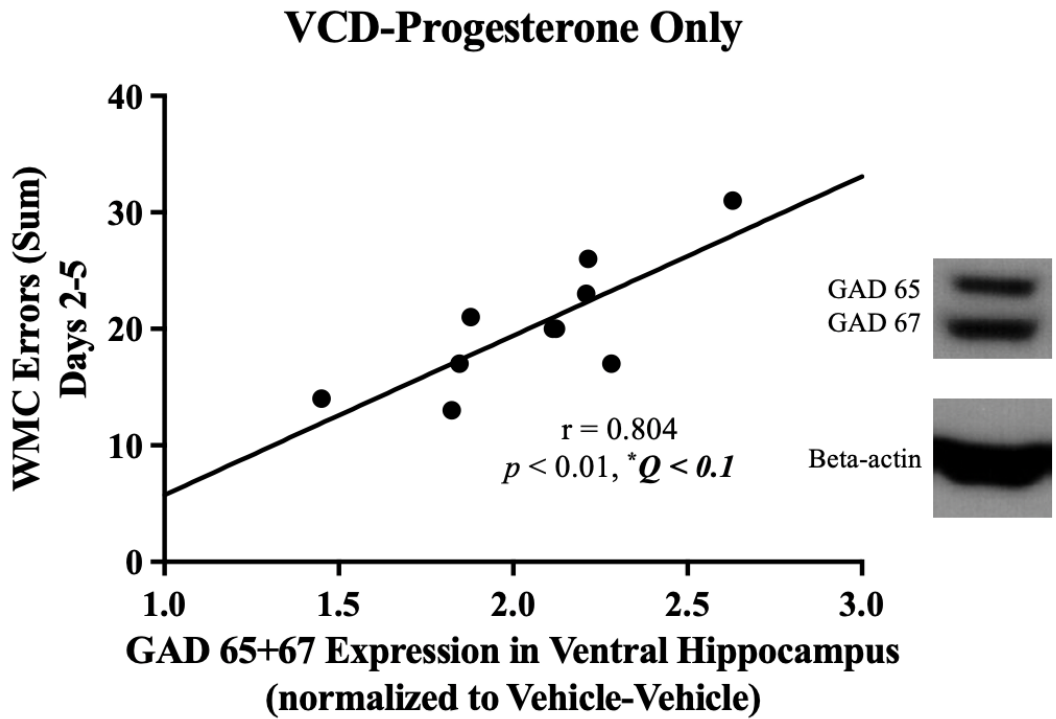
Figure 8. Pearson _r_ correlation between GAD 65+67 expression (normalized to vehicle controls) and WMC Errors during the acquisition phase (Days 2-5) for P4-treated rats in the ventral hippocampus. *$Q < 0.1$. Abbreviations: WMC – working memory correct, VCD – 4vinylcyclohexene diepoxide, GAD – glutamic acid decarboxylase.
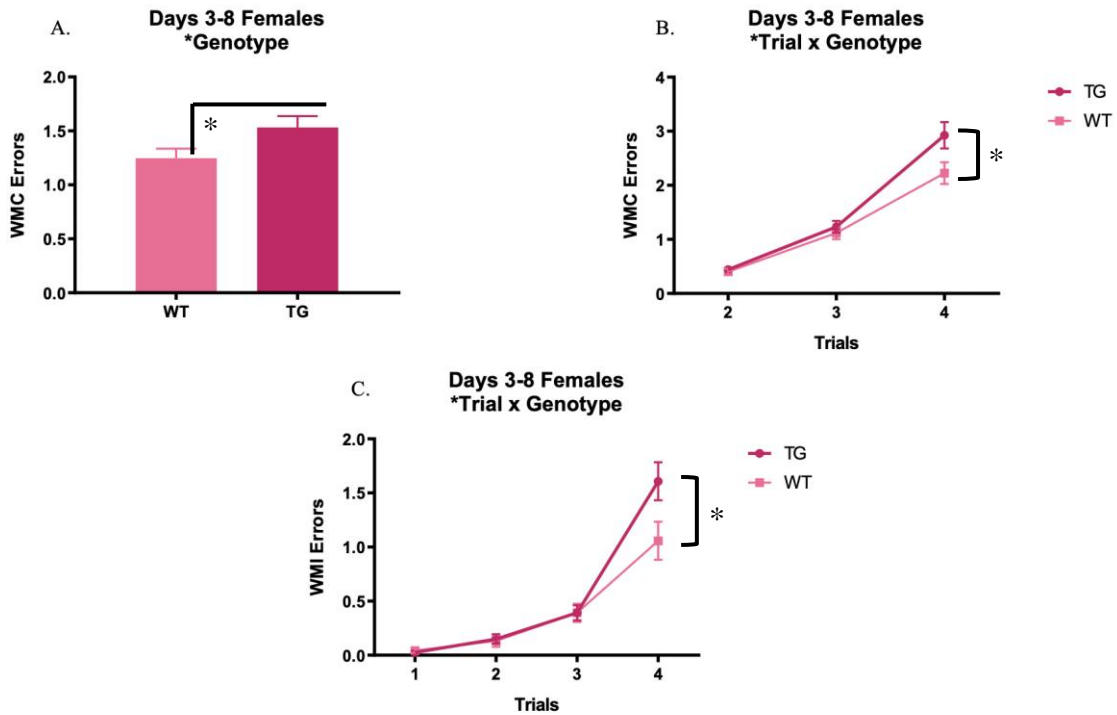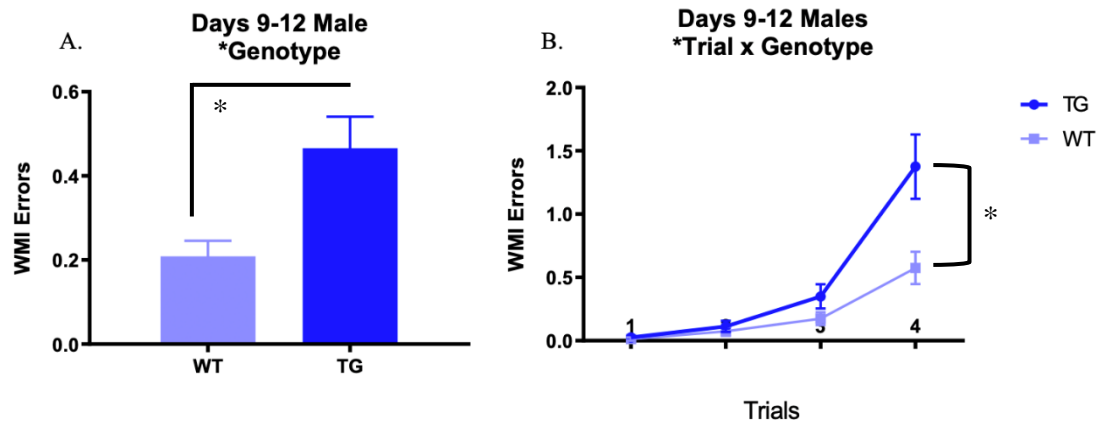
Figure 9. WMI and WMC errors during the acquisition phase (Days 3-8) on the WRAM for females. (A) TG rats made significantly more WMC errors during Days 3-8 compared to WT rats, $*p < 0.05$. (B) This effect was led by Trial 4 (highest working memory load trial), where TG rats made significantly more WMC errors on Trial 4 compared to WT controls. (C) There was a significantly Trial x Genotype interaction for WMI errors during Days 3-8 where TG rats made significantly more errors on Trial 4 compared to WT controls, $*p < 0.05$. Abbreviations: WMC – working memory incorrect; WMI – working memory incorrect, TG – transgenic, WT – wildtype.
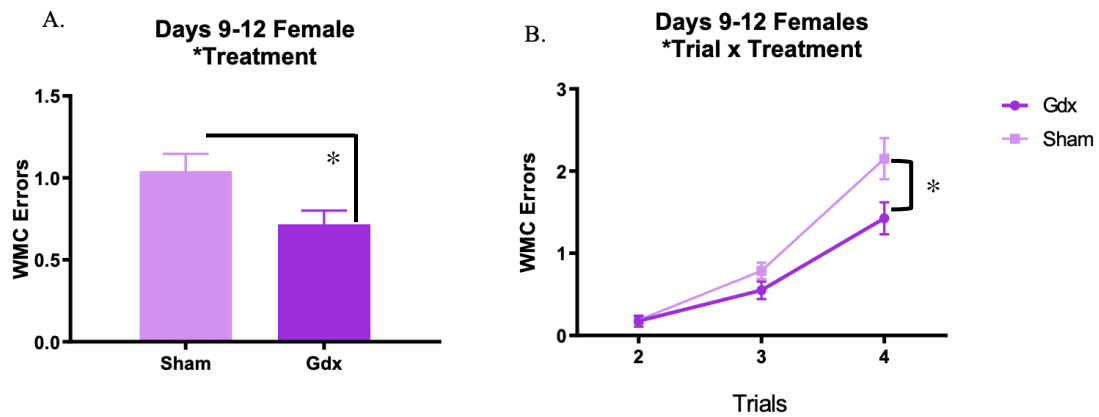
Figure 10. WMC errors during the asymptotic phase (Days 9-12) on the WRAM for females. (A) GDX rats made significantly fewer errors than Sham rats, *$p < 0.05$. (B) This effect was led by Trial 4 (highest working memory load trial), where GDX rats made significantly fewer WMC errors on Trial 4 compared to WT controls, *$p < 0.05$. Abbreviations: WMC – working memory incorrect; GDX - gonadectomy.
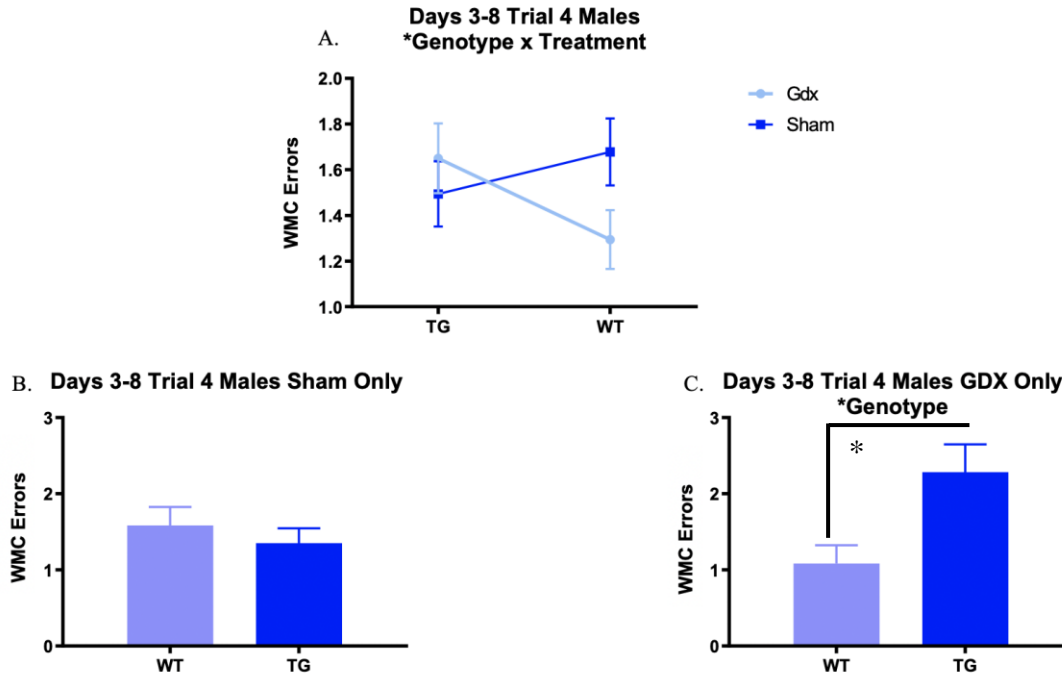
Figure 11. WMC during the acquisition phase (Days 3-8) on the WRAM for males.
(A) There was a significant Genotype x Treatment interaction whereby for GDX only rats there was a Genotype effect (C) where TG rats were impaired compared to WT controls. (B) This effect was not seen for the Sham only rats, *$p < 0.05$. Abbreviations: WMC – working memory incorrect, TG – transgenic, WT – wildtype, GDX – gonadectomy.

Figure 12. WMI errors during the asymptotic phase (Days 9-12) on the WRAM for males. (A) TG rats made significantly more WMC errors during Days 9-12 compared to WT rats, *$p < 0.05$. (B) This effect was led by Trial 4 (highest working memory load trial), where TG rats made significantly more WMC errors on Trial compared to WT controls, *$p < 0.05$. Abbreviations: WMI – working memory incorrect, TG – transgenic, WT – wildtype.
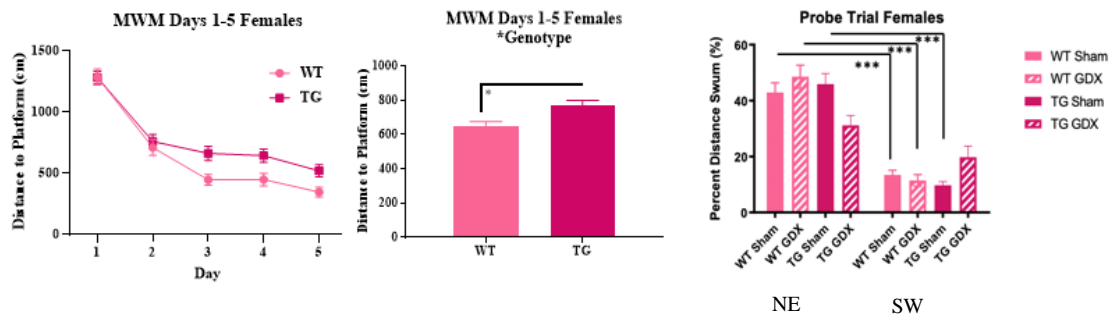
Figure 13. Morris Water Maze Performance in females. (A) TG rats had greater swim distance to platform compared to WT counterparts across all days of testing (Days 1-5), $*p < 0.05$. (B) Performance on probe trial, percent swim distance spent in NE vs SW quadrants; WT Sham, WT GDX, and TG Sham rats demonstrated increased swim distance in the NE quadrant that used to contain the platform compared to the SW quadrant indicating all rats had intact spatial localization, $***p < 0.001$. This effect was not found for TG GDX rats indicating that this groups was not able to correctly spatially localize. Abbreviations: TG – transgenic, WT – wildtype, GDX – gonadectomy, MWM – Morris Water Maze, NE – north-east, SW – south-west.
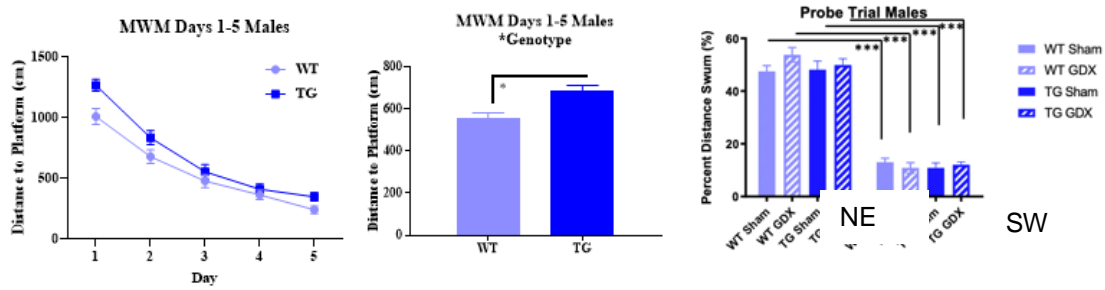
Figure 14. Morris Water Maze Performance in males. (A) TG rats had less swim distance to platform compared to WT counterparts across all days of testing (Days 1-5). (B) Performance on probe trial, percent swim distance spent in NE vs SW quadrants; all rats demonstrated increased swim distance in the NE quadrant that used to contain the platform compared to the SW quadrant indicating all rats had intact spatial localization, ***$p < 0.001$. Abbreviations: TG – transgenic, WT – wildtype, GDX - gonadectomy, MWM – Morris Water Maze, NE – north-east, SW – south-west.
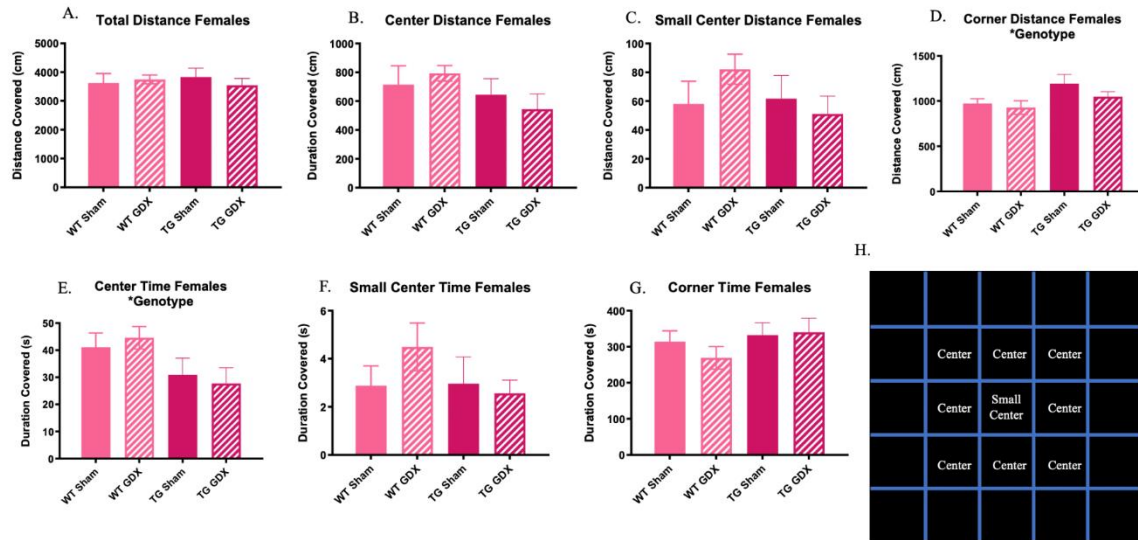
Figure 15. Open Field Performance for females. (A-C) There were no effects of Genotype or Treatment for Total distance, Center distance, or Small Center distance. (D) Corner distance; TG rats covered more distance in the corners of the arena compared to WT rats. (E) Center duration; TG rats spent less time in the center of the arena compared to WT rats. (F-G) There were no effects of Genotype or Treatment for Small Center time or Corner time. (H) Depiction of Center and Small Center in OF arena. Squares were 20cm x 20cm, *$p < 0.05$. Abbreviations: TG – transgenic, WT – wildtype, GDX – gonadectomy.
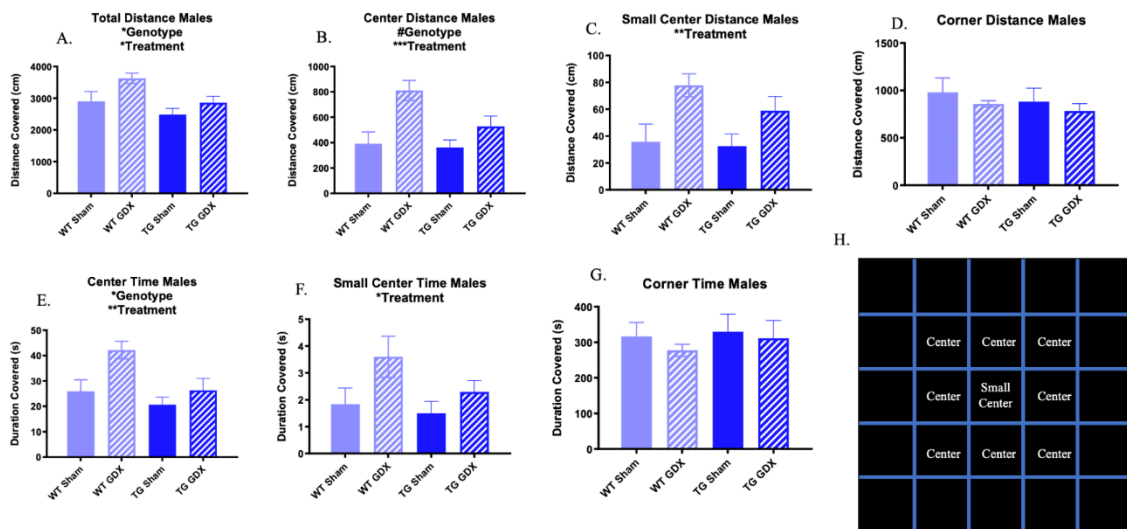
Figure 16. Open Field Performance in males. (A) Total distance; TG rats covered less distance than WT counterparts, GDX rats covered less distance than Sham counterparts. (B) Center distance; TG rats tended to cover less distance in the center of the arena compared to WT rats, GDX rats covered more distance in the center of the arena compared to Shams. (C) Small Center distance; GDX rats covered more distance in the small center of the arena compared to Sham rats. There were no effects of Genotype or Treatment for the Corner distance (D). (E) Center duration; TG rats spent less time in the center arena compared to WT rats, GDX rats spent more time in the center of the arena compared to Sham rats. (F) Small Center duration; GDX rats spent more time in the small center of the area compared to Sham rats. There were no effects of Genotype or Treatment for the Corner duration (G). (H) Depiction of Center and Small Center in OF arena. Squares were 20cm x 20cm, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, $\#p < 0.1$. Abbreviations: TG – transgenic, WT – wildtype, GDX – gonadectomy.
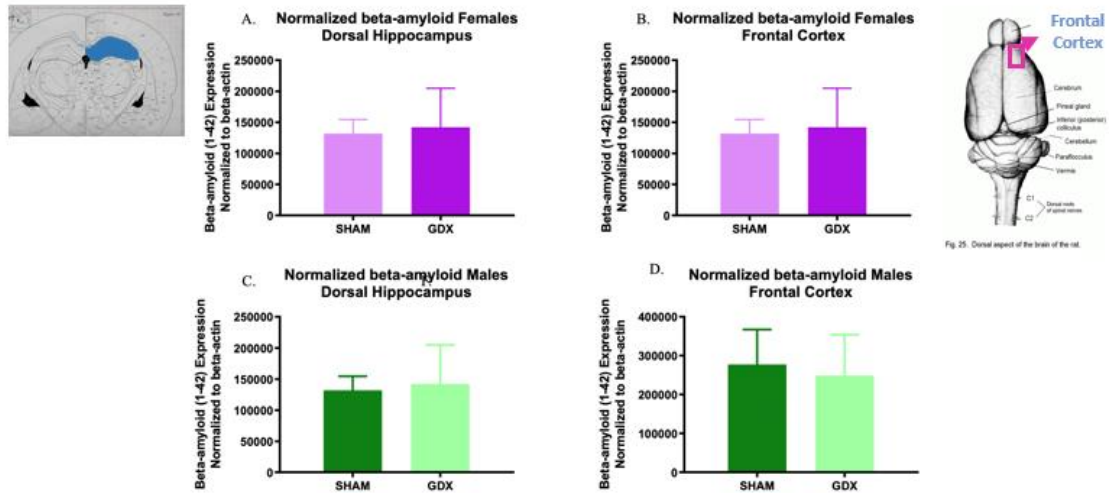
Figure 17. Beta-amyloid (1-42) expression in males and females. (A-D) There were no differences in normalized beta-amyloid expression (1-42) in either the dorsal hippocampus or frontal cortex, in male and female rats. Abbreviations: TG – transgenic, WT – wildtype, GDX – gonadectomy.
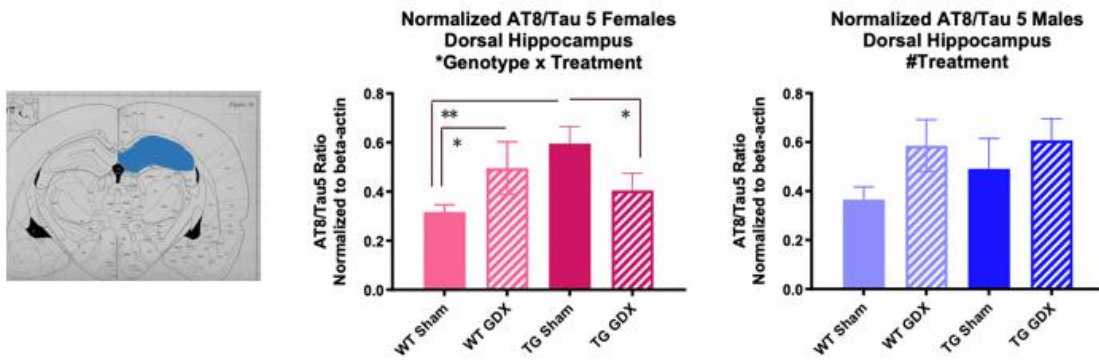
Figure 18. AT8/Tau 5 ratio in males and females. (A) There was a significant Genotype x Treatment interaction in the dorsal hippocampus for AT8/Tau 5 ratio in female rats such that there was an increase in AT8/Tau 5 ratio in WT GDX rats compared to WT Sham rats and a decrease in AT8/Tau 5 ratio in TG GDX rats compared to TG Sham rats. Additionally, for Sham rats, TG rats had an increased AT8/Tau 5 ratio compared to WT rats. (B) GDX rats tended to have higher AT8/Tau 5 ratios compared to Sham counterparts, $*p < 0.05$, $\#p < 0.1$. Abbreviations: TG – transgenic, WT – wildtype, GDX - gonadectomy.
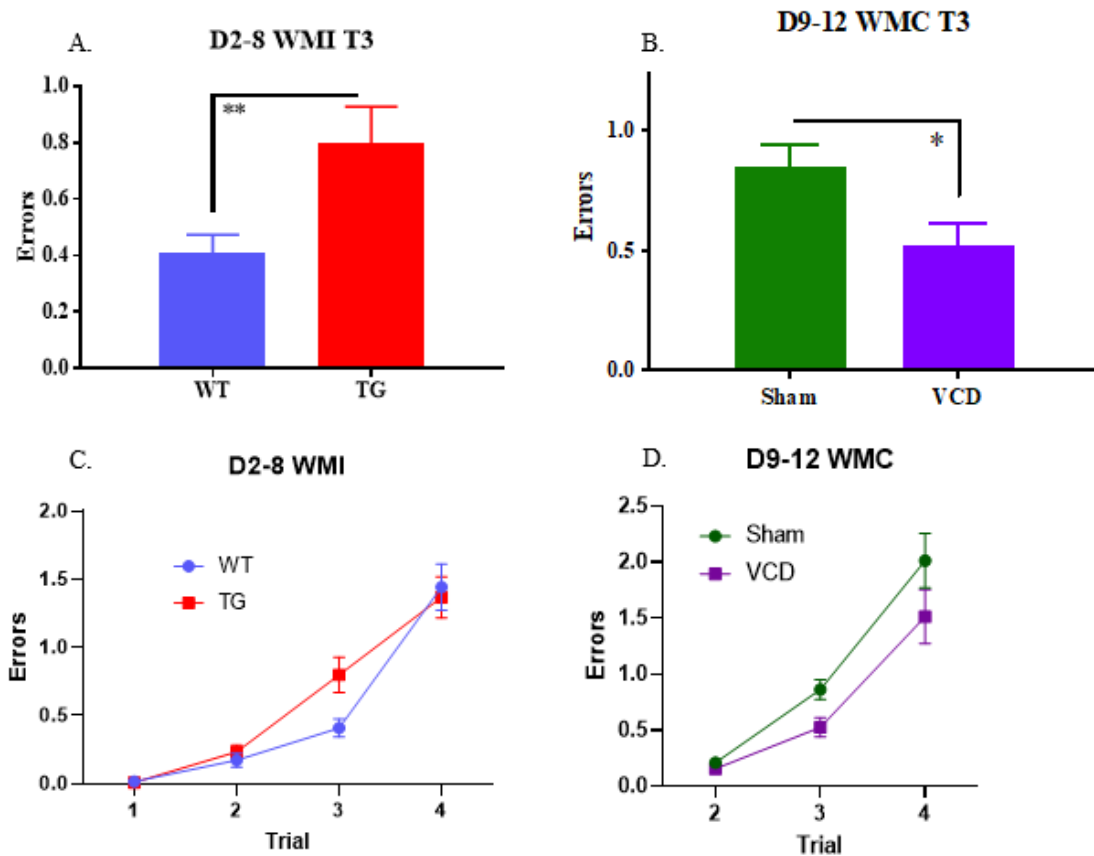
Figure 19. WMI and WMC errors during the acquisition phase (Days 2-8) and asymptotic phase (Days 9-12) for Trial 3 on the WRAM. (A) TG rats made significantly more WMI errors on Trial 3 (moderate working memory load trial) during Days 2-8 compared to WT rats, $*p < 0.05$. (B) VCD-treated rats made significantly fewer WMC errors on Trial 3 during Days 9-12 compared to Sham rats, $*p < 0.05$. Abbreviations: WMI – working memory incorrect, TG – transgenic, WT – wildtype.
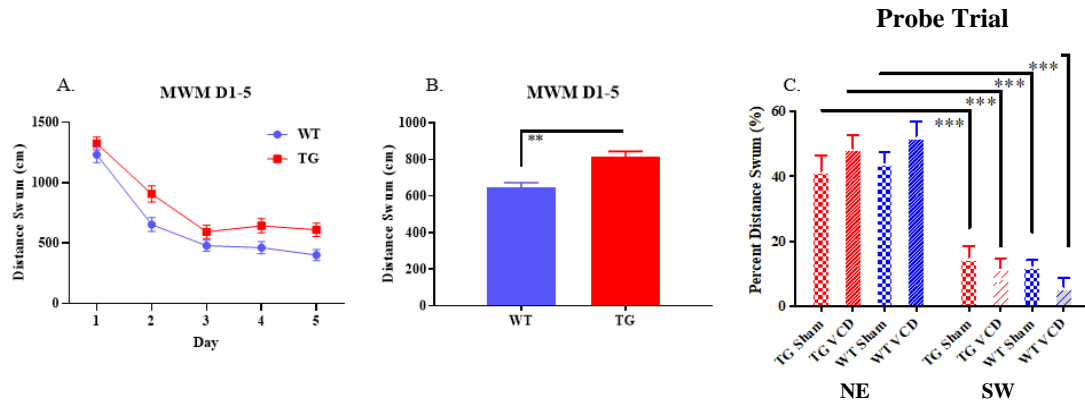
Figure 20. Morris Water Maze Performance. (A&B) TG rats had less swim distance to platform compared to WT counterparts across all days of testing (Days 1-5). (C) Performance on probe trial, percent swim distance spent in NE vs SW quadrants; all rats demonstrated increased swim distance in the NE quadrant that used to contain the platform compared to the SW quadrant indicating all rats had intact spatial localization, ***$p < 0.001$. Abbreviations: TG – transgenic, WT – wildtype, VCD – 4vinylcyclohexene diepoxide, MWM – Morris Water Maze, NE – north-east, SW – south-west.
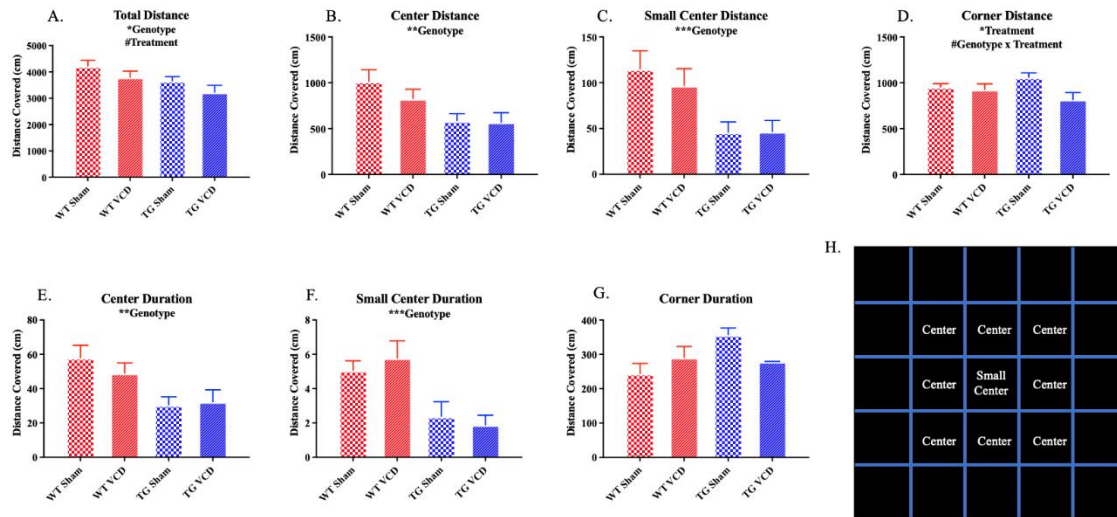
Figure 21. Open Field Performance. (A) Total distance; TG rats covered less distance than WT counterparts, VCD rats tended to cover less distance than Sham counterparts. (B) Center distance; TG rats covered less distance in the center of the arena compared to WT rats. (C) Small Center distance; TG rats covered less distance in the small center of the arena compared to WT rats. (D) Corner distance; VCD rats covered less distance in the corners compared to Sham counterparts. (E) Center duration; TG rats spent less time in the center arena compared to WT rats. (F) Small Center duration; TG rats spent less time in the small center of the area compared to WT rats. There were no effects of Genotype or Treatment for the Corner duration (G). (H) Depiction of Center and Small Center in OF arena. Squares were 20cm x 20cm, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, #$p < 0.1$. Abbreviations: TG – transgenic, WT – wildtype, VCD – 4vinylcyclohexene diepoxide, OF – open field.
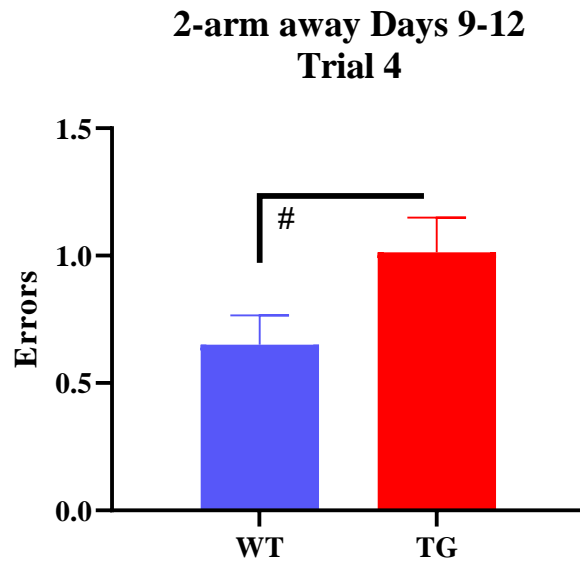
Figure 22. Precision-based WRAM analysis. TG rats tended to make more 2-arm away errors during the asymptotic phase of testing (Days 9-12) on the WRAM on Trial 4 (highest working memory load trial) compared to WT controls, #$p < 0.1$. Abbreviations: TG – transgenic, WT – wildtype.
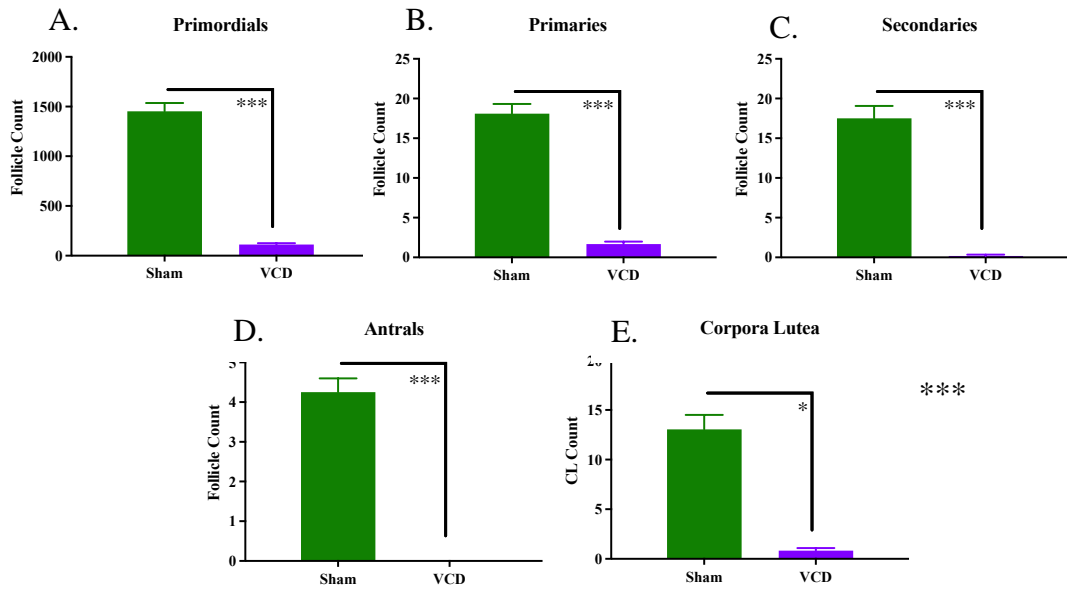
Figure 23. Ovarian follicle status. (A-E) VCD-treated rats had fewer follicles (for all follicle types) and corpora lutea than Sham counterparts, ***$p < 0.001$. There were no effects of Genotype nor any Genotype x Treatment interactions for follicle and corpora lutea count. Abbreviations: VCD – 4vinylcyclohexene diepoxide, CL – corpora lutea.
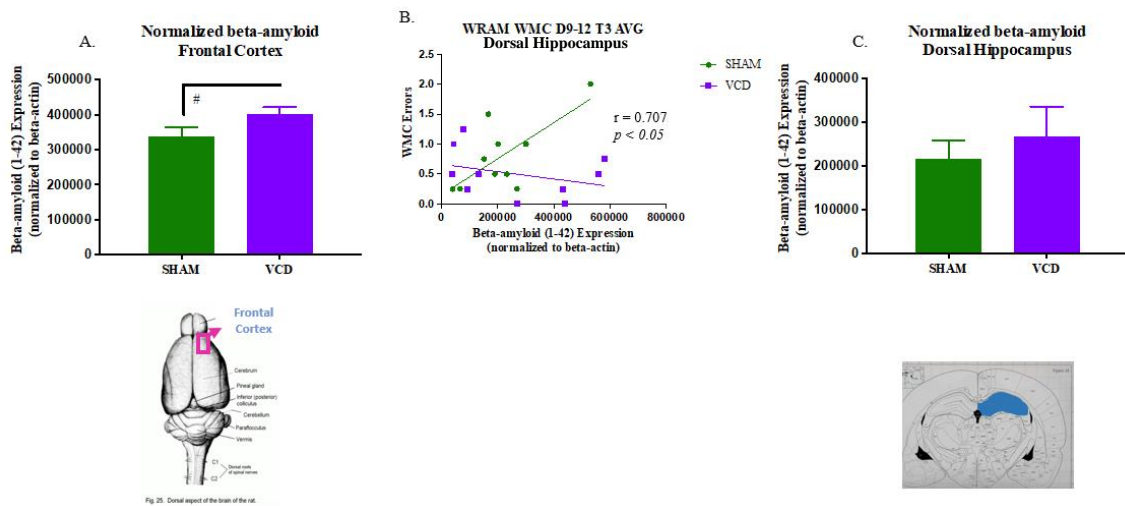
Figure 24. Beta-amyloid (1-42) expression and correlations. (A) VCD-treated rats tended to have more beta-amyloid (1-42) expression (normalized to beta-actin) in the frontal cortex compared to Sham controls. (C) There was no effect of Treatment on beta-amyloid (1-42) expression for the dorsal hippocampus. (B). Significant Pearson *r* correlation between beta-amyloid (1-42) expression (normalized to beta-actin) and WMC Errors during the asymptotic phase (Days 9-12) for Sham rats in the dorsal hippocampus, $*p < 0.05$, $\#p < 0.1$. Abbreviations: TG – transgenic, WT – wildtype, VCD – 4vinylcyclohexene diepoxide, WMC – working memory correct.

APPENDIX B

IACUC APPROVAL

Animal Protocol Review

ASU Protocol Number:	17-1580R
Protocol Title:	**Memory and Aging in the Rodent: Interventions to Improve Cognitive and Neurobiological Outcome**
Principal Investigator:	**Heather Bimonte-Nelson**
Date of Action:	5/10/2017

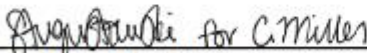The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/training/animals/levelthree.

Total # of Animals:	1,298	
Species:	Rats	**Pain Category: B-198; D-810**
Species:	Mice	**Pain Category: B-170; D-120**

Protocol Approval Period: 5/10/2017 – 5/9/2020

Sponsor:	**Multiple**
ASU Proposal/Award #:	030480 (Arizona Alzheimer's Consortium); 030166 (National Institutes of Health); 030502 (National Institutes of Health)
Title:	FY17: Arizona Alzheimer's Consortium; Variation in Hormones during Menopause: Effects on Cognitive and Brain Aging; Arizona Alzheimer's Disease Core

Signature: _____ for C. Miller   Date: 5/10/2017
IACUC Chair or *Designee*

Cc: IACUC Office
             IACUC Chair

Institutional Animal Care and Use Committee (IACUC)
Office of Research Integrity and Assurance
Arizona State University
660 South Mill Avenue, Suite 312
Tempe, Arizona 85287-6111
Phone: (480) 965-6788 *FAX:* (480) 965-7772

Animal Protocol Review

ASU Protocol Number:	20-1776R
Protocol Title:	Memory and Aging in the Rodent: Interventions to Improve Cognitive and Neurological Outcomes
Principal Investigator:	Heather Bimonte-Nelson
Date of Action:	4/23/2020

The animal protocol review was considered by the Committee and the following decisions were made:

The protocol was approved.

If you have not already done so, documentation of Level III Training (i.e., procedure-specific training) will need to be provided to the IACUC office before participants can perform procedures independently. For more information on Level III requirements see https://researchintegrity.asu.edu/animals/training.

Total # of Animals:	912	
Species:	**Rats**	**Unalleviated Pain/Distress: No**

Protocol Approval Period: 4/23/2020 – 4/22/2023

Sponsor:	NIH; Arizona Alzheimer's Consortium
ASU Proposal/Award #:	AWD00030166 (NIH); AWD00034065 (AAC)
Title:	Variation in Hormones during Menopause – Effects on Cognitive and Brain Again (NIH); Arizona Alzheimer's Consortium (ACC) FY20

Signature: _Samantha Sullivan_   Date: 4/28/2020
                IACUC Chair or *Designee*

Cc: IACUC Office
             IACUC Chair

APPENDIX C

MICHELADA RECIPE

MICHELADA RECIPE

Ingredients:

1. 12 oz Tecate or other Mexican lager
2. 2-4 oz Zing Zang Bloody Mary Mix
3. 1 lime
4. Salt or tajin for rim

Recipe:

1. Salt rim with lime and either salt or tajin
2. Pour 6oz of beer into frosted glass or beer mug
3. Pour 2-4 oz of bloody mary mix to taste
4. Juice one lime directly into drink
5. Stir and enjoy!

For a spicier version add chamoy and/or a chamoy straw